UNDERSTANDING THE SHINING RAMSHORN SNAIL, *SEGMENTINA NITIDA*: MORPHOLOGY, GENETICS AND BREEDING

by

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Abstract

The Shining Ramshorn Snail, *Segmentina nitida,* is a rare freshwater snail found predominantly in drainage ditches along field margins and in marshland. It is experiencing marked declines in distribution in the United Kingdom (UK) and mainland Europe. The species was included in the IUCN Red Data Book for Invertebrates before a guideline change in 1994 and is included on the UK Biodiversity Action Plan (BAP) as a priority species for conservation. The BAP for *S. nitida* states that further research on the species required to inform reintroduction and translocation for its conservation.

For this thesis, a modified sample evaluation method for *Segmentina nitida* was developed and evaluated. It increased sample assessment speed without significantly reducing accuracy in comparison to a traditional method of sample evaluation.

Captive breeding of *S. nitida* was explored with the aim of developing simple breeding protocols that could provide stock for potential reintroduction of the species into historical locations. Breeding proved challenging due to fluctuations in water chemistry and subsequent high mortality rates.

Geometric morphometric shape analyses were used to investigate variation in shell shape of the species across European populations from the UK, Germany, Poland and Sweden, and the Czech Republic. German and UK snails had similar shell morphologies, and Polish and Czech snails also clustered together morphologically, with the shape of Swedish snails being less distinct.

Analysis of the population genetics of German, UK, Polish and Swedish populations using nuclear (ITS, microsatellites) and mitochondrial markers (COI) revealed two distinct lineages of *S. nitida* in Europe. One comprised of populations from Poland and Sweden (East), and one represented UK populations and a Swedish population (West) with the two lineages coexisting in Germany. These two lineages show no evidence of genetic admixture and can be delimited by both genetic markers and geometric morphometrics, indicating two evolutionarily distinct units, possibly equating to species.

The genetic and shape differences between European populations has impacts the conservation of *Segmentina nitida*, especially in the UK, as previous descriptions of range may now be incorrect and the UK populations may be more significant globally than previously thought, if they are indeed *S. nitida*. Any future reintroduction plans in the UK and elsewhere would also need to take into account these genetic lineages, as they may result in the introduction of an invasive species or result in infertile offspring.

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Chapter 1- Introduction

1.1. Molluscan Classification

1.1.1 Phylum Mollusca

The Phylum Mollusca represents an incredibly diverse and varied group of organisms, second in species richness only to the Phylum Arthropoda (Bieler, 1992; Strong *et al.*, 2008). There are an estimated 80,000-100,000 described molluscan species (Fretter, 1978; Strong *et al.*, 2008). The Phylum Mollusca contains eight major lineages: Cephalopoda (squids, cuttlefish and octopi), Scaphopoda (tusk shells), Polyplacophora (chitons), Monoplacophora, Neomeniomorpha (solenogasters), Chaetodermomorpha (worm-like molluscs), Gastropoda (slugs, snails, and limpets), and Bivalvia (mussels and clams) (Kocot *et al.,* 2011). Cephalopoda, Scaphopoda, Polyplacophora, Monoplacophora, Chaetodermomorpha and Neomeniomorphaare all restricted to marine environments, while the Gastropoda and Bivalvia contain many freshwater species (Fitter and Manuel, 1994).

Molluscs have the highest number of documented extinctions, compared to other major taxonomic groups (Lydeard *et al.*, 2004). Of the 744 recorded extinctions in the Kingdom Animalia, between the years 1500 and 2016, 297 were molluscan species (40%), and of these, 265 were gastropods (IUCN, 2017). In 2016, there were 513 molluscan species listed as 'Endangered' in the International Union for Conservation of Nature (IUCN) Red List, and 2295 listed in all Red List categories except 'Least Concern' (IUCN, 2017). It is currently thought that around 9% of the 3700 described molluscan species (both terrestrial and aquatic) are considered to be endangered (Wells and Chatfield, 1992), although it is estimated that only <2% known molluscan species have been properly assessed for their conservation status (Lydeard *et al.*, 2004), so this could be an underestimate.

1.1.2 Class Gastropoda

In terms of their behaviour and form, the Gastropods are one of the most diverse groups of animals in existence (Lindberg *et al*., 2004). They form the largest group of molluscs, with between 50,000 and

100,000 described extant species, and they comprise approximately 80% of living molluscs (Chapman, 2009).

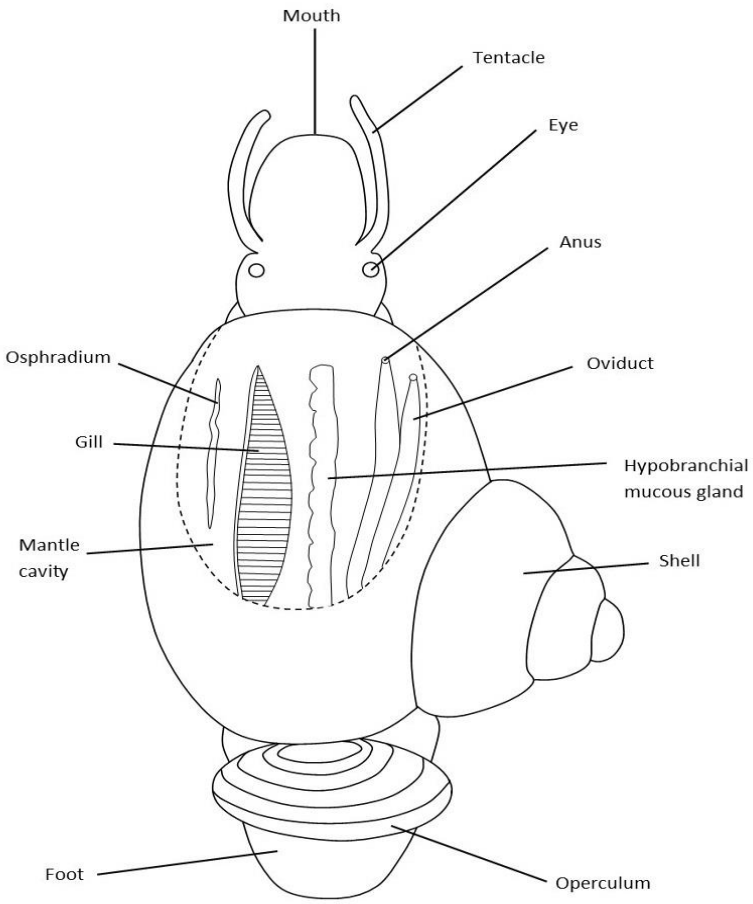


Figure 1.1. Typical gastropod body plan (adapted from Beedham 1972)

Gastropods typically have a broad, flat-soled foot used for crawling and a well-developed projecting head, bearing one or two pairs of sensory tentacles and a pair of eyes (Beedham 1972; Figure 1.1). Gastropods are distinguished from all other forms of molluscs through their basic asymmetry. This is due to a feature known as torsion. During development, the upper part of the body, including the visceral mass, shell and mantle, is twisted 180° anti-clockwise, in relation to the head. This results in the respiratory chamber being situated at the anterior end of the organism, just behind the head.

Freshwater gastropods have a wide geographical distribution and can be found in nearly all aquatic habitats, ranging from seasonal waters, such as temporary ponds, and drainage ditches, to more permanent water bodies; lakes, rivers and underground springs (Strong *et al.*, 2008). Gastropods display a range of feeding habits from micro-herbivorous (feeding on diatoms and algae) to carnivorous, with some even being suspension feeders (Gosselin and Chia, 1994; Declerk, 1995; Strong *et al.*, 2008). Individual gastropod species are typically habitat specialists, with restricted geographical ranges, long maturation times, low fecundity and relatively long life spans (Strong *et al.*, 2008). Many species of gastropods do not disperse very far, resulting in limited gene-flow between local populations (Njiokou *et al.*, 1994; Viard *et al.*, 1996).

The Gastropoda contain six subclasses: Patellogastropoda, Vegigastropoda, Cocculiniforma, Neritimorpha, Caenogastropoda, and the Heterobranchia (Bouchet *et al.,* 2017). Subclass Heterobranchia contains 13 orders: Acochlidioidea, Anaspidea, Cephalaspidea, Gymnosomata, Hygrophila, Nudibranchia, Pleurobranchomorpha, Runcinacea, Sacoglossa, Stylommatophora, Systellommatophora, Thecosomata, and Umbaculida (Ruggiero *et al.,* 2015). The Hygrophila contain two superfamilies, the Chilinoidea, and the Lymnaeoidea. Within the Lymnaeoidea are six extant families: Lymnaeidae, Acroloxidae, Bulinidae, Burnupiidae, Physidae, and the Planorbidae (Bouchet *et al.,* 2017)

1.1.3 Family Planorbidae

1.1.3.1 Phylogeny

The Family Planorbidae, commonly known as the 'Ramshorns', are the most diverse group of limnic (low-salt) pulmonates. Freshwater pulmonates are known to occur in a wide range of shallow freshwater habitats, such as streams, rivers, wetlands, and ponds (Russell-Hunter, 1978). The Planorbidae are thought to have evolved from pulmonates that transitioned from aquatic habitats to terrestrial ones and then back to aquatic habitat once again (Baker, 1945).

Whilst Albrecht *et al*. (2007) state that there are 40 recognised genera of Planorbidae, there appears to be no comprehensive list published in peer-reviewed literature. The National Center for Biotechnology Information (NCBI) taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy) lists 29 recognised genera within the family and on the World Register of Marine Species (WoRMS) there are 54 accepted genera (WoRMS Editorial Board, 2019).

Phylogenetic relationships between these genera are not well resolved and the placement and relationships between genera within the Family is a subject of current research and debate. Estimates of the number of planorbid species range between 200 and 350 (Baker, 1945; Hubendick, 1947; Meier-Brook, 2002). Traditionally, the structure of the radula and of male reproduction organs have been used to differentiate between different subfamilies and tribes within the Planorbidae (Hubendick, 1955). Recently, as with many families, the development of genetic phylogenies has begun to resolve and rearrange some of the relationships between planorbid genera and species. A study by Albrecht *et al.* (2007) used the mitochondrial DNA markers cytochrome c oxidise subunit I (COI) and 18S to assemble a molecular phylogeny of the Planorbidae (Figure 1.2).

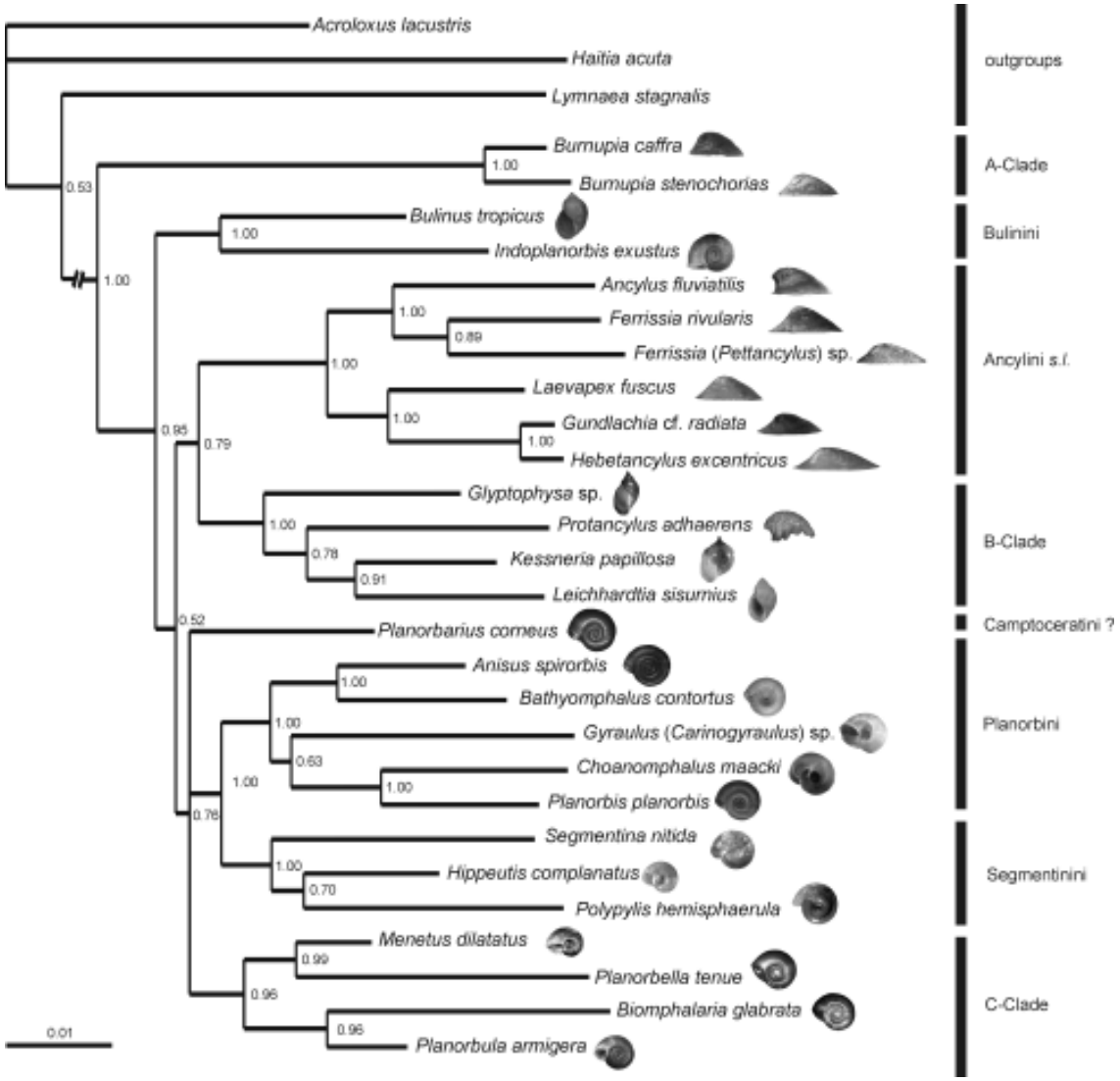


Figure 1.2. Phylogram of planorbidean taxa (Albrecht *et al.,* 2007)

1.1.3.2 Planorbid Life Cycles and Distribution

Freshwater molluscs show considerable variation in timing of reproduction and life cycle length, even between generations within species. From year-to-year there may be differences in growth rates, seasonal course of reproductive cycle, and number of generations (Russell-Hunter, 1978; Richardot-Coulet and Alfaro-Tejera, 1985). The most common life cycle pattern is univoltine, with one brood per year where the reproducing adults die after breeding, which typically happens in late spring/ early summer (Russell-Hunter 1978 and references within).

Temperature has a significant effect on the life cycle of gastropods as it dictates when the snails emerge from overwintering and begin reproduction (Terrier *et al.*, 2006). Population growth and reproduction rates are inhibited by the low temperatures experienced in the winter months in temperate regions (Russell-Hunter, 1961). Overwintered *Ancylus fluviatilis, Physa fontinalis,* and *Lymnaea peregra* adults have been found to be larger at the end of a mild winter than a cold one, indicating that growth does not completely arrest during the winter (Russell-Hunter, 1961). During the winter months there is typically no oviposition (egg laying) because of the low temperatures (Richardot-Coulet and Alfaro-Tejera, 1985). However, it has been observed that when overwintering individuals are removed from their habitat and placed in warmer conditions, they will sometimes begin to oviposit, in a time frame ranging from a few hours for *Physa gyrina* (De Witt, 1954), to three weeks for *Gyraulus crista* (Alfaro-Tejera, 1982; Richardot-Coulet and Alfaro-Tejera, 1985). Keeping individuals in constant temperatures in a laboratory setting, even with a natural photoperiod, can result in a permanent population of adults, to which growing snails are recruited via ongoing reproduction (Richardot-Coulet and Alfaro-Tejera, 1985). There is a close correlation between water temperature and life cycle pattern (Duncan, 1959; McMahon, 1975), with higher temperatures causing faster development and breeding (Richardot-Coulet and Alfaro-Tejera, 1985).

The life cycle of planorbids can also change with geographical location and the associated climatic or environmental differences. For example, Richardot-Coulet and Alfara-Tejera (1985) found that in an experimental pool in France, *Gyraulus crista* had an annual life cycle with three, overlapping breeding seasons, while in Russia, *G. crista* was observed to have an annual life cycle with only one generation per year (Tsikhon-Lukanina, 1965). In Poland the reproductive period was found to begin much earlier in the year and may involve several generations (Piechocki, 1979). In a study by Eversole (1978), three natural populations of *Helisoma trivolvis* (now *Planorbis trivolis*) had differing growth rates and maximum shell sizes, but when individuals from these three populations were reared in laboratory conditions, there were no significant differences in growth rate and maximum size (Eversole, 1978).

For freshwater snail species in general, calcium content of the water is a significant determinant of life cycle and distribution. Van Der Borght and Van Puymbroeck (1966) used calcium-45 as a radioactive tracer element and found that 80% of the calcium uptake in *Lymnaea stagnalis* was from the water, while 20% comes from food ingested. Some species that are found naturally in calcium rich environments can reproduce and survive in calcium-poor environments (Young, 1975). Generally, an adequate supply of lettuce can keep a calciphite species (one which only lives in hard water, with calcium levels over 20mg/L^{-1}) alive in calcium-poor conditions, given favourable temperatures (Young, 1975). A low level of calcium will typically also result in slower growth rates and delayed egg-laying (Dussart, 1979). In calcium poor environments, snails have thinner and less robust shells than in calcium-rich environments (Boycott *et al.,* 1932; Boycott, 1936; Hubendick, 1947; Young, 1975) and all freshwater gastropod species require the presence of calcium to successfully develop (McMahon, 1975).

In addition to affecting the life cycle of planorbids, calcium bicarbonate has been found to influence the distribution and relative densities of freshwater snails (Young 1975 and references within). However the relationship between calcium (and other ions) and snail biology seem to be very complex and vary between species and geographical area (de Azevedo *et al.*, 1967).

1.2. The Shining Ramshorn Snail*, Segmentina nitida*

1.2.1. Nomenclature

Segmentina nitida (Gastropoda: Planorbidae) was first described by Müller in 1774. Since this first description, the species has been assigned to different genera and synonymized several times. Kennard and Woodward (1926) list 43 instances of synonyms being used for *S. nitida* between 1774 and 1884. There have been further uses of synonyms and reassignment of the species since 1884, although updated lists do not exist.

Segmentina nitida is part of the tribe Segmentinini. This is a sister group to the tribe Planorbini, and it contains the genera *Segmentina, Hippeutis,* and *Polypylis*. Within the tribe, *Hippeutis* and *Polypylis* are

sister genera, with *Segmentina* basal to them, based on comparison of COI and 18S mitochondrial DNA markers (Albrecht *et al.*, 2007). The Segmentinini are regarded as a monophyletic tribe. This tribe is also supported by traditional, non-genetic phylogeny, based on the structure of the male reproductive organ and the radula (Hubendick, 1955, 1978). *Segmentina* (Fleming 1818) is the type genus of the subfamily Segmentininae, with *Nautilus lacustris* Lightfoot 1786 as the type species (Bouchet *et al.,* 2017), which is a junior synonym of *Segmentina nitida*. The syntype specimens of *S. nitida* are part of the Fabricius collection housed at the Natural History Museum of Denmark. They comprise a single lot of seven shells, with source location reported as 'Denmark' in the original literature (Müller, 1774; Nekhaev *et al.,* 2015). The only other accepted species described within this genus is *Segmentina paparyensis* (Baker 1913), of which only two specimens have been collected in Brazil. Another species, *Segmentina clessini* (Westerlund 1873), has been previously described in Europe, however, is considered by some to be a morphotype of *Segmentina nitida* instead of a distinct species. The type locality of *S. clessini* is given as: "Sweden, Island Öland, in a brook south of the village Kolstad… village near Borgholm City" (Westerlund 1873; Vinarski *et al.* 2013) and is found in Europe except for the far north and the Mediterranean region, north of Kazakhstan and Altay (Kruglov and Soldatenko, 1997). Currently, there are no genetic data to resolve which species this separate form represents, or if it is indeed a separate species.

1.2.2. General Description of *Segmentina nitida*

Segmentina nitida is a small freshwater snail with a smooth, glossy, iridescent shell with yellow-brown colouration. It grows up to 6mm in diameter and has a shell with no more than five whorls, of which the outer whorl is expanded and overlaps most of the others, resulting in a large aperture. The aperture is heart shaped, oblique and depressed. The shell is convex on its upper side and flat or concave on the lower side and resembles a contact lens in shape.

Identification of *S. nitida* is characterised by one to three white radial streaks upon the outer whorl. These are regarded as representing thickened enamel ridges that show through the shell (Figure 1.3).

These thickenings have been hypothesised to provide support to the outer whorl of the shell (Hill-Cottingham 2004) and are used to distinguish the species from a similar one, *Hippeutis complanatus* (Gastropoda: Planorbidae) (Linnaeus 1758) (Macan, 1977). A second distinct phenotype of *Segmentina nitida* has been reported in Eastern Europe, *Segmentina nitida* forma *distinguenda* (Piechocki, 1979, Piechocki and Wawrzyniak-Wydrowska 2016). This form has a more flattened shell and keel towards the centre of the shell, similar to *Hippeutis complanatus,* yet still possesses the characteristic internal thickenings of *S. nitida*. It is also reported to have more strongly developed lamellae, a narrower umbilicus, and a more yellow/green shell colouration than *Segmentina* (Piechocki and Wawrzyniak-Wydrowska 2016). There are, however, some malacologists who consider this form to represent a separate species (Stadnychenko 1990).

Historically there have been issues differentiating between *Segmentina nitida* and *Hippeutis complanatus.* Both snails grow to approximately the same size (5-8mm). Even the radial streaks (representing the internal thickenings in *S. nitida*) that are used to differentiate between the two species can be ambiguous, as shells may have been damaged and repaired throughout growth, resulting in a white repair line in *H. complanatus* similar to those seen in *S. nitida* (e.g. Figure 1.3).

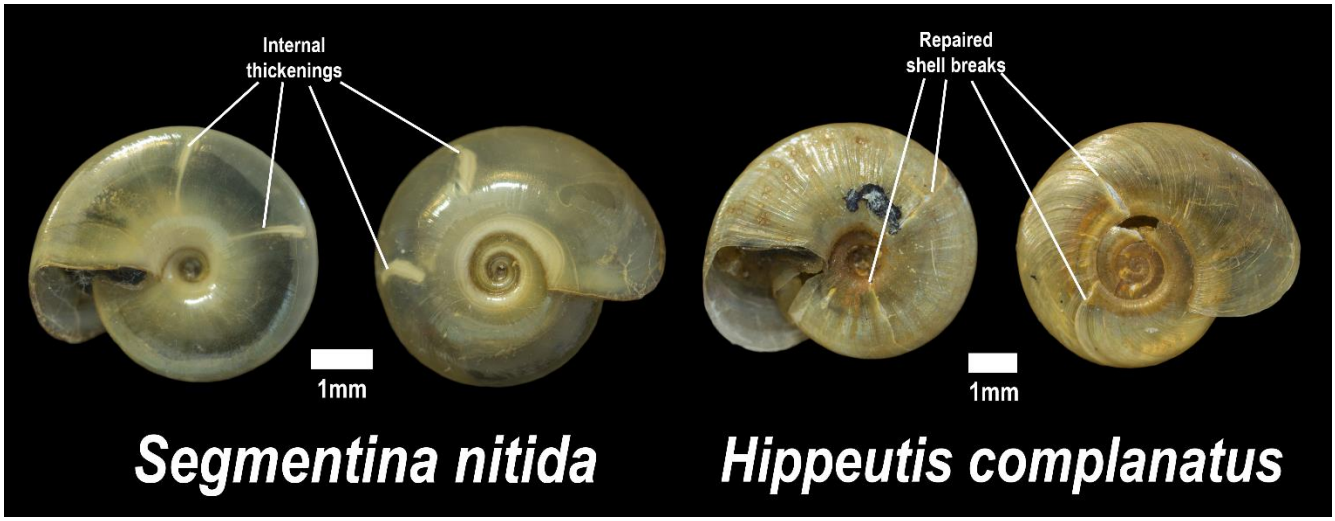


Figure 1.3. *Segmentina nitida* and *Hippeutis complanatus* individuals showing characteristic white internal thickening showing through the shells of *S. nitida*, and repaired areas of the shell on *H. complanatus* which can be mistaken for internal thickenings for identification.

The radial streaks were initially described as repairs of fractures by Müller (Reeve, 1863) and were seen to give the appearance of dividing the body whorl into segments, hence the name of the genus (*Segmentina*). These thickenings are not seen in individuals less than 1.2mm in breadth (Hill-Cottingham 2004).

The shell is described as dextral (the shell coils in a clockwise direction when viewing from the spire surface, with the aperture on the right). This means that the further projecting lip of the aperture protrudes over the animal's head, which makes it easier to obtain purchase on the substrate over the shorter, lower lip (Hill-Cottingham, 2004). In laboratory rearing, *S. nitida* is only observed with the shorter lip facing towards the substrate, when crawling along a surface (pers. obs.).

The body of *S. nitida* is a dark red colour, due to the presence of haemoglobin (Hill-Cottingham, 2004). Its tentacles are long, thin and mobile, with eyes proximal at their bases. Its movement is described as 'smooth and gliding' with the ventral side of the shell held flat against the substrate, and *S. nitida* moves rapidly when compared to other snail species (Reeve, 1863). Due to the thin nature of the shell, flesh and internal organs can be seen through the shell, with the beating heart usually visible, especially in smaller individuals.

In 2004, Hill-Cottingham observed in tank experiments that *S. nitida* feeds on *Lemna trisulca* (Ivy-Leaved Duckweed). It was hypothesised that individuals were feeding on epiphytic micro-algae on the underside of the leaves of the duckweed (Hill-Cottingham 2004). *S. nitida* has also been found to be predated upon by the leeches *Glossiphonia heteroclite, Dina lineata,* and *Erpobdella octooculta* in both lab experiments and the field (Hill-Cottingham 2004; Książkiewicz and Gołdyn 2008).

It has been reported that *Segmentina nitida* is ovoviviparous (Hill-Cottingham 2004), meaning eggs hatch within the body of the snail and juveniles are born live, though this is likely incorrect. In historical work, the egg masses have been described as a small oval-shaped capsule with a terminal tail,

containing up to 11 eggs (Bondesen, 1950). The eggs within these masses are particularly large, and arranged in 1-2 rows, occasionally overlapping (Bondesen, 1950). Gittenberger *et al.* (2004) report egg masses of 8 eggs in a gelatinous ball, which are described as free-floating. In addition to this, work by (Piechocki, 1979) has also supported the existence of egg masses for *Segmentina nitida* and states that they range between three and eight eggs in size.

1.2.3. Life Cycle of *Segmentina nitida*

The full life cycle and longevity of the species, especially in captivity, have not been studied in depth. Hill-Cottingham (2004) set up a laboratory breeding experiment, however the experiment only ran for a short time before all individuals expired due to overheating. However, in the time the experiments ran, it was found that the species is hermaphroditic, as tanks with single individuals produced young snails after several weeks, ranging from seven to 22 juveniles (Hill-Cottingham 2004). Whether this could have been from introduced egg masses on vegetation, or females storing sperm (with some freshwater snails able to store sperm for approximately two months (Nakadera *et al.,* 2014)) was not discussed. In 1975, Young reared *Segmentina complanata* in both calcium-rich and calcium-poor conditions in glass aquaria (due to repeated changes in nomenclature, it is unclear whether the species reared represents *Segmentina nitida* or *Hippeutis complanatus*). In calcium-poor conditions (3.7mg/L Ca), the individuals did not last more than two months, with no reproduction. In the calcium-rich treatment (55 mg/L Ca), the species completed its life cycle (young to young) although the time taken for this was not recorded. There are no data outside of this study for the life span of the species, although it is thought to be between one and two years. Through laboratory rearing of the species, more specific data on longevity and fecundity of the species can be obtained, informing potential reintroduction projects of *Segmentina nitida*.

In a study conducted in Poland, it was found that *S. nitida* undergoes three separate, reproductive events in the wild. These occur at the beginning of April, May, and June. It is assumed that these three events coincide with the occurrence of three generations (Książkiewicz and Gołdyn, 2008). From this,

the reproductive cycle of a single generation is thought to last approximately four weeks (with snails living through the reproductive event), resulting in three successive generations each with one reproductive event, with partial overlap between generations (Książkiewicz and Gołdyn, 2008). However, as explored in section 1.1.3.2, many planorbids have variations in life cycle under different conditions and in different countries, so the information from Poland may not apply to populations elsewhere, including the UK. Moreover, the population of *S. nitida* studied was found in a semi-permanent habitat, and the large number of reproductive events compared to other planorbid species may have been an adaption to the temporary nature of the habitat (Książkiewicz and Gołdyn, 2008).

Segmentina nitida aestivate (dormancy/hibernation) in the winter months (Książkiewicz and Gołdyn, 2008), with only large adults surviving into aestivation. When conditions become more favourable for the individuals (greater light, warmth, re-filling with water), snails emerge from aestivation ready for reproduction immediately.

1.2.4. Habitat of *Segmentina nitida*

Segmentina nitida favours shallow freshwater ditches choked with a rich, diverse flora (Watson and Ormerod, 2004b) and is found in unpolluted, usually calcareous water (water with high levels of calcium carbonate). *Segmentina nitida* is often associated with a rich variety of freshwater molluscs and gastropods (Clark 2011; Ormerod *et al.,* 2010), including other rare species, and is an indicator of species richness, composition and conservation importance of sites (Ormerod *et al.,* 2010).

Populations of *S. nitida* are associated with grassland regions used for traditional grazing, where there are low levels of both phosphate and nitrate in the water (Hingley, 1979; Killeen and Willing, 1997). The species is predominantly found in drainage ditches on the margins of grazing marshes (Kerney, 1999) and often found in dense emergent vegetation. Drainage ditches undergo cyclical changes to their structure over time when plant life and detritus are removed to aid drainage and vegetation subsequently returns. *Segmentina nitida* is regularly found in brackish, shallow water and standing water and is tolerant to some desiccation (Hill-Cottingham 2004).

Drainage ditches are relicts of a once extensive habitat (Watson and Ormerod, 2004a). They occur predominantly on lowland wetlands, often found in river valleys with impeded drainage. Drainage ditches require periodic flooding, high water tables and sensitive catchment management (Jefferson and Grice, 1998; Jenman and Kitchin, 1998). Drainage ditches surrounding agricultural or grazing land facilitate the artificial removal of water from land, and are key in wetland reclamation, erosion prevention, and are associated with irrigation of farmland (Herzon and Helenius, 2008). They are generally considered an enhancement of agricultural land use as they improve the timing and efficiency of farming operations (Herzon and Helenius, 2008). They form a link between farmlands and larger water bodies such as lakes and canals (Janse and Van Puijenbroek, 1998). Many ditches also serve the purpose of transporting water to the fields during dryer periods. A typical surface drainage system consists of field drains, ditches, a main collection ditch, and an outlet (Herzon and Helenius, 2008). The structure of the vegetation in ditches is greatly influenced by the hydroseral succession that is a product of the dredging of silt from the ditch (Caspers and Heckman, 1981; Watson and Ormerod, 2004b)

Figure 1.4. Typical UK drainage ditch alongside grazing marshland

Drainage ditches are dynamic, temporary habitats, which change by a process of ecological succession (Clark 2011) known as hydroseral succession. Hydroseral succession is a model of plant succession from wet to dry where a water body fills with sediment and detritus, which forms a marsh, fen or bog habitat, and climaxes with mature upland or forest (Tutin 1941, Clark 2011). In hydroseral succession, the flora and fauna are representative of the seral stage (Painter, 1999). *Segmentina nitida* tends to favour drainage ditches that are in the late stages of hydroseral succession with little or no flowing water (Killeen, 1996; Watson, 2002; Watson and Ormerod, 2004a; Clark, 2011). Clark (2011) reports that in each of the ditches *S. nitida* was found to inhabit in her study, the habitat was classed as Stage 4 (bog/marsh habitat with emerged plants) according to seral stages described in Jeffries and Mills (1990). Stage 4 is characterised by the presence of indicator plant genera such as *Oenanthe* (Water-dropworts), *Carex* (Sedges) and *Apium* (Fools Water-Cress). However, Stage 4 habitats also contain indicator plants of Stage 3 hydroseral succession, such as *Typha* (Bulrush) and *Juncus* (Rushes). Stage 4 indicator species were also found in habitat surveys of *S. nitida* conducted by Watson and Ormerod (2004a, 2004b).

The south eastern lowlands of the UK and associated drainage ditches are much richer in mollusc species than the non-calcareous highlands of the north and west of the UK (Kerney, 1999). Drainage ditches, unlike larger water bodies such as rivers and lakes, are subject to intense exchanges of both matter and organisms from the surrounding terrestrial environments (Herzon and Helenius, 2008). They are also comparatively shallow, with seasonally fluctuating water levels, resulting in an increased probability of drying out. To counter this, they must be regularly maintained and managed to continue efficient drainage (Foster *et al.*, 1990).

As well as serving important hydrological functions, drainage ditches have important ecological functions. Most importantly they provide habitats for many plant and animal species (Janse and Van Puijenbroek, 1998). In addition to this, the ditches can act as a source of drinking water for cattle. Due to the shallow nature of drainage ditches they are often dominated by macrophytes. In the UK,

drainage ditches are not a species rich environment; however, they do support uncommon species that are generally not found in other, larger water bodies (Williams *et al.*, 2003). Drainage ditches are among the richest habitats for molluscs in the United Kingdom. They support at least 70% of all known freshwater molluscs in the UK (Watson, 2002) and provide valuable wet vegetated non-cropped habitats (Herzon and Helenius, 2008).

In the UK*, Segmentina nitida* occurs frequently in waters with high alkalinity and high conductivity, as well as high concentrations of calcium and chlorides (Watson and Ormerod, 2004a; Książkiewicz and Gołdyn, 2008). *Segmentina nitida* is classified by Boycott (1936) as a calciphile species, restricted to freshwater habitats where calcium levels are >20 mg/L^{-1}. *Segmentina nitida* is absent from otherwise suitable ditches that have elevated levels of nitrates and nitrites, indicating a susceptibility to eutrophication (Watson and Ormerod, 2004a). *Segmentina nitida* has also been shown to be present in ditches with a significantly higher mean chloride concentration ($103mgL^{-1}$) than the UK freshwater average ($8.3mgL^{-1}$) (Watson and Ormerod, 2004a). This may be because the species requires a higher ionic concentration for osmotic regulation (Watson and Ormerod, 2004a).

There are differences in the habitats in which *S. nitida* is found in Europe and those in which they are found in the UK. In Poland, *S. nitida* inhabits temporary ponds in the middle of agricultural land (Książkiewicz and Gołdyn, 2008), with little floating vegetation, including *Lemna* spp. Some of the most abundant populations of *S. nitida* in Poland are found in small ponds choked with dead and decaying leaves from the surrounding trees, with no live vegetation (Gołdyn pers. comm.). These habitats regularly dry out during the summer, leaving *S. nitida* in aestivation, and during this time, individuals are often found in dense aggregation, often at the deepest point of the pond (Gołdyn pers. comm.). This is thought to be a behavioural adaption, allowing the snails to find mating partners quickly when the pond refills with water during the spring or autumn months to re-establish a population quickly (Dussart pers. Comm.). In Germany, *S. nitida* tolerates low pH environments, unlike UK records (Zettler *et al.*, 2006), otherwise the habitats are similar.

1.2.5. Distribution and Decline of *Segmentina nitida*

In the 19th century, *S. nitida* (then known as *Planorbis nitidus*) was reported to be common in ponds in London and found in Ireland and south Scotland (Reeve 1863). Since then, there has been a significant decline in the distribution of *S. nitida* in the UK, most notably in the last 50 years. *Segmentina nitida* is not often found in the north or west of England, Wales, and Scotland as these regions have softer water the species cannot survive in (Macan, 1977). Since 1965, *S. nitida* has only been found at a few sites in Suffolk, Norfolk, East Kent, and East Sussex and is now extinct across most of England, even in locations where it was previously recorded as common (Kerney, 1991b). Throughout the 20th century there has been an 80% decline in the range of *S. nitida* (Kerney, 1999). This has been attributed to over-frequent mechanical ditch clearance, eutrophication from fertiliser run- off and the changing of land use from grazing to arable farming with the associated lowering of the water table and increase in nutrient pollution from fertiliser run-off (Wells and Chatfield, 1992; Hill-Cottingham, 2004).

Segmentina nitida was included in the IUCN Red Data Book 3 (Invertebrates) and was described as 'Endangered'. However, in 1994, the guidelines for assessment of species on the Red List were changed, meaning *S. nitida* lost its previous status. *Segmentina nitida* has not been reassessed since this change in guidelines. *Segmentina nitida* is also included on the UK Biodiversity Action Plan (BAP). It is listed as a priority species, being a species 'which has faced marked declines, with the causes for this decline either ongoing or unknown' (JNCC, 2010b). Four action points are identified in the BAP for *S. nitida* to aid in the conservation of the species: 1) appropriate management of habitats known to support the species, with focus on ditch clearing frequency and severity, water quality stability, and adjacent land usage; 2) research into environmental factors at sites supporting *S. nitida* and post ditch clearance recovery; 3) regular monitoring of populations; 4) research into colonisation and translocation methodologies to increase extent of habitats occupied by *S. nitida* (JNCC, 2010b).

However, outside of this description, there is no protection currently assigned to *S. nitida* and it has not been reassessed for the current IUCN Red Data Book. In 1992, Wells and Chatfield collated the conservation status and level of threat to *S. nitida*. This was the last large-scale assessment of the status of *S. nitida* across Europe. The authors list the 19 countries with a reported presence of *S. nitida* and how endangered the species is in each (Table 1.1).

It appears that the distribution of this species in the UK is limited by habitat loss rather than temperature, as it is found considerably further north than it is in Britain in Scandinavia and the Baltic countries (Table 1.1). Even in the countries where it has been described as 'non-threatened' it is restricted to fragmented pockets of wetlands and marshes which are under constant threat of isolation, drainage, and pollution.

Since Wells and Chatfield (1992) compiled information on the status of *S. nitida*, there has been very limited reporting of data for the species, both in the UK and throughout Europe. This lack of data has made it difficult to reassess *S. nitida* for the IUCN Red List under new guidelines (post-1994). The most recent records of *S. nitida* in the UK are from a 2011-12 survey of marshlands in east Kent conducted by the Kent Wildlife Trust. This work was undertaken to re-assess as many ditches as possible of those surveyed in a 1999 report by Ian Killeen for Natural England (Killeen, 2000), and to expand on the survey. The Kent Wildlife Trust survey (2012) found that *S. nitida* was present in 30 of 131 surveyed ditches, in comparison to the 48 of 104 surveyed ditches in the 1999 survey (Killeen, 2000).

One of the issues that *Segmentina nitida* may be facing is that it occupies a very specific ecological niche. In several studies, *S. nitida* has been found to favour ditches of late stage hydroseral succession (Watson and Ormerod, 2004a, 2004b; Clark, 2011). Succession is a continuous event, and it typically requires careful management to maintain a specific stage of succession (Clark 2011). As drainage ditches become more choked with dense vegetation, they start to lose their functionality for farmers, namely draining water from the surrounding land. To counter this, landowners must repeatedly dredge and clear the drainage ditches surrounding their land.

Table 1.1. Level and threat and conservation efforts for *Segmentina nitida* in 17 countries throughout Europe, adapted from Wells and Chatfield (1992).

Country	**Level of Threat**	**Conservation**
Austria	Endangered	Listed in Red Data Book (RDB)
Belgium	Uncommon, rare in Ardennes	None
Bulgaria	Unknown	Unknown
Czech Republic	Unknown	None
Denmark	Not Threatened	None
Finland	Not Threatened	None
France	Vulnerable	None
Germany	Rare/ Vulnerable	Listed in RDB and Lists for the West, Hessen, Bavaria, Baden-Württemberg, and Nordrhein-Westfalen
Great Britain	Endangered	Occurs in five sites of special scientific interest (SSSI)s and one National Nature Reserve (NNR); listed in RDB
Hungary	Not Threatened	None
Italy	Indeterminate- Information lacking.	None
Liechtenstein	Unknown	None
Netherlands	Not Threatened	None
Norway	Rare	Only known locality is a reserve
Poland	Not threatened	None
Romania	Unknown	None
Sweden	Rare	Listed on national list of threatened species; data sheet compiled for National Swedish Environment Protection Board
Switzerland	Vulnerable	Listed in RDB
Russia (USSR)	Probably not threatened	None

1.2.6. Habitat Loss

Hydrological management has led to significant losses and changes to grazing marshes throughout the UK (Watson and Ormerod, 2004a). Between 1930 and 1980 they declined in area by 64% in the Greater Thames Basin, 48% in the Romney Marshes, and 37% in the East Anglican Broadlands (HMSO, 1995; RSPB *et al.,* 1997). The once extensive network of drainage ditches is now facing fragmentation and losses, which in turn is threatening relict communities of organisms that were once widespread (Jefferson and Grice, 1998; Drake, 2004). Many European countries are replacing open surface drainage ditches with more efficient and easier to maintain subsurface piping (Herzon and Helenius, 2008; Stoate *et al.*, 2009). Drainage ditches are also being increasingly threatened by conversion of grassland to arable land, and nutrient enrichment through run-off from agricultural land (Driscoll, 1985; Hicklin, 1986; Palmer, 1986; Williams and Hall, 1987; Janse and Van Puijenbroek, 1998).

Nutrient enrichment, or eutrophication, is a crucial factor affecting drainage ditches as it can cause significant changes to their ecology. Eutrophication results from increased inputs of nitrates and phosphates to a water system. The increased phosphate levels have been found to increase the biomass of floating plants, while leaving rooted plants largely unaffected (Roelofs *et al.*, 1984; Daldorph and Thomas, 1991). Increased nitrates cause algal blooms, which cause submerged plants to lose biomass (references in Daldorph and Thomas 1991).

Drainage ditches with moderate eutrophication are often characterised by dominant submerged vegetation and phytoplankton blooms in spring (Veeningen, 1982). Further eutrophication can also stimulate filamentous and/or epiphytic algal blooms (Janse and Van Puijenbroek, 1998). These blooms decrease light availability, which causes a shift from species with vertical growth strategies to species with a horizontal growth strategy (Bloemendaal and Roelofs, 1988)

When there is very high nutrient loading present in a drainage ditch, a surface layer of pleustophytic plants (plants that are not attached and float freely in the water) (Symoens, 1988) dominates the vegetation, such as duckweeds (Lemnaceae), while the submerged plants disappear. This results in all

the oxygen produced by the vegetation to be released into the atmosphere instead of the water. Combined with decomposition continuously extracting oxygen from the water, this means water can become anoxic and mineralisation occurs mainly anaerobically (Marshall, 1981; Portielje, 1994; Janse and Van Puijenbroek, 1998). This leads to the loss of aerobic life in the ditch and anaerobic species becoming dominant. This is then followed by a replacement of macrophytes by phytoplankton (References in Daldorph and Thomas 1991) and reduction in aquatic vegetation overall (Zhang *et al.*, 2016).

Pulmonate snails are associated with freshwater macrophytes (References in Daldorph and Thomas 1991); therefore, the collapse of a macrophyte community causes marked declines in pulmonate snail communities (Daldorph and Thomas 1991). In a study by Daldorph and Thomas (1991), in experimental enclosures in drainage ditches, numbers of snails declined significantly as phytoplankton chlorophyll-values increased due to increased phosphate and nitrate loading. This may be partly caused by the reduction of available resources such as epiphytic algae, oxygen supply, and access to the surface caused by the death of macrophytes (Thomas, 1987, 1990; Thomas and Daldorph, 1994).

In addition to the lack of macrophytes, snails can also be affected by toxins released by phytoplanktonic algal blooms (Carmichael, 1980). These blooms can also cause low levels of oxygen during the night, and increased pH levels during the day. This can enhance the toxicity of excretory ammonia, further harming the snails (Green *et al.*, 1986)

1.2.7. Management of Drainage Ditches

Drainage ditches require regular maintenance, such as the removal of vegetation and detrital layers, to ensure water flow (Janse and Van Puijenbroek, 1998). Clearance of drainage ditches was traditionally done by hand, but in recent years there has been a shift towards less labour-intensive mechanical methods of clearing (Van Strien *et al.*, 1991).

A study by Van Strien *et al.* (1991) demonstrated that regular cleaning of ditches resulted in a lower floristic richness, with an optimal frequency of clearing (for species richness) of every 2-3 years. Over-frequent clearing of drainage ditches reduces species richness and favours the growth of a few common species of vegetation and invertebrates. Whilst annual clearing of ditches provides better drainage, it does result in lower vegetative richness, due to smothering of vegetation, damage to plant life, and a high supply of nutrient-rich sludge from the benthos (Van Strien *et al.*, 1991). However, under-frequent clearing of these banks (under once every three years) also resulted in a lower species richness, and this reflects the effects of succession (Van Strien *et al.*, 1991).

For effective management of drainage ditches, it is generally thought that the best approach is a dynamic management regime, providing vegetation at various stages of hydroseral succession. This requires rotational vegetation clearing and removal of silt, retention of vegetation and refuges, and management conduction outside of key breeding seasons (Foster *et al.*, 1990; Twisk *et al.*, 2000; Defra, 2002). Harvesting the plant biomass from a ditch can reduce the amount of phosphorus released during the dormant season (Osborne and Kovacic, 1993)

1.2.8. Conservation of *Segmentina nitida*

The conservation of *S. nitida* is best achieved through the management of habitat (Killeen, 2000; Watson and Ormerod, 2004a; JNCC, 2010b; Ormerod *et al.*, 2010). As a result of a survey for *S. nitida* in the Kent marshland in 2012 by Kent Wildlife Trust (KWT), a series of ditch management recommendations was released to landowners (Table 1.2). These recommendations were developed to preserve the molluscan assemblages contained within and aim to aid the continued survival of *S. nitida*.

Table 1.2. Recommended management of waterways containing *Segmentina nitida* given by Kent Wildlife Trust (KWT) to landowners (Sadler 2012)

Never clearing entire ditches at any one time.
Clearing alternate 10-15m stretches of ditches, leaving 10-15m untouched lengths in between.
On wider ditches (over 4m wide), clearing from one side to the middle of the ditch, leaving the other half undisturbed.
Creating and leaving occasional ditch 'spurs' and side sections untouched for much longer periods than the main ditch to act as a reservoir for *S. nitida.*
Creating ponds to act as reservoirs for the snail, which are left undisturbed for at least 10-15 years, and never cleared all at once.

These recommendations have also been stated in the species specific Biodiversity Action Plan (JNCC, 2010b), emphasising the need for land owners to work with Natural England and the Environment Agency to regulate ditch management frequency.

In the report by Killeen in 2000, *S. nitida* was found in a number of ditches that fell outside of sites of special scientific interest (SSSIs) or land with ESA (Environmentally Sensitive Area) status, so management of these for the conservation of *S. nitida* would be hard to implement and coordinate.

The main recommendations of this study for the conservation of *S. nitida* were increased engagement with landowners, potentially through Countryside Stewardship (CS) schemes; monitoring programmes; as well as potentially limiting potential winter flooding at current *S. nitida* sites (specifically Stodmarsh National Nature Reserve) to minimise fluctuations in water levels and hydrochemistry (Killeen, 2000).

Despite all these recommendations, there is no legal protection for this species. Whilst some of its range lays within SSSIs, which confer some amount of protection, on private land there is no legally binding protection. The species has not been re-evaluated for the IUCN Red List since 1994. This is most likely due to the patchy knowledge of the status of the species throughout Europe, with limited-to-no records of the species from several countries, making it difficult to understand declines and trends of the species as a whole, rather than just in the UK.

1.3. Aims and Objectives of Study

The aims of this study were designed around two of the action points identified for the ongoing conservation of *Segmentina nitida* in its Biodiversity Action Plan (JNCC, 2010b), detailed in section 1.2.5., specifically the need for increased monitoring and surveying for the species (action point 3), and research on translocation and reintroduction (action point 4). Therefore, this study aimed to:

- Develop a quicker sample assessment method for field surveying of *Segmentina nitida* and compare the effectiveness to a traditional sampling method.
- Determine the optimal conditions for laboratory rearing of *Segmentina nitida.*
- Gather information on genetic structure and phenotypic adaptions of European populations *S. nitida* to inform potential reintroduction or translocation through:
    - *i.* Geometric morphometrics to understand ecological pressures and habitat differences that may be influencing phenotypic change in *S. nitida* populations.
    - *ii.* Population genetics of *S. nitida* to identify genetic divergence between populations.
    - *iii.* Comparison of genotypes of samples from both the UK and Europe to understand dispersal history and biogeographic patterns of *S. nitida*.

Chapter 2- Evaluating a quicker sampling technique for *Segmentina nitida* and other freshwater gastropods

2.1. Introduction

Monitoring natural populations is often an important and necessary step in improving management decisions for a species, and assessing its conservation status (Yoccoz *et al.*, 2001; Martin *et al.*, 2007). However, monitoring for species, especially those at low abundances or cryptic species, can be difficult. Whilst presence of a species at a location can be confirmed it is impossible to confirm a species' absence (Mackenzie, 2005; Mackenzie and Royle, 2005), as the sampling method may just have failed to detect the species whilst it is indeed there. One of the main issues with surveys of aquatic invertebrate assemblages in particular is that they can be biased if protocols are used inconsistently or inappropriately (Kerans *et al.,* 1992). Therefore, having appropriate and consistent methodologies in place is essential for surveying freshwater species, such as *Segmentina nitida*.

Over the last 20 years there have been numerous surveys of drainage ditches within marshes around the UK for *Segmentina nitida* and other gastropods (Killeen, 1996, 2000; Killeen and Willing, 1997; Hill-Cottingham, 2004; Watson and Ormerod, 2004a). Many of these surveys were undertaken to give insight into the current status of populations of *S. nitida* and *Anisus vorticulus* for the UK Biodiversity Action Plan (BAP) (JNCC, 2010b, 2010a) as well as monitoring for associated Red Data Book Species such as *Valvata macrostoma* (Kerney, 1991c) and *Pisidium pseudosphaerium* (Kerney, 1991a). Many of these surveys used different methods and covered different ranges of distribution, and this can make the results of these surveys difficult to compare.

Killeen and Willing (1997) and Killeen (2000) conducted surveys to find *S. nitida* in the east Kent grazing marshes. For these surveys, at each site ten scoops of the vertically distributed vegetation between the sediment and water surface were taken with a kitchen sieve. All samples were combined and tipped into a plastic sample container. Snail free vegetation (mainly *Lemna trisulca*) was removed from this container. A possible issue at this stage of the sampling process is that especially small snails, such as *S. nitida,* may be missed if hidden within the vegetation, thus not reported at the site or at an incorrect population size. In Killeen (2000), the vegetation was agitated at this stage to wash snails

from vegetation prior to removing it to counter this issue. Samples were then either examined in the field or preserved in 80% alcohol and examined in the laboratory. Numbers of snails were estimated using an ACFOR scale (Crisp and Southward, 1958) for each species (Abundant= ≥101 individuals, Common= 51-100 individuals, Frequent= 16-50 individuals, Occasional= 6-15 individuals, Rare= 1-5 individuals). This method of 'semi-quantifying' abundance of a species is a way to save time during surveying by removing the need to count every individual in a sample.

Watson (2002) surveyed for *S. nitida* and other gastropod species in the Arun Valley, Ouse Valley, Lewes Brooks, Pevensey Levels and the Stour Valley. Sediment, vegetation and water were sampled with a sieve net, and then preserved with 70% Industrial Methylated Spirits (IMS) for identification of snails in the laboratory with a light microscope.

Hill-Cottingham (2004) sampled specifically for *S. nitida* on the Catcott North Reserve in Somerset. This study focussed on a single ditch with repeated sampling throughout the year. Unlike Watson (2002), her method avoided sampling sediment by only sampling the floating and submerged vegetation with a sweep net. She then went through all vegetation by hand, using site water and agitation to wash off smaller snails from vegetation in a white plastic tray. This method was based on that described by English Nature on the Somerset Levels and Moors as the standard method for environmental contracts (Hill-Cottingham *et al.*, 1991) and did not involve preservation of snails in ethanol. This method was considered to have less of an impact on populations within sampled ditches, as extensive destructive sampling could reduce the number of snails and/or the gene pool of isolated populations in an unsustainable way, especially when surveying for rare species and in locations in which intensive research is being conducted (Jocque *et al.*, 2010).

In 2012, Kent Wildlife Trust surveyed the East Kent Marshes for *Segmentina nitida* and other associated freshwater molluscs (Sadler, 2012). This study involved similar methods to those used by Killeen and Willing (1997), sampling at ten locations per ditch, avoiding the ends of the ditch, and

sorting through all vegetation in white trays to find gastropod species, and then returning snails and vegetation to the ditch.

Segmentina nitida has been observed detaching from vegetation and falling through the water column when vegetation is agitated in water, and this behaviour has been used to help distinguish it from the highly similar species *Hippeutis complanatus,* which does not display this behaviour (Hill-Cottingham, 2004). During sampling of East Kent drainage ditches in 2015 by the author, it was also observed that when an entire sample (plant life and water) was kept in a container whilst the vegetation was sifted through in a white tray in smaller portions, the greatest number of snails was found in the water at the bottom of the container, rather than in the vegetation being removed.

Exploiting a specific behavioural trait of a target species in field sampling can potentially increase detection rate and decrease the amount of time needed to survey. The latter point is very important, as a less time-intensive sample assessment method can facilitate an increase the scope and range of an ecological survey targeting a specific organism. For example, Hill-Cottingham *et al.* (1991) found that species of *Pisidium* were mostly found in sediment at the bottom of a ditch, so targeting the species by focusing on the sediment rather than labour-intensively sifting through a sample including vegetation is likely to be more time effective.

Developing effective sampling protocols and sampling design for species is important to conservation (Carlson and Schmeigelow 2002; Azevedo *et al*., 2013; Bosch *et al*., 2017). One of the key ways of assigning management and conservation effort to species is the International Union for Conservation of Nature (IUCN) Red List. Red Lists are used to assess the risk of extinction of a species (Mace *et al.*, 2008), ranging from Critically Endangered to Least Concern. The IUCN Red List is an important tool in conservation efforts (such as raising awareness of imperilled species), enables setting of priorities for protection, influences environmental policies and legislation and is effective at enabling the monitoring of biodiversity and species trends (Gärdenfors *et al.*, 2001; Baillie *et al.*, 2008; Mace *et al.*, 2008; Martín-López *et al.,* 2011)

There is currently a considerable taxonomic bias in the assessment for species listed on the IUCN Red List. Around 90% of known mammalian, avian and amphibian species have been assessed, whilst only ~3-4% of molluscan species have been assessed (Baillie *et al.*, 2008; Cardoso *et al.*, 2011). As of 2017, 46,092 vertebrates were assessed for the IUCN Red List, compared to only 21,130 invertebrate species (IUCN, 2017). An important aspect of the IUCN Red List, as well as biodiversity conservation in general, is understanding trends in the distribution, range and abundance of species (Gaston, 1994; Baillie *et al.*, 2008). Unfortunately, the monitoring of populations of rare species can be challenging, especially in aquatic environments, due to their dynamic nature and three-dimensional complexity (Stork *et al.*, 1996). For invertebrates, accurately measuring the abundances of species can be particularly difficult (Cardoso *et al.*, 2011). Invertebrates as a whole are important for conservation, as they are often the first to experience population declines and become extinct due to disturbance and habitat loss (Cardoso *et al.*, 2010). An effective sampling procedure that quickly and efficiently estimates the abundance of freshwater gastropod species could therefore support the development of accurate distribution ranges for IUCN Red List assessment of these species, at least at a regional level (Maes *et al.*, 2012). Gastropod diversity can also be used as an indicator of various environmental factors, such as the impact of anthropogenic disturbances (Lange *et al.,* 2013), hydrological gradient (Ilg *et al.*, 2012), and eutrophication levels (Karydis and Tsirtsis, 1996; Janse and Van Puijenbroek, 1998; Rosset *et al.*, 2014), amongst others.

The aim of this chapter was to develop and evaluate a modified (hereafter referred to as 'quicker') method for assessing samples of gastropod invertebrates from standing freshwater habitats by combining the method of agitating vegetation in a sample (Killeen, 2000; Hill-Cottingham, 2004) with the method of removing plant life from the sample (Killeen and Willing, 1997). The quicker method was compared to the traditional method of sampling (Hill-Cottingham *et al.,* 1991; Sadler, 2012) that involves sifting by hand through all the vegetation in a sample. Development of this quicker method was aimed at facilitating the required action of increased surveying and monitoring of populations of *S. nitida* in the UK, as raised in its Biodiversity Action Plan (JNCC, 2010b).

First, the quicker method was compared to the traditional method by evaluating its effectiveness in detecting *S. nitida* and other gastropod species in samples. To this end, samples taken from ditches were surveyed by washing the vegetation in water twice and then sorting through the vegetation, recording gastropod individuals at each stage. The first wash represented the quicker method of assessing the sample (one wash only), both washes combined with the vegetation represented the traditional method of surveying (full sample). The hypotheses tested were that (i) the quicker sample assessment method (wash 1 only) detects as many *S. nitida* as the traditional method (wash 1+ wash 2+ vegetation); (ii) the quicker sample assessment method reports the same number of gastropod species, individuals and species' richness (Menhinick's Index) for sampled sites as the traditional method, and (iii) the quicker sample assessment method reports comparable ACFOR scores for species to those given for species using the traditional sample assessment method.

Second, the quicker method was compared with the traditional method when used by an experienced and inexperienced surveyor. In this experiment, the length of time it took to complete a sample using each method was also compared. The hypotheses tested were that (i) TIME- the quicker sample assessment method (wash 1 only) takes as long, or less time, than the traditional method (wash 1+ wash 2+ vegetation) per sample, for both experienced and inexperienced surveyors; (ii) DETECTION RATE- the quicker sample assessment method detects *S. nitida* as frequently as the traditional sample assessment method for both experienced and inexperienced surveyors; and (iii) ABUNDANCE- the quicker sampling technique reports the number of gastropod individuals and different gastropod species as well as the traditional sampling method.

2.2. Materials and Methods

2.2.1. Field sites for the testing of quicker sample assessment method

Field sites were chosen to include sites that contain both large and small populations of *S. nitida,* based on the most recent survey of *S. nitida* conducted by Kent Wildlife Trust (Sadler 2012), as well as a preliminary survey by the author in 2015. For the experiments in this chapter, the Ash Level and

Preston Marshes were sampled, as they contained broadly similar habitats (ditches surrounded by grazing land) and had records of *S. nitida* in 2012 (Sadler, 2012).

Preston Marsh

National Grid Reference: TR 234605

Size: 42.9ha

Preston Marsh is located in Preston Valley, and is to the east of the Little Stour river. The majority of the site consists of sheep-grazed grassland, however there are some areas of cattle-grazing and arable farmland (Killeen 2000). Fields are surrounded by drainage ditches in a late stage of hydroseral succession.

Preston Marsh was designated as a Site of Special Scientific Interest (SSSI) in 1984, due to the presence of populations of the Red Data Book plant, *Potamogeton acutifolius,* and it is the last remaining area of fen vegetation in the Little Stour Valley. The Preston Marshes contain two habitats, lowland neutral grassland, and lowland fen, marsh, and swamp. This area is regarded as an 'unfavourable habitat that is recovering' (Natural England, 2017).

The Ash Level

National Grid Reference: TR 292628

Size: 1200ha

The Ash Level is to the south of the River Stour, in East Kent, in the Richborough area. Much of the land in this area is used for intensive farming for a range of crops. The drainage ditches surrounding these fields have previously shown signs of algal blooms and eutrophication, probably due to run-off from the fields (Killeen and Willing 1997). These ditches have also been found to show a low diversity of molluscan life (Killeen and Willing 1997).

In the north-east of this area there are small areas of grazing marshland. The drainage ditches surrounding these areas here are minimally managed, allowing a late stage of hydroseral succession. These diches are generally shallow and choked with a rich diversity of flora dominated by *Hydrocharis sp., Lemna trisulca,* and *Berula erecta* (Killeen 2000).

There are a number of historical records for *S. nitida* in this region, however there is currently no protection for the habitats contained within, unlike the SSSIs of Preston and Stodmarsh. It was previously recommended that the areas containing *S. nitida* be designated as a SSSI (Killeen and Willing 1997).

Sampling was conducted during 2016 in May and July-August. This allowed an assessment of whether seasonal variations on gastropod abundance and community vegetation density and composition influenced the accuracy of the proposed quicker sample assessment method, to see if dense vegetation would affect the efficacy of the washing technique. These time periods were chosen to coincide with one of the spring reproductive events and just after the summer breeding event of *S. nitida* (Książkiewicz and Gołdyn, 2008).

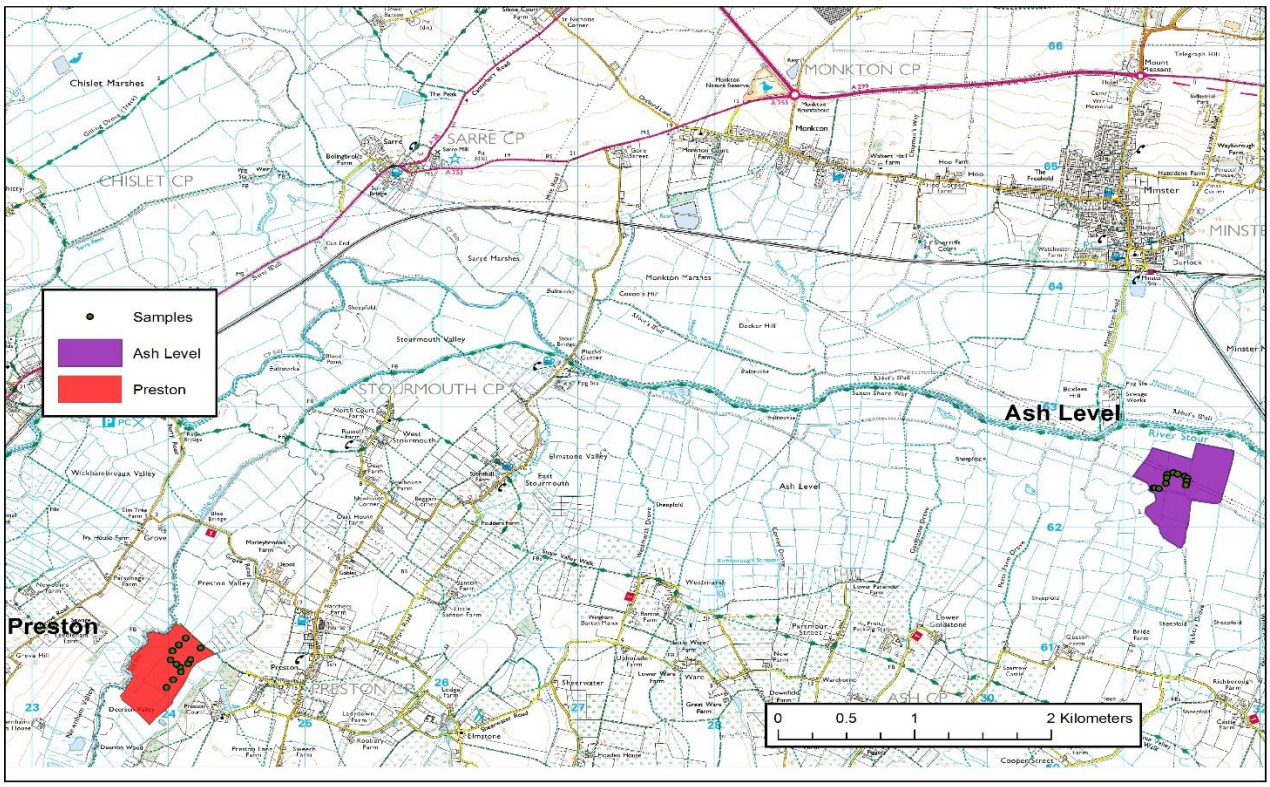


Figure 2.1. Map showing locations of samples and boundaries of sites in the Ash Level and Preston Marshes, sampled in 2016.

2.2.2. Sampling procedure

2.2.2.1. Comparing quicker and traditional sample assessment method for number of gastropod species, number of gastropod individuals and number of *S. nitida* individuals

Samples were collected from each site by sweep netting the ditch for 15 seconds at each sampled location using a 250mm diameter sweep net with 1mm mesh. The area sampled included submerged, floating and emergent vegetation up to 0.5m from the bank. Sampling was undertaken at three locations for each drainage ditch, the centre of the ditch and at each end of the ditch (roughly ¼ distance of the ditch at each end). Four ditches were sampled per site (Preston Marsh and the Ash Level) and three samples were taken from each ditch, resulting in 24 samples in total for eight ditches at each sampling time (May and August).

Figure 2.2. Fieldwork equipment used in the surveying of *Segmentina nitida*. L-R: White tray, ice cube tray for isolating individual specimens for identification, buckets for washing vegetation, sweep net, lidded container for transportation of samples to lab.

Figure 2.3. Typical drainage ditch at Preston Court, with dense emergent vegetation, and very little open water.

Global Positioning System (GPS) coordinates were recorded for each location using a Garmin eTrex 10. Each sample was transferred to a 10L bucket and site water was added to approximately 5L to facilitate the washing step of the quicker sample assessment method. Water from the ditch was then washed through the pond net into the bucket and the net was examined to ensure all snails and vegetation were transferred to the bucket.

The sample was then processed in three stages, comprising two washes of the vegetation in each sample and identification of the snails washed from vegetation after each wash, followed by identification of all snails remaining in the vegetation of the sample after the two washes. For the first wash the vegetation was vigorously agitated in the water in the bucket for 30 seconds and left to settle for five minutes, to allow snails to sink to the bottom of the bucket. The vegetation was then transferred to a second identical 10L bucket with approximately 5L of ditch water, agitated vigorously for 30 seconds and left to stand for five minutes. The water in the first bucket was then poured into white plastic trays (30cm x 22cm) in portions and all gastropods identified down to species level. This represented the record for 'wash 1'.

The vegetation in the second bucket was then removed and transferred to a third bucket filled with ditch water. The water in the second bucket was sifted through and gastropods were identified as described above for 'wash 1'. This represented the record for 'wash 2' For the third bucket, both the water and vegetation were transferred to a white plastic tray in portions and examined for gastropods, which were recorded as described above. This represented the record for 'vegetation'.

2.2.2.2 Comparison of ACFOR scores for *S. nitida* and associated gastropods obtained using quicker and traditional method

ACFOR scores for gastropod species were assigned according to the scale used in Killeen and Willing (2002) (A= 101+ individuals, C= 51-100 individuals, F= 16-50 individuals, O= 6-15 individuals, R= 1-5 individuals). The ACFOR scale has been used in surveys to avoid the need to count every individual

(with some samples containing around 2000 individuals), whilst still giving precise data for species with low densities (Killeen and Willing, 1997).

The ACFOR score was determined for each species in the first wash of each ditch (hereby referred to as 'wash 1 ACFOR'), and for both washes and the vegetation combined ('total ACFOR'). Wash 1 ACFOR scores were then compared to total ACFOR scores to identify any ditches where they did not match for *Segmentina nitida*, as well as for all other species.

2.2.2.3. Sampling time efficiency comparison of quicker method versus traditional method

Samples were taken at each site (Ash Level and Stodmarsh) by an experienced surveyor with the same sweep net method as described above, and then divided approximately into halves between two surveyors, one experienced (the author) (with experience in assessing >50 ditch samples for gastropod species) and one a novice (with no experience is surveying ditch samples for gastropod species) in relation to the identification of freshwater gastropods. Each surveyor then examined their sample using either the traditional method or the quicker method described above.

Each surveyors identified all gastropod individuals they collected to species level using the freshwater gastropod key by Hill-Cottingham (2008). This key was chosen as it is very simple and easy to use for inexperienced surveyors. Every five minutes the number of individuals and new species found during that time were recorded for both the experienced and inexperienced surveyor. After 30 minutes, sampling was stopped and for any samples not completed it was estimated what proportion of the sample had not yet been assessed.

The order in which the methods were used (quicker and traditional) was alternated between samples, to minimise the error caused by the experience gained by the novice surveyor from one sample to the next.

2.2.3. Data Analysis and Statistics

All data analysis was carried out in Statistics Package for the Social Sciences (SPSS) (IBM, 2017) unless otherwise stated.

Cumulative species graphs for wash 1 & 2 and the vegetation were produced for each ditch across the two sites (Ash Level and Preston) to assess how many new gastropod species were detected in each of the three steps of the procedure (wash 1, wash 2, vegetation). Separate cumulative graphs were produced for *Segmentina nitida* individuals in each of the ditches across the two sites.

Menhinick's index (Menhinick, 1964) is a diversity index that describes species richness in a sample.

$$D = \frac{S}{\sqrt{N}}$$

Where D= Menhinick's index score, S=Number of species, N=Number of individuals.

Menhinick's index is based on the presumed linear relationship between species richness and the total number of individuals (Camargo, 1992). Menhinick's index is effective in distinguishing between eutrophication levels in marine habitats, showing strong partitioning of community structure between oligotrophic, mesotrophic, and eutrophic water systems (Karydis and Tsirtsis, 1996). It is also a sensitive measure for assessing invertebrate responses to changes in environmental conditions, such as eutrophication (Camargo, 1992). Eutrophication has been proposed as one of the leading causes in decline in *S. nitida* (Kerney, 1991b), so determining the impact the quicker method of sampling has on this index could be important for future surveys.

Menhinick's index was calculated for each ditch using the data for the quicker method (wash 1 only) and the traditional method (wash 1 + wash 2 + vegetation). A linear regression model (MiniTab 17) was used to predict the Menhinick's index for an individual sample calculated from data for the traditional sample assessment method from the index calculated using data for the quicker sample assessment method (wash 1 only) (n = 47). Since the index values for traditional and quicker method

were dependent (wash 1 data was also included in traditional method), a significant relationship was assumed. Data were not normal initially (Anderson-Darling test; α=0.05), so were transformed using the reciprocal (1/x) to achieve normality. Data for one sample were identified as an outlier with an unusually large residual in the model (standardized residual > 2) and was excluded from statistical analysis but included in the scatterplot illustrating the data for completeness. The linear regression model was used to test for a zero-offset in the relationship (indicated by a model constant significantly different from zero), evaluate the gradient of the relationship (model coefficient) and to quantify the amount of variation explained by the model (adjusted R2 value). The model fit was confirmed by testing residuals for normality (Anderson-Darling test; α=0.05).

Continuous data (number of gastropod species and gastropod individuals) for the experiment comparing the time needed using the quicker method to the traditional method were tested for normal distribution using a Shapiro-Wilk test (α= 0.05), and data were then tested for equal variance with Levene's test (α= 0.05). To test for effects and interaction of sample assessment method and surveyor on the number of individual snails and of species recorded, a repeated measures General Linear Model (GLM) was run in Minitab 19, with site used as the repeated measure (since samples were split between surveyors at each site), and sample method (quicker or traditional) and surveyor (experienced or inexperienced) used as fixed factors.

The number of *S. nitida* found per sample using the quicker sample assessment method and the traditional sample assessment method for experienced and inexperienced surveyors was compared using a Mann-Whitney U test.

2.3. Results

2.3.1. Comparison of quicker and traditional sample assessment methods for number of gastropod species and number of individuals

Of the 24 samples taken from Preston Marshes across the two sampling times, six were found to contain *S. nitida*. Of these, in five samples *S. nitida* was found in the first wash, in two it was found in the second wash, and in one it was found in the vegetation (Table 2.1).

Of the 24 samples taken from the Ash Level at the two sampling times, eight contained *S. nitida*. In all of these, *S. nitida* was found in the first wash, in four it was found in the second wash, and in four it was found in the vegetation (Table 2.1). In total, of the 8 ditches sampled at the Ash Level and Preston Marshes, four had *S. nitida* present in them in May, and four in July/August. This accounted for six of the eight sampled ditches over the two seasons (Table 2.1).

Segmentina nitida was not detected using the quicker method (1st wash only) when it would have been detected using the traditional method (wash 1 + wash 2+ vegetation) in only one sample (collected at Preston Marshes in May). It was, however, detected in the second wash for this sample. All of the eight ditches containing *S. nitida* would have been identified using the quicker sample assessment method (wash 1 only).

Table 2.1. Summary of percentages of *Segmentina nitida* individuals found in each of the washes and the vegetation sample in the eight ditches surveyed at Preston Marshes and the Ash Level. Only sites containing *S. nitida* included.

Site	Season Month	Percentage of *S. nitida* individuals found in Wash 1 (number of individuals)	Percentage of *S. nitida* individuals found in Wash 2 (number of individuals)	Percentage of *S. nitida* individuals found in vegetation (Number of individuals)
P1	May	100 (1)	0	0
P4	May	66.7 (4)	16.65 (1)	16.65 (1)
AL2	May	88.5 (23)	7.7 (2)	3.8 (1)
AL3	May	66.7 (2)	0	33.3 (1)
P2	July/August	66.7 (2)	33 (1)	0
P4	July/August	100 (1)	0	0
AL1	July/August	100 (1)	0	0
AL2	July/August	80 (92)	15.7 (18)	4.3 (5)

Wash 1 consistently contained the highest proportion of *S. nitida* individuals in samples from both the Ash Level and Preston Marshes, with few additional individuals found in the subsequent wash or when sifting through vegetation (Figure 2.4).

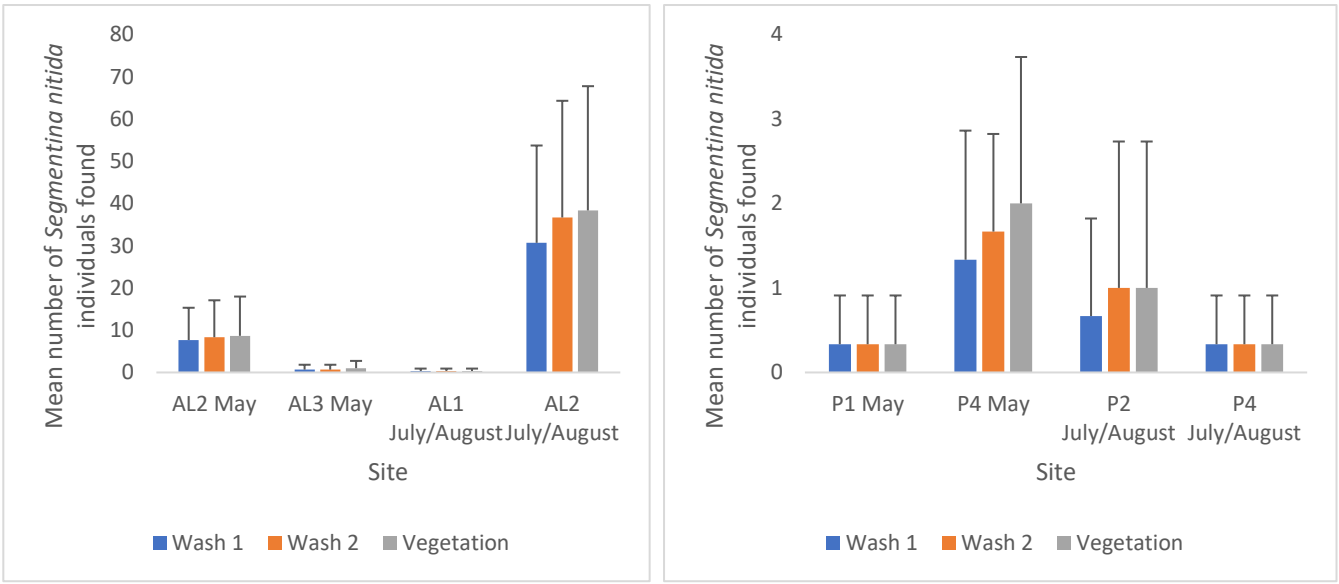


Figure 2.4. Cumulative mean number of *Segmentina nitida* individuals in samples collected at the Ash Level and Preston Marshes, showing mean number of individuals found in the first wash, first and second wash combined, and the first and second washes combined with the vegetation for each sample. Only data from ditches with *S. nitida* present was included. AL(n) and P(n) refer to 'Ash Level' and 'Preston', respectively, followed by the number of the ditch surveyed. Error bars represent standard error.

A total of 18 different snail species were found at both the Ash level and Preston across the spring and summer sampling seasons. The highest number of gastropod species was found in wash 1, and only in one sample was a species found in the vegetation that had not been previously found in wash 1 or 2 in any of the three samples of the same ditch. The majority of gastropod individuals in a sample was consistently found in the first wash, which typically contained approx. 80% of gastropod individuals in a sample. The number of individuals in the second wash and the vegetation of a sample were similar to each other (Figure 2.7).

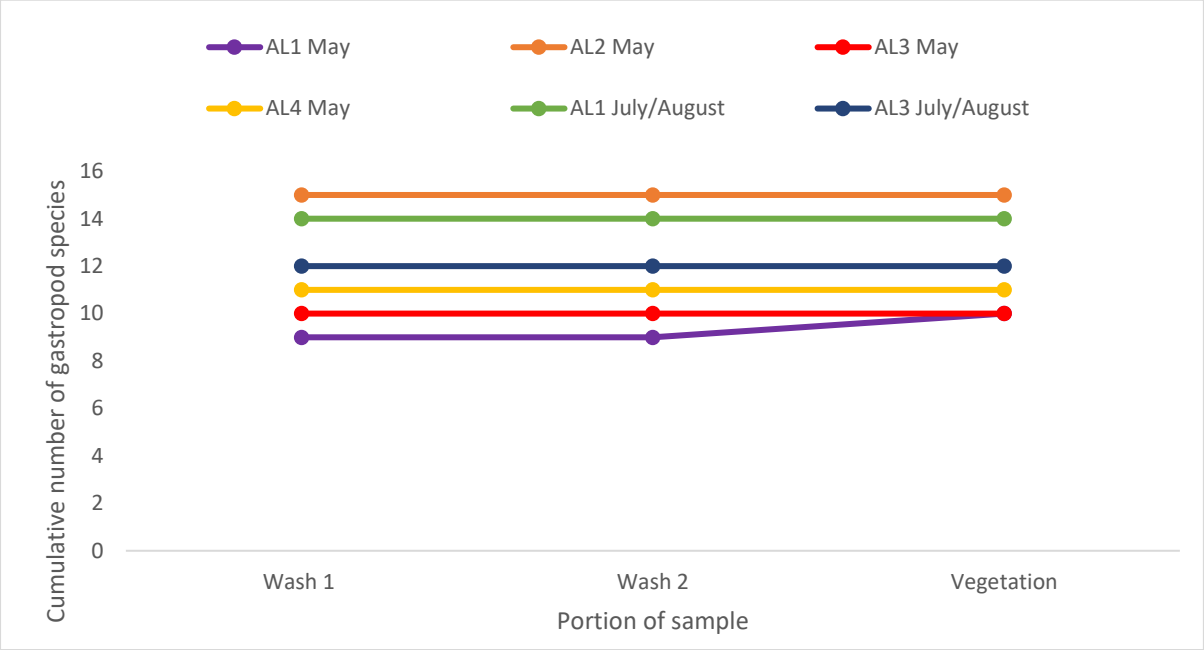


Figure 2.5. Cumulative number of gastropod species in samples collected at the Ash Level, showing number of unique species (accounted for once over the three samples per ditch) found in the first wash, first and second wash combined, and the first and second washes combined with the vegetation for each sample. AL(n) refers to 'Ash Level', followed by the number of the ditch surveyed.

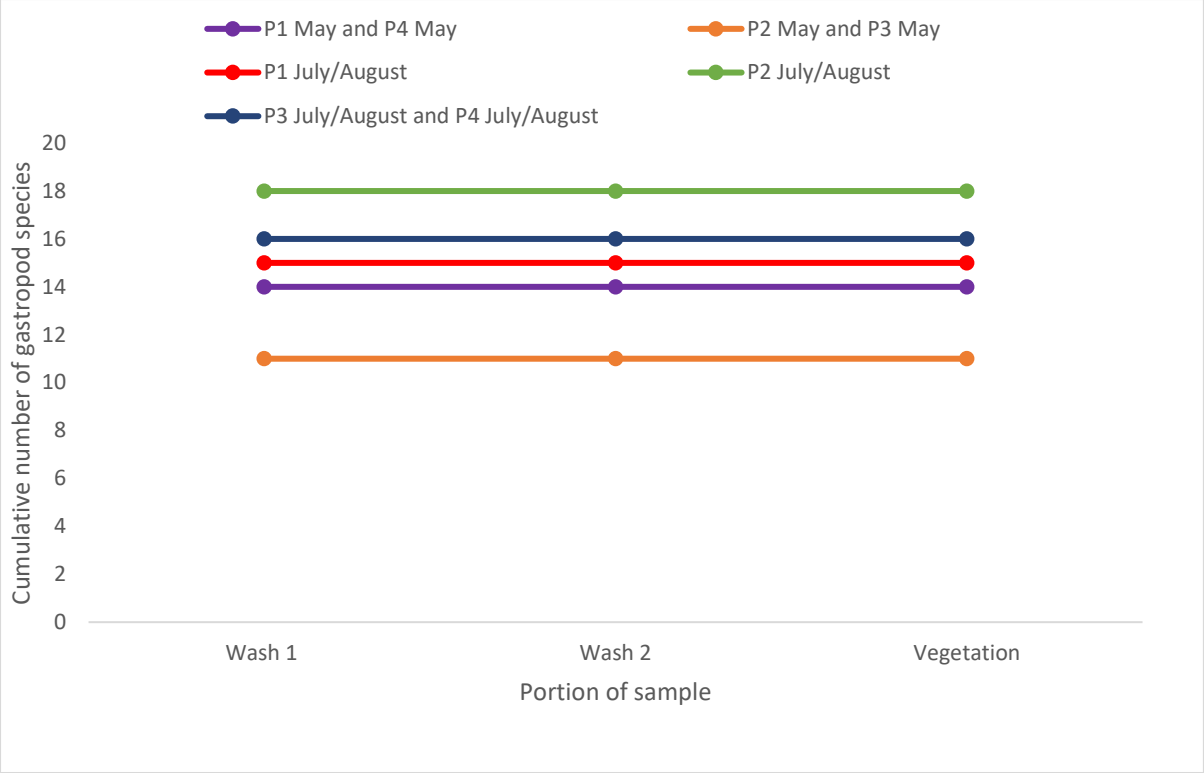


Figure 2.6. Cumulative number of gastropod species in samples collected at Preston Marshes, showing number of unique species (accounted for once over the three samples per ditch) found in the first wash, first and second wash combined, and the first and second washes combined with the vegetation for each sample. P(n) refers to 'Preston', followed by the number of the ditch surveyed.

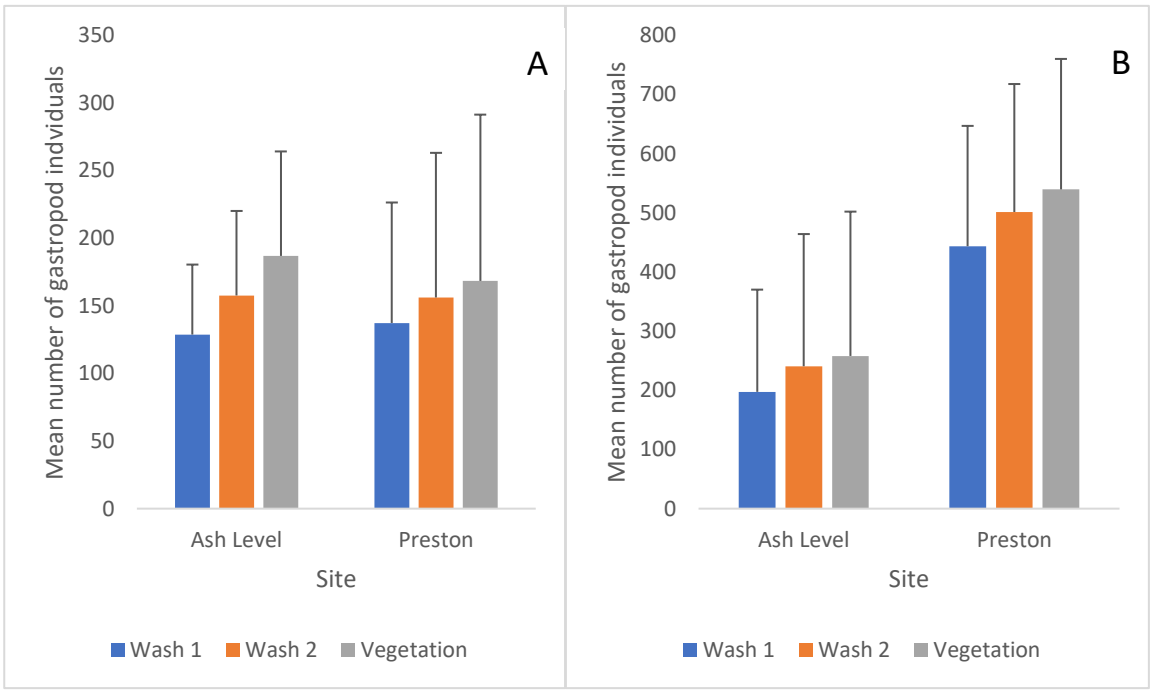


Figure 2.7. Mean cumulative number of gastropod individuals in wash 1, wash 2 and the vegetation of samples for each site (Preston and the Ash Level) in the two sampling seasons. Error bars represent standard error. A= May, B= July/August.

2.3.2. Comparison of ACFOR scores obtained using quicker and traditional methods

In May, for 78% (75/96) of gastropod species recorded, ACFOR scores obtained per sample using the quicker sample assessment method (wash 1 only) were the same as would have been obtained using the traditional method (wash 1 + wash 2 + vegetation). In July/August the same was true of 84% (98/116) of recorded species. Overall, 173/212 of the ACFOR scores obtained for all species found when using the quicker sample assessment method were the same to those that would have been obtained with the traditional method.

For 75% (6/8) of ditches in which *S. nitida* was recorded, the ACFOR score obtained for this species was the same for the quicker method (wash 1) compared to the traditional method (wash 1+ wash 2+ vegetation). Both of the other ditches had ACFOR scores one scale bracket lower than the traditional method (Table 2.2).

Table 2.2. Frequency of ACFOR scores for *Segmentina nitida* using the quicker method and the traditional method of surveying for the Ash Level and Preston combined.

Method	Rare	Occasional	Frequent	Common	Abundant
Quicker (wash 1 only)	6	0	1	1	0
Traditional (wash 1+ wash 2 + vegetation)	5	1	1	0	1

2.3.3. Comparison of species richness (Menhinick's index) between quicker method and traditional method

The median Menhinick's Index for each ditch total sample species richness (wash 1 + wash 2 + vegetation) was significantly lower than the median Menhinick's index calculated only for data from wash 1 (Wilcoxon test; Z= -3.516, P<.001).

As expected, the linear relationship between the inverses of the Menhinick's index calculated for the traditional method and the quicker method was significant (F=746.19, DF = 1, P <0.001) (Figure 2.8). The model constant was not significantly different from zero (T=-1.01; P=0.317) and the model explained 93.96% of variance in the data (adjusted R2). The gradient of the relationship was close to 1 (1.13). Scatterplots of model residuals plotted against each of the predictor variables indicated that variation of data points from the model was consistent across the range of data included (Figure 2.9).

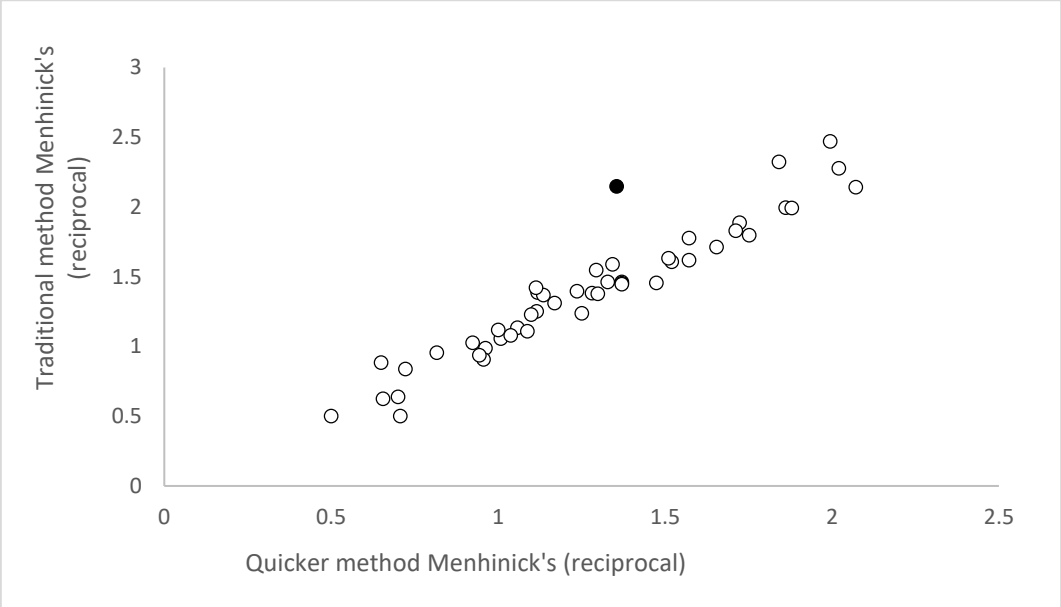


Figure 2.8. Reciprocal (1/x) of the Menhinick's index for the traditional sample assessment method (wash 1 + wash 2 + vegetation) plotted against the reciprocal of the Menhinick's index for the quicker sample assessment method (wash 1 only). Each point represents one sample (n=48). The data point in black was identified as an outlier based on the size of its residual in the linear model (>2) and was therefore excluded from final linear regression analysis.

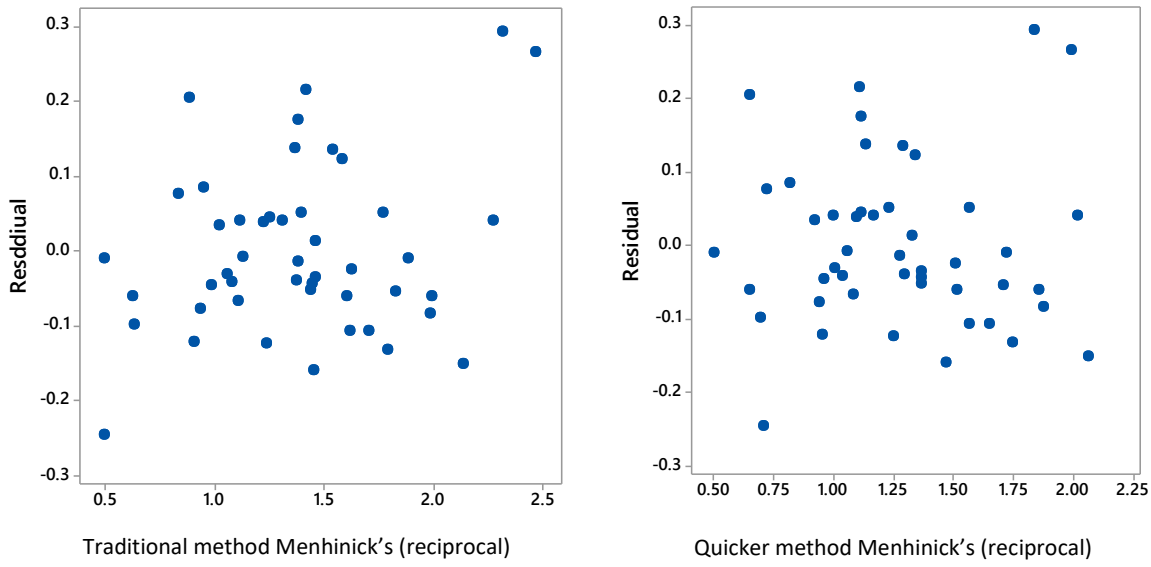


Figure 2.9. Scatterplots of linear regression model predictor and response variables (Menhinick's index) against model residuals (n=47).

2.3.4. Sampling time efficiency comparison of quicker and traditional sample assessment methods

For the inexperienced surveyor, of the four samples assessed with the traditional sample assessment method, none were completed within 30 minutes. For the quicker method only one of the samples was not completed within 30 minutes. The remaining three samples were completed within 20, 25 and 30 minutes respectively, using the quicker method (Table 2.3). Similar numbers of gastropod individuals were found using both methods, though they were found in a shorter amount of time using the quicker method.

For the experienced surveyor (surveyor with experience of assessing >50 ditch samples for gastropod species), all samples were completed within the 30-minute time limit with times ranging between 20 and 30 minutes for the traditional method, and 15 and 25 minutes for the quicker method (Table 2.4). For both surveyors the mean time it took to complete a sample was 3.75 minutes shorter compared to the traditional method.

Table 2.3. Inexperienced surveyor time expenditure of quicker sample assessment method (N) versus traditional sampling (T) two ditches at Ash Level (1.1 and 1.2) and two at Preston (2.1 and 2.2). The quicker method is based on identifying gastropods at the bottom of a bucket of water that were removed from vegetation by a single wash, the traditional method involves sifting through all vegetation in a sample in a plastic tray. Table A: New species recorded per 5-minute interval; Table B: New individuals recorded per 5-minute interval.

New species recorded- Inexperienced surveyor

A	Time							
Site	5	10	15	20	25	30	Total	Completed? (approx. percentage of sample remaining)
1.1 N	2	1	2	2	2	1	10	No (10)
1.1 T	3	2	0	0	3	0	8	No (30)
1.2 N	4	3	1	1	0	1	10	Yes
1.2 T	5	2	2	1	0	1	11	No (20)
2.1 N	6	3	1	0	1	NA	11	Yes
2.2 T	5	2	2	1	0	0	10	No (20)
2.2 N	6	1	1	0	NA	NA	8	Yes
2.2 T	5	1	2	0	0	0	8	No (15)

New individuals recorded- Inexperienced surveyor

B	Time							
Site	5	10	15	20	25	30	Total	Completed? (Percentage of sample remaining)
1.1 N	3	3	5	11	11	9	42	No (10)
1.1 T	4	6	4	8	5	7	34	No (30)
1.2 N	8	11	19	24	26	22	110	Yes
1.2 T	8	5	3	8	12	11	47	No (20)
2.1 N	17	15	18	31	33	NA	114	Yes
2.2 T	15	12	9	17	28	26	107	No (20)
2.2 N	30	19	37	19	NA	NA	105	Yes
2.2 T	19	13	26	21	19	32	130	No (15)

Table 2.4. Experienced surveyor time expenditure of quicker sample assessment method (N) versus traditional sampling (T) at two ditches at Ash Level (1.1 and 1.2) and two at Preston (2.1 and 2.2). The quicker method is based on identifying gastropods at the bottom of a bucket of water that were removed from vegetation by a single wash, the traditional method involves sifting through all vegetation in a sample in a plastic tray. Table A: New species recorded per 5-minute interval; Table B: New individuals recorded per 5-minute interval. NA = Not applicable.

New species recorded- Experienced surveyor

A	Time							
Site	5	10	15	20	25	30	Total	Completed?
1.1 N	7	2	0	0	1	NA	10	Yes
1.1 T	7	1	1	1	1	0	11	Yes
1.2 N	7	2	1	NA	NA	NA	10	Yes
1.2 T	6	1	1	2	2	NA	12	Yes
2.1 N	6	4	2	0	NA	NA	12	Yes
2.2 T	5	2	1	1	0	NA	9	Yes
2.2 N	5	2	3	1	NA	NA	11	Yes
2.2 T	6	1	0	2	NA	NA	9	Yes

New individuals recorded- Experienced surveyor

B	Time							
Site	5	10	15	20	25	30	Total	Completed?
1.1 N	16	27	33	51	26	NA	153	Yes
1.1 T	14	19	10	17	31	6	97	Yes
1.2 N	22	25	41	NA	NA	NA	88	Yes
1.2 T	9	16	17	22	23	NA	87	Yes
2.1 N	28	36	39	21	NA	NA	124	Yes
2.2 T	19	24	27	44	12	NA	126	Yes
2.2 N	48	65	50	76	12	NA	251	Yes
2.2 T	25	19	32	57	NA	NA	133	Yes

There was no significant difference in the number of *Segmentina nitida* individuals found with the quicker method (median=8) and the traditional method (median=1) when data for both surveyors were combined (MW U-test; U=22.0, P = 0.328). There was also no significant difference when data were analysed separately for experienced surveyor (U=6.0, P=0.686) and inexperienced surveyor (U=5.0, P=0.486).

The mean number of gastropod species found per sample using the quicker method was higher than for the traditional method, though not significantly (repeated measures GLM; $F_{(1,3)}$=0.462, P=0.546). The mean number of gastropod species found per sample by the experienced surveyor was higher

than the number found by the inexperienced surveyor, though again not significantly (repeated measures GML; $F_{(1,3)}$=4.800, P=0.116).

There was no significant interaction between the factors 'surveyor' and 'method' ($F_{(1,3)}$=0.0, P= 1.000), suggesting that the quicker method resulted in detecting a greater number of gastropod species per sample for both surveyors within 30 minutes of sampling.

The mean number of gastropod individuals found using the quicker method was greater than for the traditional method, though not significantly (repeated measures GLM; $F_{(1,3)}$=9.350, P=.055). The mean number of gastropod individuals found by the experienced surveyor was also higher than the number found by the inexperienced surveyor, though not significantly (repeated measures GLM; $F_{(1,3)}$=5.288, P=0.105).

There was no significant interaction between the factors 'surveyor' and 'method', ($F_{(1,3)}$=0.468, P=0.543), suggesting that the quicker method resulted in a higher number of gastropod individuals for both surveyors within 30 minutes of sampling.

2.4. Discussion

2.4.1. Detection of *Segmentina nitida* using quicker and traditional sampling methods

Many of the gastropod species found in the late-stage hydroseral succession drainage ditches in this study can be classified as 'micromolluscs', species which have an adult size of less than 5mm (Geiger *et al.*, 2007), e.g. *Pisidium* sp*., Gyraulus* sp*., Hippeutis complanatus,* and *Segmentina nitida*. Micromolluscs can be time-consuming to collect, sort, and identify unless specific sampling methods or genetic identification tools are used (Middelfart *et al.*, 2016). In surveys such as the ones previously targeting *S. nitida*, which included over 100 sampling sites (Killeen, 1996, 2000; Killeen and Willing, 1997; Watson, 2002; Watson and Ormerod, 2004b; Sadler, 2012), specialised sampling collection and sample assessment methods that facilitate detection and identification of small snails in the field are beneficial..

The efficiency of this sampling approach is reflected in the results of the present study. The quicker gastropod sample assessment method proposed here, using only one wash of vegetation in a sample, was as effective at detecting the presence of *Segmentina nitida* in a sample as the traditional method of sifting through the vegetation by hand for all but one of the investigated samples. The quicker method also allowed both experienced and inexperienced surveyors to find more snails than the traditional method in the same amount of time. This indicates the quicker method allows quicker sampling for *S. nitida* with comparable accuracy to traditional field methods. The quicker method also allowed the detection of the presence of small (<5 individuals per sample) populations of *S. nitida* as efficiently as the traditional method.

It is important to understand the current distribution, population trends and abundance of *Segmentina nitida* for its IUCN Red List assessment. Many of the associated gastropod species found in this experiment have already been assessed for the Red List yet *S. nitida* has not, which is likely due to the lack of information about the state of its populations throughout Europe (JNCC, 2010b). Classed as an endangered species before IUCN guideline changes (Kerney, 1991b) and as a priority species on the UK Biodiversity Action Plan (JNCC, 2010b), the Biodiversity Action Plan for *S. nitida* assessment states that there needs to be further work on assessing and monitoring current populations of *S. nitida*. With the proposed quicker sample assessment method, the species can be effectively detected even when present at low abundances (e.g. <5 individuals) in less time than when using the traditional method that has been used in previous surveys of the species (e.g. Hill-Cottingham 2004; Sadler 2012). Of note was the high abundance of *S. nitida* at ditch AL2 in July/August. Like the other sites at the Ash Level it was surrounded by grazing land, however it contained a greater quantity of *Enteromorpha* sp., a tubular algae normally found in saltwater. The presence of this species in a freshwater habitat may be due to the high input of salt from the grazing animals, and *S. nitida* may thrive either in the presence of high salt levels or the alga, though this would need to be explored in depth before a link can be made between the two.

The quicker sample assessment method proposed here allows for quick surveying of rare gastropod species, with no significant loss of accuracy against a more time-intensive traditional method of surveying, thus allowing surveyors to survey more sites and therefore potentially more habitats in the same amount of time. A key aspect of surveying for rare species and accurately assessing its distribution is being able to survey all micro-habitats within a survey area that it may occupy (Newmaster *et al.*, 2005; Bowering *et al.*, 2018). Building accurate distribution maps of species influences management and conservation decisions, especially if it includes so-called 'priority species' (Ormerod *et al.*, 2010). It can also indicate impacts on habitats (such as degradation of habitat or water quality) (Thomas, 2005) and provides information on habitat requirements of these species and acts as a baseline for future monitoring projects (Killeen and Willing, 1997).

2.4.2. Comparison of ACFOR scores between quicker and traditional sample assessment methods

The ACFOR scale results obtained with the quicker and traditional sample assessment methods were compared as this scale has been used in many of the historical surveys for *S. nitida* (Killeen, 1996, 2000; Killeen and Willing, 1997; Sadler, 2012) and offers a semi-quantifiable measure of population size for each species that aims at speeding up sampling (Crisp and Southward, 1958). In the present study, the ACFOR category obtained for *S. nitida* with the quicker sample assessment method was the same as the category that would have been obtained with the traditional method for each of the 16 ditches sampled over the two seasons (8 per season), except for two ditches where the score was underestimated by one category using the quicker method. Occasional underestimation of the ACFOR score of *S. nitida* populations are unlikely to represent a significant issue, especially when populations are larger, as the quicker method still accurately detects presence/absence of the species.

For gastropods species in general, 81.6% of the ACFOR scale scores obtained in the first wash (quicker sample assessment method) were the same as those that would have been obtained using the traditional method (wash 1, wash 2 and vegetation combined). Contrary to previous surveys, this study

quantified all gastropod individuals present and assigned the ACFOR score for each species based on the exact number of individuals found, whilst many of the surveys for *S. nitida* and other gastropod species estimate population sizes in the categories included on the scale to speed up sampling, especially for species with a high number of individuals present in a sample (Killeen, 1996, 2000; Killeen and Willing, 1997; Sadler, 2012). Using the quicker sample assessment method in combination with population estimation according to the ACFOR scale could further speed up sampling of *S. nitida* and other associated freshwater gastropods.

2.4.3. Sampling speed and detection of gastropod species

With the quicker sample assessment method, an experienced surveyor (with prior experience of assessing over 50 ditch samples for gastropods) and an inexperienced surveyor (with no prior experience in assessing ditch samples for gastropods) required less time to find the same number of gastropod individuals and species when using the quicker sample assessment method compared with the traditional method. The inexperienced surveyor likely rapidly gained experience in the process of assessing samples that would allow them to assess samples more quickly, however the use of the quicker and traditional methods was alternated between samples to control for this effect. Geiger *et al.* (2007) recommend for marine samples that after a washed sample is allowed to settle, it is then run through a sieve to catch floating molluscs. Including this additional step in the washing methodology presented here would remove the need for sifting through sediment and debris for snails at the bottom of the bucket, potentially further increasing the sampling speed.

Inexperienced surveyors without detailed taxonomic knowledge or familiarity of the organisms being surveyed are often involved in conservation surveys and monitoring, for example as 'citizen scientists' (Dickinson *et al.*, 2010; Theobald *et al.*, 2015). Citizen science is an effective way to develop public understanding and support of scientific projects, promoting Earth stewardship and environmental protection (Dickinson and Bonney, 2012; Shirk *et al.*, 2012). Citizen science has been used in a range of ecological projects, such as monitoring the effects of climate change on species range (Parmesan

and Yohe, 2003; Root *et al.*, 2003), tracking the spread of diseases (Dhont *et al.,* 1998), and monitoring the decline in target species (Ward-Paige *et al.*, 2010). Studies utilising trained volunteers can cover a greater area and produce a larger dataset than those undertaken only by trained ecologists. For example, Ward-Paige *et al.* (2010) conducted a survey utilising trained volunteer divers that included 76,340 dives over 15 years, far more than a small number of professional ecologists could complete in that time. Therefore, the quicker sample assessment method may provide a significant benefit, especially in freshwater conservation projects and monitoring of species that involves 'citizen scientists'.

Utilising citizen science could also be a useful tool for looking at geographical sampling bias for some species. *Segmentina nitida* is currently only reported as present in a few locations around the UK, but it is not clear if this represents their actual distribution or is a result of sampling bias due to past survey results and assumptions about the biology and habitat requirements of *S. nitida* (Sastre and Lobo, 2009). Additionally, some *S. nitida* populations in other parts of Europe seem to occupy different habitats to those in the UK, both in terms of structure (e.g. Poland in semi-permanent ponds, Książkiewicz and Gołdyn (2008)), and water chemistry (e.g. Germany Zettler *et al.,* (2006)) so there is potential for widening the scope of surveys aimed at evaluating the distribution of *S. nitida* in the UK. Promotion of a citizen science project that has a greater scope and looks for *S. nitida* in other locations than the SSSIs it is currently found in could give a greater idea of its current geographical range in the UK, and reduce the effect of 'geographical bias' (Dennis *et al.*, 1999; Dennis and Thomas, 2000; Hassall, 2012). Performing more random sampling, instead of subjective sampling that focusses only on where a species is 'expected' to be can also give new information about the ecology and distribution of species, potentially changing its conservation status (e.g. Martikainen 2002).

Kent Wildlife Trust often uses volunteers in their ecological surveys and research projects, including the recent survey of *S. nitida* (Sadler, 2012). These volunteers often have little or no prior experience in ecological surveys. With the quicker sample assessment method proposed here and the time

savings it can provide especially for inexperienced surveyors, citizen scientists could potentially survey more sites within a sampling season, which is especially important for surveys targeting specific species of conservation interest like *S. nitida* and changes in their distribution over time.

2.4.4. Relationship of Menhinick's index between quicker and traditional sample assessment methods

The relationship between the inverse Menhinick's index for traditional and quicker sample assessment method was linear and explained almost 95% of the variance in the data. Moreover, there was no significant zero-offset of the linear relationship. Residuals indicated a consistent deviation of data points from the regression line across the data range, suggesting that there was no systematic deviation of data from the linear model. Although there was one outlier sample, this suggests that, while the quicker sample assessment method tends to overestimate species richness as expressed by Menhinick's index, it provides a proportional and consistent predictor of the Menhinick's index for a sample that can be used to compare species richness between samples and sites.

2.4.5. Effectiveness of the quicker sample assessment method to detect gastropod populations across seasons

An important concept in effective species monitoring is that of 'temporally adaptive surveying'. This is where sampling coincides with times of the year when greater activity or abundance is expected, allowing more effective detection of a species (Charney *et al.*, 2015). The quicker sample assessment method was as effective at detecting *S. nitida,* other gastropod species and the number of gastropod individuals as the traditional sampling method during both spring and summer months. It does therefore not appear that differences in seasonal temperature, vegetation density or water chemistry affect the efficiency of the washing process. The quicker sample assessment method can therefore be used at different times in the year and effectively detect gastropod species and be timed to coincide with potential breeding events or other significant periods. In Poland, *Segmentina nitida* have been found to have three reproductive events across the spring and summer, with partially overlapping

generations (Książkiewicz and Gołdyn, 2008) but little is known about the voltinism (number of generations of an organism in a year) of *S. nitida* in the UK. The quicker sample assessment method may be useful in studying this in more detail, with multiple surveys throughout the year.

The quicker method proposed here was effective in detecting gastropods in the spring and summer. Ecological monitoring can be used to identify trends at a habitat or ecosystem level over time, such as the effect of management on diversity and population size (Yoccoz *et al.,* 2001; Lindenmayer and Likens, 2010). In addition to this, yearly single-season studies of ecosystems can underestimate biodiversity (Winter and Gittenberger, 1998; Cameron *et al.*, 2003). Comparing surveys using this method over multiple seasons and multiple years may allow conservation managers and land-owners to track the effect management and conservation initiatives have on *S. nitida* and other associated gastropod species, thereby facilitating adaptive management (Johnson *et al.*, 1993; Williams, 1996; Nichols and Williams, 2006).

2.4.6. Limitations of the proposed quicker sample assessment method for freshwater gastropods

Before the quicker sample assessment method can be recommended for sampling freshwater gastropod habitats other than the drainage ditches in which it was trialled for this study, there should be further testing of the method in habitats with different vegetation and structural composition. In terrestrial habitats, utilisation of identical sampling methods across multiple vegetation types and geographical locations may yield inconsistent and unrepresentative datasets for ecological analyses (Liew *et al.*, 2008). The structural diversity and stratified nature of terrestrial vegetation is often reflected in submerged and emergent freshwater vegetation, such as that in late-stage hydroseral succession. Stratified surveys investigating terrestrial meso- and micro-habitats have been found to be more effective at detecting species, especially rare species, than randomised sampling approaches but they do not estimate abundance well (Newmaster *et al.*, 2005; Bowering *et al.*, 2018). It remains to be seen whether the proposed methods for assessing samples is equally effective across a variety

of habitat types and micro-habitats within them, e.g. when washing sediment or detritus samples rather than vegetation.

The procedure used to represent the traditional method for assessing a sample in this study likely overstated its power. This is because the procedure combined the results of the first and second wash of vegetation in a sample with the results of sifting through the vegetation by hand. In previous surveys, no washes were undertaken before sifting through vegetation by hand (Killeen and Willing, 1997; Hill-Cottingham, 2004; Sadler, 2012). Assessing a sample only by sifting through the vegetation by hand means a surveyor is probably more likely to miss smaller snails – like *S. nitida* - amongst the vegetation. The data presented in this chapter suggest that washing the vegetation removes a significant proportion of small gastropods from the vegetation, therefore probably making them more likely to be found and recorded.

2.5. Conclusion

The data presented here indicate that the quicker sample assessment method detects *S. nitida,* and other gastropods as effectively as a traditional field method of sifting through all the vegetation in a sample. In addition, the quicker method was shown to save between five and ten minutes of sample assessment time per sample for both inexperienced and experienced surveyors. This could save up to 100 minutes per sampled ditch (assuming ten samples per ditch as in Killeen and Willing (1997). This quicker method of surveying would be most suited for targeted surveys of *S. nitida* and other species of similar size, especially those that that share the behavioural trait of readily falling away from vegetation when agitated in water.

The quicker sample assessment method requires no additional field materials except for a bucket (that can replace the white tray used traditionally). It has the potential to speed up surveys for freshwater invertebrates that utilise citizen scientists. This could be especially beneficial for rare gastropod species in need of IUCN Red List assessment, providing data for generating accurate species range maps and for monitoring populations. For *S. nitida* in particular, quicker surveying would also aid in

addressing the need for increased monitoring of the species in the UK, as detailed in its Biodiversity Action Plan.

Chapter 3- Evaluating laboratory breeding methods for *Segmentina nitida*

3.1. Introduction

Due to a variety of impacts, both natural and as the result of human activity, habitats can become unsuitable to sustain populations of rare species, and therefore *ex situ* captive breeding programmes are required for their conservation (Griffith *et al.*, 1989; Robert, 2009; Thomas *et al.,* 2010). The need for captive breeding will undoubtedly increase if the loss and degradation of wildlife habitats continues at the present pace (Johnson *et al.*, 2017). However, captive breeding and reintroduction programmes can only work if there is suitable habitat to reintroduce to, so it must happen in conjunction with protecting and improving remaining habitats (Ralls and Ballou, 2013) and should be carried out in combination with *in situ* conservation (Thomas *et al.* 2010). Captive breeding programmes typically aim to provide species with a benign, stable environment (Robert 2009) with the goal of increasing fecundity and survival rates in comparison to those that would be experienced in the wild (Ricklefs and Scheurlein, 2001). An important challenge for the conservation of some threatened and endangered species lies in developing cost-effective, efficient captive breeding strategies and meeting the requirements required for the species of interest to survive and reproduce in captivity (Lysne *et al.*, 2008).

Conservation-oriented captive breeding has been mostly focussed on birds and mammals (e.g. Alroy, 2015), with invertebrates grossly underrepresented in relation to the proportion of endangered species they encompass (Sarrazin and Barbault, 1996; Seddon *et al.*, 2005). Dolman *et al.* (2015) argue that the efficacy of captive breeding as a conservation strategy relative to an *in-situ* conservation approach must be considered on a case by case basis. Moreover, programmes for captive breeding and reintroduction as conservation measures must be carefully planned because of various associated risks, including the possibility of changing natural behaviours (McPhee, 2004), introduction of disease into wild populations through released individuals, and loss of captive populations after removal of individuals from the field for breeding, putting strain on already depleted populations (Snyder *et al.*, 1996). Additional challenges involved in the planning, setup, and execution of effective captive breeding programmes revolve around securing adequate and suitable broodstock (IUCN/SSC, 2013),

identifying suitable release sites (Lyles and May, 1987; Griffith *et al.*, 1989; IUCN/SSC, 2013) and determining optimal environmental requirements for growth and reproduction (DeWitt, 1967; Russell-Hunter and Eversole, 1976; Jarne and Delay, 1990). There are also concerns associated with the genetic bottleneck created by inbreeding of small populations in captivity (Witzenberger and Hochkirch, 2011; Ralls and Ballou, 2013). This can result in reduction of genetic diversity in captive-bred populations and inbreeding depression (Jarne and Delay, 1990; Witzenberger and Hochkirch, 2011), especially when working with small and closed founder populations (Ralls and Ballou, 1986). All these aspects should be explored and considered before individuals produced in a captive breeding programme are used for reintroduction to the wild.

In freshwater snails, water pollution, competition and predation from invasive species, habitat loss and habitat alteration have resulted in one of the best documented declines of a group of organisms worldwide (Lydeard *et al.*, 2004). Snails are ecologically important as they hold a disproportionately large role in the transfer of energy through aquatic ecosystems (Newbold *et al.*, 1983; Richardson *et al.,* 1988; Brown, 2001) due to their numerical abundance. However, freshwater gastropods are becoming increasingly imperilled by river regulation, habitat loss, poor water quality and quantity, and competition from invasive species (Lysne *et al.*, 2008). It has been estimated that approximately 40% of freshwater snail species are negatively affected by anthropogenic factors (Neves *et al.*, 1997). The range of *Segmentina nitida* for example has declined by around 80% over the last 100 years due to land drainage, eutrophication and poor habitat management (JNCC 2010b). The UK Biodiversity Action Plan for *S. nitida* states that research into translocation methodologies may be needed to increase the extent of occupied habitat (JNCC 2010b), in conjunction with appropriate management of water bodies known to host or have previously hosted the species.

Whilst many snails thrive in a variety of habitats and environmental conditions in the wild, these conditions can be difficult to duplicate in the laboratory (Berrie, 1970; Webbe and James, 1971; Eveland and Haseeb, 2011). New captive breeding programmes often require substantial research on

behaviour, reproductive biology, nutrition, genetics and disease (Ralls and Ballou, 2013) and research that has been conducted on related species is often helpful if species-specific data are unavailable. Several studies have assessed the effect of different variables on the breeding of snails in captivity to establish populations for various scientific applications, ranging from the assessment of the effects of inbreeding and crossbreeding fitness (Jarne and Delay, 1990), to the consequences of starvation on tissue de-growth (Russell-Hunter and Eversole, 1976). Laboratory-reared snails have also been used as model organisms for neurobiology and behavioural ecology research (Liddon and Dalesman, 2015). Many studies on snail breeding are focussed on medically important species in the *Biomphalaria* genus that act as vectors for schistosomiasis in African and South American countries (Pointier, 1991; Eveland and Haseeb, 2011). Although these vector species are tropical in origin, aspects of the literature may apply to *Segmentina nitida*, as they are classified within the same family (Planorbidae) and *Biomphalaria* sits within a sister clade (C-Clade) to *S. nitida* (Segmentinini) (Albrecht *et al.,* 2006).

Segmentina nitida has several characteristics that may make it suitable for effective captive breeding. Adult individuals are small (c. 5mm in diameter) so don't require a lot of resources or space in a captive programme. Its habitat is predominantly stagnant water bodies (Kerney, 1991b, 1999) which should not be as challenging to recreate in a laboratory setting as dynamic habitats (e.g. flowing water). The natural habitats of *S. nitida* are declining and difficult to replace, and their protection requires intensive management strategies (Sadler, 2012), so captive breeding may be effective in relation to maintaining wild habitats and can be used to expand the range and reintroduce captive-bred individuals to sites with historic records.

To breed freshwater snails in captivity there are several considerations to be made regarding water chemistry and other abiotic factors. In snails growth rate, shell weight, and fecundity all tend to increase with increasing calcium concentrations of the water (Thomas *et al.*, 1974). Snails tend to develop small, thin shells when reared in water with low calcium concentrations (Mishkin and Jokinen, 1986). Snail mortality in captivity has been reported at both high and low calcium concentrations, with

an optimal calcium concentration of around 30mg/L promoting fecundity for *Biomphalaria glabrata* (Mishkin and Jokinen, 1986). There are several options to increase the amount of calcium available to snails, either via the water or in their diet**.** Small pieces of calcium carbonate ($CaCO_3$) can be added to aquaria as a calcium source to promote snail growth and shell hardening (Ulmer, 1970). Alternatively, some breeding programmes have used Romaine lettuce (*Lactuca sativa*) as a foodstuff, which has been found to provide the necessary calcium required for snail growth (Boston *et al.*, 1994).

Some studies have found that including a water circulation system involving pumps can encourage earlier egg laying and increased snail growth (Richie *et al.*, 1963). However, including water circulation in a breeding programme requires more infrastructure and sophisticated design and is not necessarily representative of stagnant water habitats. It has also been reported that there is better fecundity of freshwater snails (*Biomphalaria pfeifferi*) closely related to *S. nitida* in stagnant systems with weekly water changes (Frank, 1963), but these water changes can become time-consuming in comparison to a circulating system, especially when large numbers of microcosms are used in a breeding programme.

There has been only one attempt at developing a captive breeding programme for *Segmentina nitida* to date, undertaken by Hill-Cottingham (2004). Hill-Cottingham conducted a laboratory breeding experiment and an outdoor breeding experiment for the species. The laboratory breeding programme was set up in glass aquaria with 14-hour lighting provided by a triphosphor lamp, imitating spring conditions, and the external breeding programme was setup as a small plastic box filled with rainwater in a hole in the ground. The laboratory rearing experiment failed due to the lighting causing elevated temperatures (>20^{o}C) that resulted in snail mortality. The external experiment failed due to elevated water levels and insecure net lids to the microcosms, many of the snails escaped from the microcosms resulting in this breeding experiment being abandoned. To date there have been no other *ex situ* breeding experiments on *Segmentina nitida*.

The aim of this chapter was to design and assess *ex situ* rearing experiments for *Segmentina nitida* to develop an effective protocol for breeding the species in captivity for potential reintroduction,

identified as an action in its Biodiversity Action Plan (JNCC, 2010b). Based on known habitat requirements, the life cycle of the species and results of the previous breeding programme for *S. nitida*, an initial microcosm-scale breeding experiment was designed. Microcosms were initially chosen due to the small size of *Segmentina nitida* (approx. 5mm) as well as its low abundance in the wild. Microcosms allow a small number of snails to be in close proximity, which may increase mating probability. Additionally, having the experiment in a laboratory setting prevented snails from escaping the microcosms, a problem experienced in the external breeding experiment conducted by Hill-Cottingham (2004). Based on the results of this experiment, further breeding experiments were created, iterating on the initial design.

3.2. Materials and Methods

3.2.1. Breeding *Segmentina nitida* in Microcosms

3.2.1.1. Source of *S. nitida* founding population

Segmentina nitida individuals were collected from drainage ditches with historical records of *S. nitida* in the Preston Marshes (TR2407960839) and the Ash Level (TR3124162320) in East Kent. These sites were chosen in consultation with Kent Wildlife Trust and Natural England because they contained comparatively large populations of *Segmentina nitida*, and snails were only removed from samples with n>20 individuals to minimise the impact this might have had on the population. General information about these two regions can be found in Chapter 2 (Section 2.3.1). All samples were collected in July 2015. Site water was also collected from the Ash Level sampling location for use in the experiment.

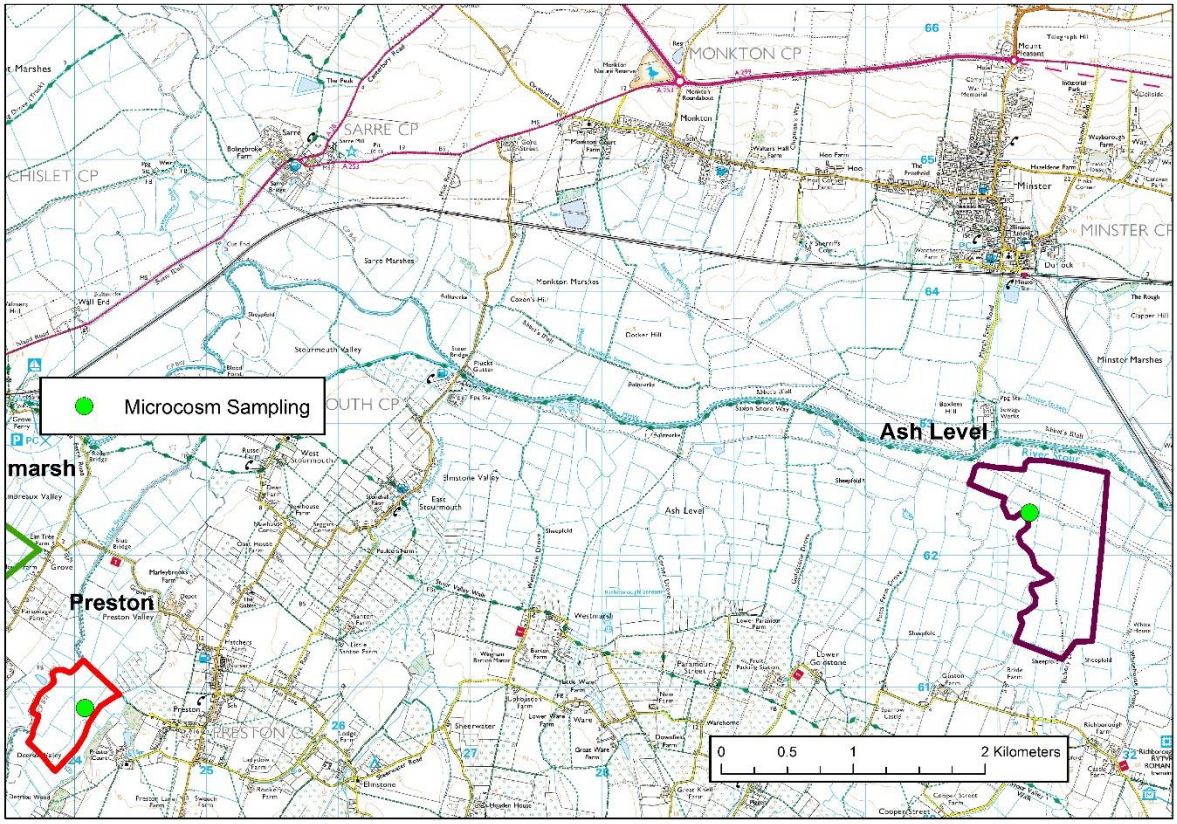


Figure 3.1. Map showing locations of samples taken for microcosm experiments and boundaries of sites in the Ash Level and Preston Marshes.

Samples were collected by sweep netting with a 250 mm diameter sweep net with 1mm mesh, and the netted vegetation was sorted through by hand to find *S. nitida* individuals. Snails were transported in plastic containers to a temperature and light controlled room (18°C; 14:10 hours light: dark) and were allowed to acclimatise to the laboratory conditions for a week before being introduced into the experiment. Collected site water from the Ash Level was filtered using a vacuum pump and Whatman Qualitative 4 filter paper (30 µm pore size), before being transferred to a lidded black water container (approximate volume of container: 1.35×10^5 cm^3).

3.2.2.2. Experimental Setup

Thirty-five 60 mm x 45 mm x 48 mm (approx. 130 cm^3) plastic microcosms were soaked in Decon 90 (Decon Laboratories Ltd.) for 24 hours and then rinsed thoroughly with distilled water six times to remove any residue. The microcosms were then soaked in filtered site water for 24 hours as a pre-treatment.

The microcosms were then filled with filtered site water and thin layer of *L. trisulca* and allowed to acclimatise for 24 hours before inclusion of *S. nitida* individuals. Initially, five microcosms were set up 2/7/15 with a total of 15 *S. nitida* individuals. Upon discovery of large stable populations of *S. nitida* through a field survey, an additional thirty microcosms were set up 10/7/15 with a total of 147 *S. nitida* individuals. All microcosms contained between two and five *S. nitida* individuals (1 x two snails, 4 x three snails, 2x four snails, and 28 x five snails).

Every 2-3 weeks the detritus and faecal matter was removed from the microcosms with a plastic Pasteur pipette, and observed with a low-powered microscope (Leica EZ4D) to check for egg masses or juveniles. These were recorded and, if present, were then returned to the microcosm. The microcosm was then topped up with filtered site water. All live and dead individuals were counted in the microcosms every 2-3 weeks when cleaning was carried out. The shells of the dead snails were removed. pH was monitored weekly using a Hanna probe (HI9024) for the final four weeks of the experiment.

3.2.2. Effect of *Enteromorpha* sp., natural sponge and paper as additional substrates on survival and fecundity of *Segmentina nitida* in microcosms

This experiment explored whether providing various substrates in microcosms would improve the survivability and fecundity of *S. nitida*. In field studies conducted by the author in 2015, high numbers of *S. nitida* were often found attached to a tubular species of algae, *Enteromorpha* sp. that may be a foodstuff of *S. nitida.* Natural sponge pieces were used in the experiment to act as a substrate for alga,

as it was previously hypothesised that *S. nitida* may feed on epiphytic algae (Hill-Cottingham, 2004). Due to the late hydroseral succession of the ditches in which *S. nitida* is typically found in the UK, often with woody plants and a lot of dead plant matter, paper was included as a substrate in the experiment as an easy to control substitute for dead plant material, as well as an additional algal growth substrate (Georges Dussart, pers. comm.).

3.2.2.1. Collection of samples for testing substrate influence on survival and fecundity of *S. nitida* in microcosms

Samples were collected from the same drainage ditch at the Ash Level (TR3124162320) in East Kent as in experiment 3.2.1 and brought to the laboratory and kept in the same conditions as in the previous experiment whilst setting up experiment. The snails were isolated from each other in individual microcosms for 24 hours as they acclimatised to the laboratory conditions. This helped to eliminate the possibility of mating just before the experiment.

3.2.2.2. Experimental setup for testing substrate influence on survival and fecundity of *S. nitida* in microcosms

The same microcosms as the previous experiment were used and were cleaned and pre-treated as in experiment 3.2.1. Temperature and photoperiod were also the same as in the previous experiment.

Four substrates were used in the microcosms: paper, *Lemna trisulca*, *Enteromorpha* sp. and natural sponge, in all possible combinations with three replicates of each combination (Appendix A1). The paper was cut up into equal pieces (2cm x 2cm) and then torn up into smaller pieces, to increase surface area. The natural sponge was torn into roughly equal pieces (around 2cm x 2cm), with two pieces added to appropriate microcosms.

Microcosms were set up in a randomised grid in the laboratory (Appendix A2). One *S. nitida* individual placed in each microcosm. A solitary individual was used to limit the removal of *S. nitida* from the field

as well as providing an opportunity to confirm self-fertilisation occurs in this species, as with all planorbid species (Baker, 1945). In previous laboratory breeding experiments containing one *S. nitida* individual additional snails were observed (Hill-Cottingham, 2004), from self-fertilisation, so therefore it was expected that solitary individuals would be suitable for this experiment.

The pH in each microcosm was monitored twice a week using a Hanna Probe (HI9024). Stock water in the covered water container used to replenish microcosms was monitored twice a week for dissolved oxygen, conductivity and pH using Hanna Probes (HI9143, HI9835, and HI9024, respectively). On 16/11/15, fresh water was brought in from the field site at the Ash Level, filtered with Whatman Qualitative 4 filter paper using a vacuum pump, and used to replenish the stock water. Pieces of calcium carbonate were added to the stock water to increase calcium levels in the water and improve conditions for snails in the microcosms.

Twice a week half of the water from each microcosm was removed and replaced with fresh filtered site water from the stock lidded water container. Numbers of live and dead *S. nitida* per microcosm were recorded every two months.

Figure 3.2. Experimental setup of microcosms with added substrates.

3.2.3. Breeding *Segmentina nitida* in mesocosms

3.2.3.1. Collection of samples for assessing survival and fecundity of *S. nitida* in mesocosms

Samples were collected from the same location at the Ash Level as before and brought to the laboratory as in experiment 3.2.1. The mesocosms were cleaned and pre-treated with Decon 90 and filtered site water as in experiment 3.2.1.

3.2.3.2. Experimental setup for assessing survival and fecundity of *S. nitida* in mesocosms

Four mesocosms (H=10 cm, D=16 cm, W=28 cm; approx. 1300 cm^3, ten times the volume of microcosms) were set up in plastic containers (Figure 3.2). Each was filled with filtered site water and distilled water at a 3:1 ratio to ¾ full and nine *S. nitida* individuals were added to each. The distilled water was added to buffer the water and ensure filtered site water contained in the laboratory lasted longer. *Enteromorpha* and *Lemna trisulca* collected from the field site in the Ash Level were added to the mesocosms and a 1mm layer of detritus was added so it covered the bottom of the mesocosm. Photoperiod and temperature were the same as the previous experiments in this chapter.

Ammonia, conductivity, pH, and dissolved oxygen were monitored every 2-3 weeks, using a HN_3/NH_4^+ water test kit (API: Aquarium Pharmaceuticals) and Hanna probes as in the previous experiments. After one month, a quarter of the water in the mesocosms was removed and replaced with fresh filtered site water. After three months the mesocosms were cleaned out and all live and dead snails were counted.

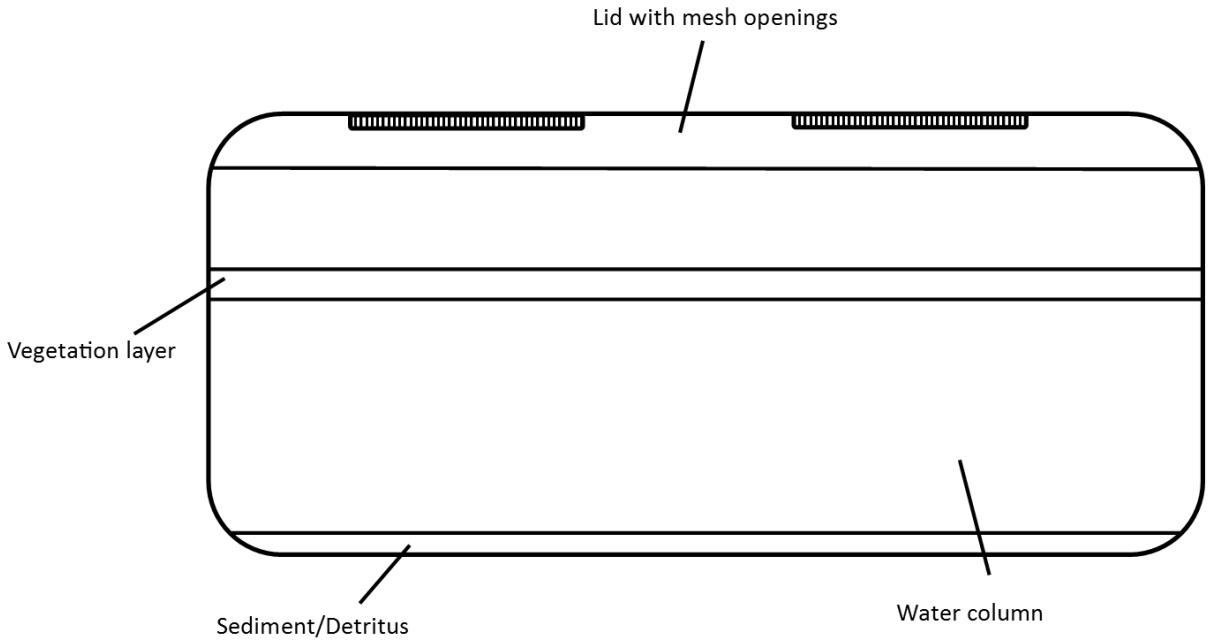


Figure 3.3. Diagram showing the setup of a single mesocosm for the breeding of *Segmentina nitida* in experiment 3.2.3. Approximately 5mm sediment, 1cm vegetation, 7.5cm water.

3.2.4. Breeding *Segmentina nitida* in outdoor macrocosms

3.2.4.1 Sample collection of *S. nitida* individuals for outdoor macrocosm experiment

Samples were taken from the site at Ash Level used in the previous experiments in the summer of 2017. All *S. nitida* in the samples were counted using the quicker sample assessment method described in Chapter 2 (Table 3.1).

Table 3.1. Number of *Segmentina nitida* individuals added to each of the macrocosms in the external breeding experiment.

Macrocosm	Initial number of *Segmentina nitida* individuals
1	72
2	152
3	132
4	136

3.2.4.2. Experimental setup for assessing external macrocosms for the rearing of *S. nitida*

Four 25 L plastic barrels that had previously contained distilled water were rinsed thoroughly with distilled water, then cut vertically (Figure 3.4) to produce 'troughs' measuring H=23 cm, D= 29 cm, W= 43 cm (approx. 28680 cm^3; 22 times volume of mesocosms). These were then filled with unfiltered water and sediment collected from the field site at the Ash Level. The aim was to mimic the wild habitat of *S. nitida* as closely as possible. Vegetation and associated gastropods in samples collected in the field were added to the water butts.

At the beginning of August 2017, the four troughs were placed in four shallow holes in the ground created on the Canterbury Christ Church University campus. The ditches were in the shade of a hedge, reducing the amount of direct sunlight and reducing evaporation of the water contained within the macrocosms. Placing the containers outside meant that temperature and light levels fluctuated seasonally.

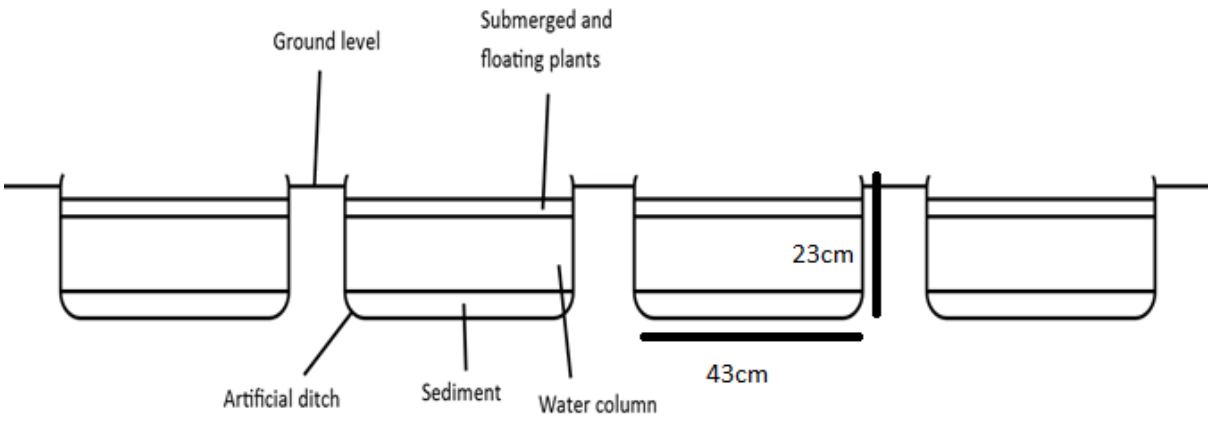


Figure 3.4. Diagram showing experimental setup of the four artificial ditches for experiment 3.2.4. H= 23 cm, W=42 cm, D= 29 cm

Figure 3.5. Two of the macrocosms at Canterbury Christ Church University. Macrocosms were located in the shade of the hedge to the left to avoid prolonged exposure to direct sunlight.

The conductivity and pH of the external macrocosms were monitored in August, September, October and December 2017, and June 2018. The number of *S. nitida* individuals in each trough were counted in April 2018 to coincide with potential seasonal reproductive events for *S. nitida* (Książkiewicz and Gołdyn, 2008). All live and dead *S. nitida* individuals were counted in the water column and on the vegetation, sampled with a sweep netting of the water and vegetation. In June 2018, at the conclusion of the experiment, pH, conductivity and dissolved oxygen were measured in each macrocosm (Hannah probes, HI9143, HI9835, and HI9024). All vegetation and sediment was washed and sifted through by hand in white trays to record all live and dead *S. nitida*.

3.3. Results

3.3.1. Rearing of *Segmentina nitida* in microcosms.

For both groups of microcosms (initial five microcosms and additional 30 microcosms) setup throughout the 2015 summer sampling period, all but one of the *Segmentina nitida* individuals contained within died within two months (Figure 3.6).

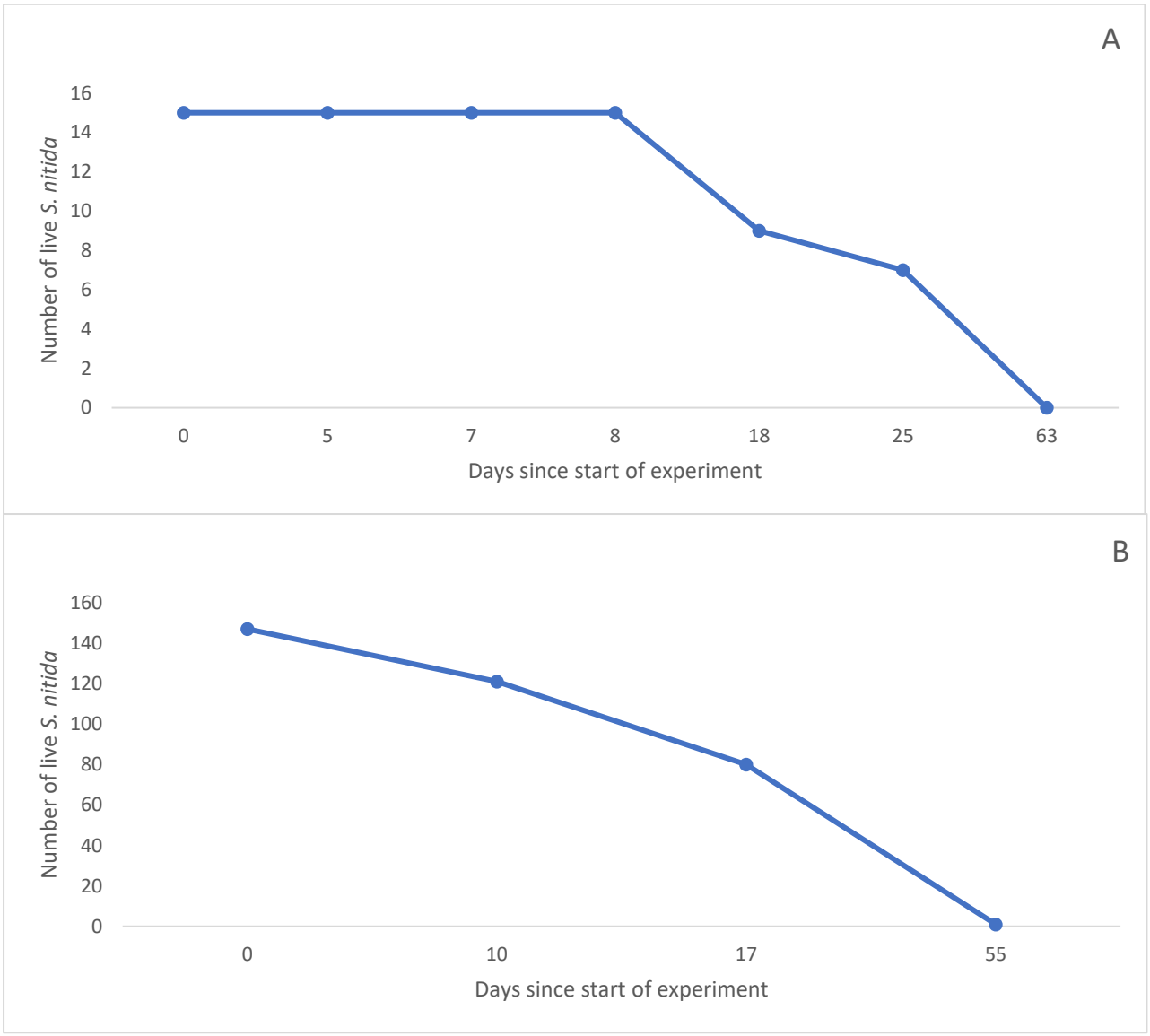


Figure 3.6. Total number of *Segmentina nitida* individuals alive in microcosms for the two sets of microcosms set up over a field season in 2015. Graph A: Five microcosms set up 2/7/15 with a total of 15 *S. nitida* individuals. Graph B: Thirty microcosms set up 10/7/15 with a total of 147 *S. nitida* individuals.

3.3.2. Effect of substrate on survivability and fecundity of *Segmentina nitida* in microcosms

Only two of the 16 treatments contained live *Segmentina* present at the end of this experiment, seven months after the start of the experiment (Table 3.2). These treatments contained a mixture of either *Enteromorpha* sp. and paper or *Lemna trisulca* and paper. Five other treatments lasted six months with at least one live snail remaining, four of which contained paper and mixtures of other substrates, and one of which contained no substrates or vegetation.

Only in four treatments was there an increase in the number of *S. nitida* individuals at any point during the experiment, with an increase of between one and two individuals (Table 3.2). The increases were all recorded two months into the experiment, and all treatments where they were recorded had a decrease in individuals at the next time point when individuals were counted. All these treatments contained either *Lemna trisulca* or *Enteromorpha* sp.

Table 3.2. Number of live *Segmentina nitida* in microcosms for each treatment (N=3) with combinations of substrates present (E= *Enteromorpha* sp. P= Paper L= *Lemna trisulca* S= Sponge) on the dates checked throughout the experiment. A dash indicates no live *S. nitida* remaining in the treatment.

Substrates						**Date**					
E	P	L	S	Average Min. pH	Average Max. pH	07/10 /15	04/12 /15	18/02 /16	28/03 /16	14/04 /16	25/05 /16
•	•			7.39	8.70	3	4	2	2	2	2
	•	•		7.97	8.80	3	3	1	1	1	1
	•			7.35	8.63	3	3	1	1	1	-
•	•	•		7.26	8.59	3	4	2	2	2	-
•	•		•	7.30	8.59	3	3	3	2	1	-
•	•	•	•	7.72	8.73	3	3	3	3	1	-
				8.20	8.59	3	3	2	2	1	-
•				8.14	9.46	3	4	1	-	-	-
		•		8.11	9.36	3	1	1	-	-	-
			•	8.05	8.64	3	2	1	-	-	-
•			•	7.76	9.40	3	3	-	-	-	-
•		•	•	8.42	9.29	3	1	-	-	-	-
•		•		7.61	9.41	3	5	-	-	-	-
	•		•	7.45	8.59	3	3	-	-	-	-
	•	•	•	8.11	8.60	3	3	-	-	-	-
		•	•	8.50	9.31	3	2	-	-	-	-

The overall mean pH range across all microcosms was 1.09. The stock water used for this experiment showed rising pH and conductivity over time. When fresh site water was added to the water container on 16/11/15 (40 days after the beginning of the experiment), there was a drop in the pH from 8.4 to 7.4, however within 7 days the pH had reached a value of approximately 8 again (Figure 3.7A). When the fresh water was added the conductivity rose from 920 µS to 1003 µS and then gradually declined,

followed by a slight increase at the end of the experiment (Figure 3.7B). Dissolved oxygen levels remained comparatively constant throughout the experiment (Figure 3.7C).

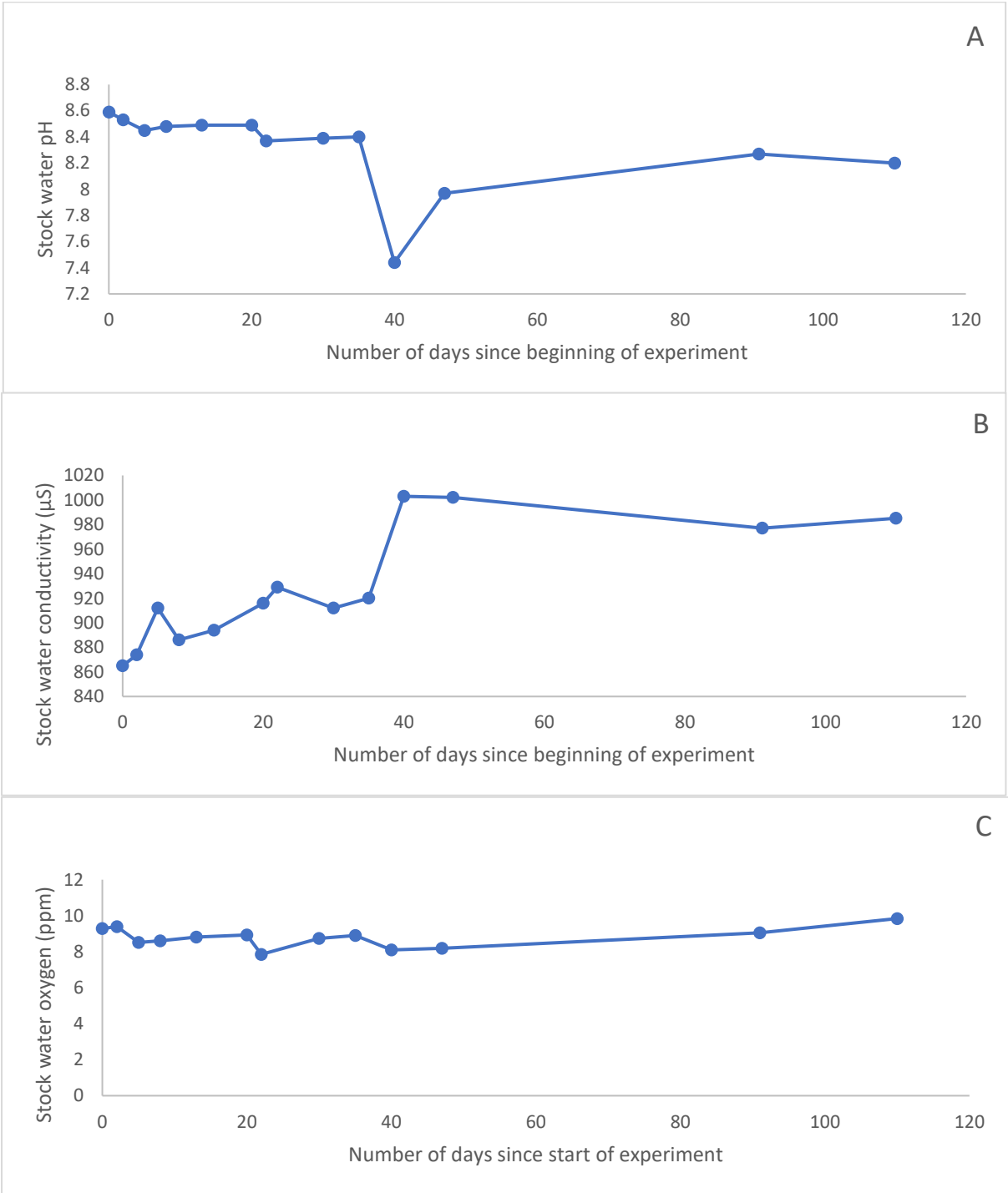


Figure 3.7. Water chemistry parameters of stock water used in substrate microcosm experiment. A: pH. B: Conductivity. C: Dissolved oxygen.

3.3.3. Rearing *Segmentina nitida* in mesocosms

Within three months of experimental setup most of the *S. nitida* individuals in the mesocosms had died (Table 3.3). Only five live snails were found across all four mesocosms at the end of the experiment, and of these, four were juveniles. Including dead snails found, there was only an increase of between 1-3 snails per microcosm before the snails died.

Table 3.3. Number of live *Segmentina nitida* included at the start of the mesocosm experiment, the number of live *S. nitida* present at the end of the experiment, the number of empty *S. nitida* shells found in each mesocosm at the end of the experiment, and overall change of the number of *S. nitida* from the starting number compared to the final number of both live and dead individuals.

Mesocosm	Initial live *S. nitida* (12.12.16)	Final live *S. nitida* (21.3.17)	Final dead *S. nitida* (21.3.17)	Final *S. nitida* total (21.3.17)	Increase in number of *S. nitida*
1	9	2	8	10	1
2	9	1	9	10	1
3	9	2	10	12	3
4	9	0	10	10	1

The water chemistry parameters in the mesocosms fluctuated less than they did in the stock water as measured in the previous experiments (Table 3.4). Across all mesocosms the pH remained below 9, with the highest value recorded being pH 8.89, and stayed within a comparatively narrow range of values (difference between minimum and maximum pH per mesocosm ranges from between 0.29 to 0.49; Table 3.4). Conductivity varied by approximately 200-300 µS in each mesocosm, and dissolved oxygen varied by approximately 34-46 ppm in each mesocosm.

Table 3.4. Minimum and maximum values for pH, conductivity (μS) and dissolved oxygen (ppm), and their range. Water parameters measured eight times throughout experiment.

	Mesocosm 1	Mesocosm 2	Mesocosm 3	Mesocosm 4
pH Max	8.86	8.89	8.88	8.77
pH Min	8.37	8.6	8.45	8.44
Difference	0.49	0.29	0.43	0.33
Conductivity Max	1106	1124	1174	1191
Conductivity Min	822	935	968	896
Difference	284	189	206	295
Oxygen Max	124.7	133.7	130.3	128.7
Oxygen Min	90.7	95.5	82.3	82.8
Difference	34	38.2	48	45.9

3.3.4. Rearing *Segmentina nitida* in external macrocosms

Ten months after the beginning of the experiment, at which time a total of 492 live *S. nitida* individuals were distributed across the four macrocosms, only 33 live snails were found across all macrocosms (Table 3.5). In November, when the macrocosms were sampled as a mid-point before the winter, 462 live *S. nitida* individuals were found in the water and vegetation (sediment was not sampled), and 146 dead individuals were found and removed. A further five live individuals were removed from macrocosm three on 01.11.17 as samples to allow the design of microsatellite primers for the population genetics analyses detailed in Chapter 5.

Table 3.5. Number of live and dead *Segmentina nitida* individuals found in the four external macrocosm throughout the experiment. All dead individuals were removed from macrocosms when sampled. All live individuals were returned after sampling.

	15.08.17	**01.11.17**		**27.06.18**	
Macrocosm Number	**Initial number of *S. nitida***	**Number of live *S. nitida***	**Number of dead *S. nitida***	**Number of live *S. nitida***	**Number of dead *S. nitida***
1	72	1	25	0	29
2	152	39	46	14	68
3	132	156	35	7	125
4	136	266	40	12	129
Total	**492**	**462**	**146**	**33**	**351**

Overall, a total number of 535 *S. nitida* individuals were removed from the four macrocosms over the course of the experiment (both alive and dead). This was an increase of 43 snails from the 492 initially used in the experiment.

Table 3.6. Minimum and maximum values for pH and conductivity (µS) and their range for the macrocosm experiment. Water parameters measured seven times throughout experiment. Dates: 15.08.17; 30.08.17; 14.09.17; 17.10.17; 30.10.17; 12.12.17; 02.07.18.

	Macrocosm 1	Macrocosm 2	Macrocosm 3	Macrocosm 4
pH Max	8.04	8.05	8.09	7.98
pH Min	6.91	7.08	6.95	6.76
Difference	1.13	0.97	1.14	1.22
Conductivity Max	1836	1906	1722	1826
Conductivity Min	833	634	679	669
Difference	1003	1272	1043	1157

The water chemistry parameters in the macrocosms fluctuated more than in the mesocosms used in the previous breeding experiment (Table 3.6). Across all macrocosms the pH remained well below 9, with the highest value recorded being pH 8.09, 0.8 lower than the maximum value of pH 8.89 in the previous mesocosm experiment. However, each macrocosm experienced a large range of pH values over the ten months of the experiment, with pH ranges between 0.97 and 1.22. Conductivity fluctuated by up to 1272 µS, with the lowest values all experienced in December. During December 2017 and between February and March 2018 protracted and severe cold weather and snowfall, caused all macrocosms to freeze over. Between December 2017 and July 2018 the conductivity of all macrocosms increased by 208-641 µS. Oxygen levels were measured at the end of the experiment, and levels fell to between 5.1-14.5% oxygen.

3.4. Discussion

The first microcosm experiment was set up as a preliminary experiment informed by previous literature on breeding of *Segmentina nitida* (Hill-Cottingham, 2004). Whilst the issues previously encountered in breeding *Segmentina nitida*, over-heating and over-crowding (Hill-Cottingham, 2004),

were avoided, only a single *S. nitida* individual survived for two months in this experiment. *Segmentina nitida* is thought to have a 2 year life cycle (Hill-Cottingham, 2004) so the deaths are unlikely due to the individuals living their entire life cycle. Literature shows that substrate and water chemistry parameters are important considerations when developing captive breeding for snails (Wagner and Chi, 1959; Brönmark, 1989; Ewald *et al.*, 2009). From this it was hypothesized that the microcosms were missing some essential aspect to the survival and/or successful reproduction of *S. nitida*, or that the water chemistry of the microcosms was unsuitable.

The next experiment was therefor designed to include artificial substrates and vegetation collected from the field, to see if these would improve survival or facilitate reproduction in captive *S. nitida*. This experiment contained additional potential food sources, as well as substrates for the growth of algae. In the study conducted by Hill-Cottingham (2004), *S. nitida* was observed on numerous occasions on the surface of *Lemna trisulca* in both the field and the lab, and it was thought that *S. nitida* could be feeding on algae present on the surface of this plant (Hill-Cottingham, 2004). In addition to this, paper was included in the experiment not only as an algal growth substrate, but also to mimic the structure of plant detritus in late-stage hydroseral succession conditions experienced by *S. nitida* in the field (Watson and Ormerod 2004; Clark 2011; Georges Dussart, pers. comm.).

Of the four treatments in this experiment where an increase in the number of *S. nitida* was observed, all of them contained at least one species of plant also typically found with *S. nitida* in the field. The increase in number of snails could have been because of self-fertilisation. In the literature, *S. nitida* has been described as laying egg masses of between three and eleven eggs per mass (Bondesen, 1950; Piechocki, 1979). There was never more than an increase of one snail in any of the microcosms. Whilst an egg mass may have been laid, hatched, and produced juveniles of which only one survived, no empty shells were found in the microcosms, so it is most likely that small juveniles were attached to the vegetation that were missed when setting up the experiment.

The reason for the death of the snails in the first experiment is unlikely to have been because of a lack of suitable foodstuff. In the treatment that contained no substrates or vegetation at all, only filtered site water, snails were still alive and actively grazing in microcosms six months into the experiment. This shows that *S. nitida* is most likely feeding on algae or diatoms precipitating from the water column or growing on the sides of the microcosms. Hill-Cottingham (2004) observed *S. nitida* feeding on the surface of *L. trisulca* but was unsure whether it was feeding on the leaf itself or on epiphytic algae growing on the surface. Epiphytic algae and bacteria often provide a rich food source for grazing invertebrates (Soszka, 1975; Brönmark, 1989). Most freshwater snails are herbivores and scrape a complex of algae/bacteria/detritus from surfaces, such as macrophytes, with their radula (Brönmark, 1989). Taking this into account, as well as the survival of *S. nitida* in vegetation-free treatments, it is most likely that *S. nitida* feeds on algae growing on the surface of vegetation and any other surfaces in their natural habitat. Analysis of faecal matter may allow identification of specific algal/bacterial/diatom species in their diet. An additional substance that could be of importance to the feeding of *S. nitida* is that of the detritus and sediment found at the bottom of their natural habitat. The stomachs and crops (an enlarged portion of the oesophagus) of snails have often been found to contain fine sand, probably to help with the grinding of food before entering the intestine (Baker 1945), so it would be interesting to see if substrate contributes to the survival of *S. nitida* in captivity by facilitating the feeding of the species in captivity. However, in the wild, *S. nitida* are not found as much in the sediment/ detritus layer of drainage ditches (Watson and Ormerod, 2004b) and predominantly found in the water column and on floating and submerged vegetation, so this may counter the importance of this sediment/detritus layer.

The most probable cause for the high mortality observed in the second microcosm experiment seems to be the water chemistry of the microcosms. pH was monitored throughout the experiment and ranged from a mean minimum pH of 7.26 to a mean maximum pH of 9.46. Even in microcosms with vegetation in them, mimicking the natural environment of *S. nitida* more closely, the range in pH values recorded was large, with a mean 1.16, higher than the overall mean pH range of 1.09 across all

treatments, showing that the vegetation didn't prevent excessive changes in pH. Throughout the experiment, the pH of the stock water mostly stayed above pH 8, starting at pH 8.59 and slowly dropping over time, before rising towards the end of the experiment. Field measurements for pH in the same location where the captive *S. nitida* were taken later the year and ranged from pH 6.95-8.06 (personal observation by author). The water in dark storage had not been checked consistently before, and there was no reason to expect the water chemistry to rapidly or significantly change to the point where it would have an effect on the survival of *S. nitida.* When fresh water was brought in from the field the pH of the stock dropped from pH 8.4 to 7.44, but then rose again quickly by approximately pH 0.5 within a week. In the wild, the pH of natural water systems can fluctuate considerably, both daily and seasonally, and most freshwater animals are tolerant to a range of pH, with most aquaculture ponds recommended to have a range of between pH 6 and 9 (Tucker and Abramo, 2008). However, stress or death can occur when rapid changes in pH occur or extreme pH values are reached, even if that pH value is within the species' tolerance (Tucker and Abramo, 2008). For many of the microcosms in this experiment, the pH changed by 0.5+ pH in seven days in multiple microcosms and this rapid change may explain the high mortality in this experiment. However, this may be due to the snails coming to the end of their natural life, but aren't reproducing, as the age of the snails taken from the field was unknown. In some closed aquarium systems with seawater, calcareous filtrants have been used to buffer water pH, but a lot of this research was focussed around countering gradual acidification rather than increasing alkalinity (Bower *et al.* 1981). Adding small amounts of easily decomposable organic matter (such as corn, or soy-bean meal) can be used to reduce the pH of a water body in the long term (Mandal and Boyd, 1980; Tucker and Abramo, 2008). In the wild habitat of *S. nitida* this process is most likely facilitated by the thick layer of detritus and sediment sitting at the bottom of the ditch, and the decomposing vegetation there. Therefore, to better mimic the natural habitat of *S. nitida*, some of this detritus was added in a thin layer to the mesocosms used in the next experiment.

In addition to rising pH in the microcosms the conductivity of the stock water increased throughout the experiment. Due to time constraints, the conductivity of each individual microcosm was not monitored, only the stock water container. The change in the stock water would also apply to the microcosms receiving water from the stock container. Additionally, throughout the experiment the water was topped up periodically to replace evaporated water. This could have resulted in the accumulation of salts in the water increasing the conductivity over time, which may also have contributed to the mortality of the snails. High salinity (4.5-6%) has been found to contribute to mortalities of molluscs in freshwater systems (Ercan and Tarkan, 2014) and inhibit growth and reproduction of freshwater snails (Kefford and Nugegoda, 2005), though intermediate salinity levels (100-1000µS) has been found to promote fecundity in *Physa acuta*, a species of freshwater snail (Kefford and Nugegoda, 2005). Other freshwater animals have been observed to undertake little or no reproduction in high salinity conditions (Mills and Geddes, 1980; Williams and Williams, 1991). Rare species such as *Segmentina nitida,* whose decline has been attributed to changes in water chemistry (eutrophication) (Kerney, 1991b), may be much more susceptible to changes in salinity, and experience lethal effects earlier, so the rising salinity may be the cause for the death of *S. nitida* individuals in this experiment. However, in the field *S. nitida* had been found in water bodies at conductivity of up to 1480 µS at the Ash Level, so seem quite tolerant of this (personal observation).

The rise in pH may also have increased the toxicity of ammonia in the water, which could have resulted in the death of the snails. Ammonia is a toxicant that is both produced by, and poisonous to animals. Ammonia is naturally present in freshwater ecosystems as the product of the biological degradation of organic matter (Alonso and Camargo 2003) and is more toxic to aquatic animals than other nitrogen containing compounds, such as nitrates and nitrites (Russo 1985; Scott and Crunkilton 2000). In aquatic environments, pH has a marked effect on the toxicity of ammonia and alkalinity of water increases this toxicity (Ip *et al*., 2001). In water, two species of ammonia occur at equilibrium, ionized (NH_4^+) which is non-toxic, and unionized (NH_3) which is toxic (Thurston *et al*., 1981; Alabaster and Lloyd 1982; Mummert *et al*., 2003). When pH increases the equilibrium shifts towards NH_3, with an increase

of one pH increasing the concentration of NH_3 approximately ten-fold due to the logarithmic nature of the pH scale (Thurston *et al*., 1981). In aquatic invertebrates, ammonia toxicity causes damage to respiratory surfaces by diffusing across cellular membranes, causing damage to gills and changes in haemolymph pH (Colt and Armstrong 1981; Alabaster and Lloyd 1982; Rebelo *et al*., 2000, Camargo and Alonso 2006). In laboratory experiments, snails have been found to be particularly sensitive to ammonia toxicity (Hickey and Vickers 1994) and increases in ammonia concentration and time exposed to ammonia increasing snail mortality (Alonso and Camargo 2003). Juvenile and senescent (old) snails are more susceptible to the toxic effects of ammonia than prime adults (Watton and Hawkes 1984). Ammonia toxicity would explain why only a small increase in snail numbers was observed, with all recently hatched snails likely to be affected by the toxicity of ammonia caused by the pH increase. With the small volume of water used in these microcosm experiments, buffering the pH to avoid increasing ammonia toxicity would present a significant challenge and require intensive and continuous water management.

The third experiment was designed to address the issues with water chemistry observed in experiment 2, especially the rising pH and conductivity, by using larger and therefore more buffered mesocosms (10x more water in the mesocosms than the microcosms). In the field, *S. nitida* has been found in water of around pH 6.8-7.2 so that should indicate what they are adapted to and experience in the wild, so pH 8-9 would probably be a stress on individuals, and minimising this was thought to be important for the survival of the species in the laboratory. This experiment was run as a low maintenance setup, requiring less frequent water replacement and topping up with distilled water when levels began to drop through evaporation. With fewer tanks to monitor (four instead of 48 as in the last experiment) all water chemistry parameters were monitored throughout the experiment. The water was more buffered than in the previous microcosm experiment, with a mean pH range of 0.385 in the mesocosms and mean pH range of 1.09 in the microcosms. The pH of all mesocosms stayed

below pH 9, an improvement on the second microcosm experiment, however there was still variation in the pH values (ranging from 8.37-8.89) and they did increase throughout the experiment.

The mesocosm experiment still did not result in successful breeding of *S. nitida* in *ex situ* laboratory conditions. From the literature it was expected that it would take around 2 months for reproduction to occur (Książkiewicz and Gołdyn, 2008), which dictated the time the mesocosms were left between counting live *S. nitida* present on the sides of the mesocosm or on the vegetation. Putting the snails in mesocosms that attempted to more closely recreate the ditches they had been collected from (deeper water column, a layer of detritus, and a layer of vegetation) did not fully encompass the complex conditions the snails would experience in the wild, such as variable light conditions, varying temperatures and weather conditions. Recreating such complex conditions in a laboratory setting would be almost impossible (Berrie, 1970; Webbe and James, 1971; Eveland and Haseeb, 2011). Therefore, the fourth and final breeding experiment was conducted outdoors. This meant snails would experience natural seasonal conditions and daily temperature and light fluctuations, water would be topped up through rainfall, thereby potentially better reproducing the conditions for *S. nitida* to survive and reproduce (Książkiewicz and Gołdyn, 2008).

The final experiment involved external macrocosms that were intended to reproduce the ditches *S. nitida* occupies in the wild, including having a greater volume of water to buffer the water chemistry. However, after ten months only 33 live *S. nitida* were found across all macrocosms, a reduction of 93% from the beginning of the experiment. There are two probable causes for this decline in macrocosm populations: 1) freezing conditions experienced in December 2017, and February-March 2018. Over the experimental period a particularly cold winter caused all macrocosms to freeze over and caused the conductivity to drop by between 781 and 906µS per macrocosm. A high rainfall in the autumn and high snowfall winter may have contributed to this decreasing conductivity. 2) Low oxygen levels at the end of the experiment. All macrocosms were found to contain hypoxic levels of dissolved oxygen, between 5.1 and 14.5%. Hypoxia is often experienced in freshwater habitats in the wild, and can be

associated with decomposition of organic material, amongst other factors (Whitworth *et al.*, 2012; Small *et al.*, 2014). Hypoxia has been found to have both sub-lethal and lethal effects on aquatic fauna, though most of the research has focussed around fish species (Gehrke *et al.,*, 1993; La and Cooke, 2011; Whitworth *et al.*, 2012; Small *et al.*, 2014). The low levels of oxygen found in the macrocosms was likely caused by decomposition of organic matter (primarily leaf litter from the adjacent hedge) that had fallen into them. However, an increase in 38 *S. nitida* individuals was recorded over the course of the experiment, based on the number of dead and live snails recorded. Repeating this breeding experiment with better controls for temperature fluctuation and organic input may show if this type of breeding experiment would be effective in the future for *S. nitida,* especially if snails are intermittently removed from the macrocosms to prevent overcrowding. The best time to set up this experiment would be in the spring, allowing for at least two reproductive events previously described in *S. nitida* (Książkiewicz and Gołdyn, 2008), and use the potential increase in individuals for reintroduction of *S. nitida.*

However, these experiments also highlight more general issues for breeding snails in captivity for reintroduction, especially on a small scale. For the micro-, meso-, and macrocosms the stability and controllability of the water conditions within them were likely affected by the 'edge effect'. Edge effects occur between two conjoining habitats where interactions between the two produce biotic and abiotic conditions that are not present in each habitat separately (Gehlhausen *et al*., 2000; Potts *et al*., 2016). Edge effects have primarily been studied in terrestrial habitats, e.g. woodland, forest, and heathland habitats (Gehlhausen *et al*., 2000, Piessens *et al*., 2006, Zurita *et al*., 2012), with some work done on marine and freshwater habitats, such as bogs, marshes, and seagrass beds (e.g. Lachance and Lavoie 2004; Tanner 2005 Cooper *et al*., 2012). Adjacent habitats transition into each other along their edges in 'ecotones' (Forman 1995) which usually differ from either of the two adjacent habitats in their conditions. In the micro- and mesocosms used in the present study, the ratio of edge length to the total water volume was high, so ecotones would proportionally extend further into the habitat. It is likely that for the microcosms, their entire contents were under the influence of

edge effects. This makes controlling the water chemistry and utilising them as viable rearing containers difficult. In a study on edge effects on marshland, Cooper *et al*. (2012) found that there were physical and chemical gradients from the edge of the habitat to the interior with depressed macroinvertebrate richness towards the edges of the marsh habitats (Cooper *et al*., 2012). If unfavourable environmental conditions exist at the edge of the microcosms, there is like no 'interior' habitat for individuals to retreat to and would hinder success of the rearing program. Using larger containers would reduce edge effect proportionally and help ameliorate these issues.

For rare species, especially small sized organisms such as *Segmentina nitida*, rearing them in large macrocosm containers present separate challenges. The removal of large numbers of snails from wild habitats to stock a sufficient density of individuals in a macrocosm could have impacts on existing populations (Armstrong and Reynolds 2012) and these impacts could outweigh the potential benefits of captive rearing. Some studies have modelled the effect of taking small and large numbers of individuals from source populations, and predict that taking too many individuals can result in the extinction of the source population, whilst reintroducing or translocating too few can result in the extinction of the new population (Kohlmann *et al*., 2005, Tocher *et al.*, 2006). The removal of individuals from source populations needs to be sustainable in terms of the dynamics of that population, i.e. the population needs to be able to recover from the reduction in population size and maintain its genetic diversity (Armstrong and Reynolds 2012). Low densities of individuals in a captive rearing program can cause Allee effects. Allee effects are the suppression of vital rates in the presence of low population densities, manifested through multiple mechanisms, one of which is low contact rates of potential mates (Courchamp *et al.,* 2008). In reintroduction biology Allee effects can be attributed to reintroduced populations failing when at too low a density (Armstrong and Reynolds 2012) and the same would apply to founder populations in breeding programmes.

3.5. Conclusion

Captive rearing has historically proved difficult for *Segmentina nitida,* and the iterative experiments presented in this chapter proved no exception, though they did provide clues as to factors preventing successful breeding and survival of the species *ex situ. Segmentina nitida* individuals appear to have been susceptible to water chemistry fluctuations, particularly rising pH values, in variously sized micro- and mesocosms in a laboratory setting. The hermaphroditic nature of *S. nitida* could not be confirmed in these experiments, with no increases in numbers consistent with previous literature on egg mass sizes in *S. nitida.*

An outdoor experiment using macrocosms provided no improvement in long-term breeding success. Low oxygen concentration caused by organic input and an unusually long and cold period of weather in winter seemed to have been responsible for high mortality levels of the *S. nitida* populations being reared outdoors. This experiment did suggest an overall increase in the number snails in macrocosms at some point during the experiment (based on the number of dead snails retrieved in November and at the end) and some individuals did survive for ten months in the macrocosms. However, throughout all experiments, fluctuation in water chemistry due to the relatively small volume of water and the influence of subsequent edge effects highlight the issues with breeding aquatic invertebrates at a small scale for reintroduction or population management. At a larger scale, mimicking wild abiotic conditions showed the risk of extreme conditions on survivability in the system. For small, rare invertebrates, especially freshwater snails, captive breeding on small scales may not be a viable conservation option in the presence of Allee effects, and removal of large numbers of individuals from source populations may also have significant negative effects. Breeding small, rare species at a small or large scale is therefore inadvisable, especially if impacts and population dynamics in response to population changes are not known or understood.

Chapter 4- Geometric morphometric analysis of *Segmentina nitida* shell morphology

4.1. Introduction

Morphometrics is the quantitative analysis of shape and variation in shape and has applications in almost every branch of biology (Liew and Schilthuizen, 2016), including in the study of evolution and development (Roth and Mercer, 2000), systematics (Rohlf, 1990), and developmental and ecological patterns of shape (Roth and Mercer 2000).

'Traditional' morphometrics, also known as multivariate morphometrics (Blackith and Reyment, 1971), consists of multivariate statistical analyses on morphological variables (Marcus 1990) such as linear measurements (distances between two points), as well as counts, ratios and angles (Adams *et al.*, 2004)). Traditional morphometrics allows covariation in morphological measurements to be quantified and assesses patterns of variation between samples (Adams *et al.,* 2004). One of the main issues with traditional morphometrics is its use of linear measurements (such as the maximum width or maximum height). Linear distance measurements are usually highly correlated with size (Bookstein *et al.*, 1985), and therefore methods for size correction had to be developed, to ensure shape difference was analysed independent of size (e.g. Sundberg 1989; Jungers *et al.*, 1995). Many methods of size correction have been proposed, though with little agreement on which method should be used (Adams *et al.*, 2004). Different size correction models yield different results, making it difficult to compare datasets. In addition to this, homology of linear distances can be difficult to assess because many distances, such as maximum width, are not defined by exact points and can therefore be quite subjective. Finally, distance measurements between two points will often not adequately represent the actual shape of the object (Figure 4.1). Sets of linear distances are usually unable to represent the geometry of the object, losing aspects of the shape (Adams *et al*., 2004).

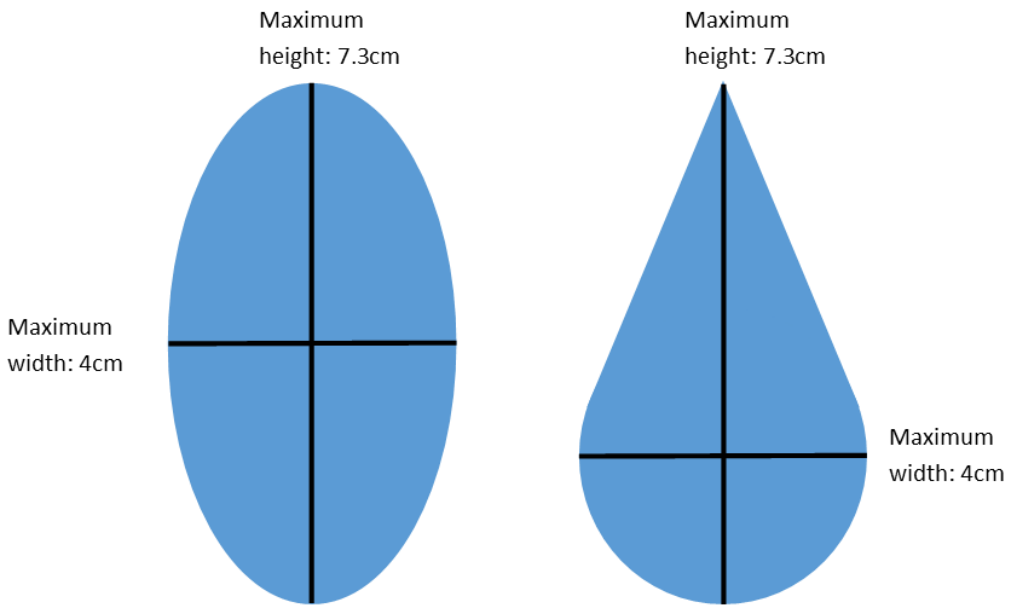


Figure 4.1. Diagram showing linear measurements as used in traditional morphometrics on two different shaped objects with identical maximum heights and widths.

In the 1970s-80s the field of morphometrics was revolutionized by the introduction of geometric morphometrics. Geometric morphometrics is the statistical analysis of form based on landmarks that can be given Cartesian coordinates in two- or three-dimensional space. Landmarks are discrete anatomical loci that can be recognised in all objects in the study (Zelditch *et al.*, 2004), and generally can be named, described and given Cartesian coordinates (e.g. 'tip of the spire', 'bridge of the nose' etc.) (Bookstein, 1991). The names are intended to imply correspondence (biological homology) among forms. Landmark-based geometric morphometrics utilise data collected as two- or three-dimensional coordinates of distinct, quantifiable, and biologically important points on organisms (Roth and Mercer, 2000; Adams *et al.*, 2004), therefore landmark-based geometric morphometrics provide a standardised, consistent and comprehensive analysis of shape variation (Buchanan *et al.*. 2014). Geometric morphometric analysis focusses purely on shape, the geometric information of an object after removal of location, orientation and size (Kendall, 1977).

When using landmark-based geometric morphometrics the landmarks must be present on all objects included in the analysis (Viscosi and Cardini, 2011). Choice of landmarks and features under analysis (e.g. anatomical, topographical, developmental etc.) must be meaningful in terms of the specific

hypothesis being tested (Klingenberg *et al.* 2002; Oxnard and O'Higgins, 2009). Landmark choice is a crucial step in the analysis of shape (Robinson *et al*., 2002) and landmarks should be chosen so that they (i) record homologous anatomical locations, (ii) do not change their topological positions relative to other landmarks (iii) provide adequate representation of morphology (iv) can be identified consistently and reliably, and (v) lie within the same plane (for 2D geometric morphometrics) (Zelditch *et al.*, 2004).

Bookstein (1991), defines three types of landmark used in geometric morphometrics, each defined by which features they represent:

Type I: Landmarks on points in space at which structures meet (e.g. "a local pattern of juxtaposition of tissue types or a small patch of some unusual histology").

Type II: A landmark that is on a point that is supported by geometric evidence (E.g. tips of teeth or claws).

Type III: Landmarks at diverse, finitely separated locations, such as end points of diameters at the bottom of a concavity.

Type I and II landmarks are ones that represent anatomical features on an object, whilst Type III landmarks are regarded as 'mathematical' landmarks. Type I landmarks are the preferred type of landmark in geometric morphometrics, but very few locations on an object fit this definition, and when comparing multiple species at higher levels of classifications, for example, even fewer such locations will exist across all specimens under investigation. Type II and Type III landmarks are concessions to practicality in terms of using mathematical points to describe complex geometries. Type II landmarks are difficult to locate precisely and consistently from form-to-form, but in principal are locations that can be represented by a single point. Type III landmarks are even more problematic because they depend either on the orientation of the object being measured, or the placement of other landmarks, or both.

The most common methods for analysis of landmark-based geometric morphometrics utilises 'Procrustes superimposition' (Sneath, 1967; Gower, 1975; Siegel and Benson, 1982; Rohlf and Slice, 1990). Procrustes superimposition minimises the sum of the squared distances among landmarks of each object under analysis by translation (shifting the landmarked samples together in a fixed direction), rotation ("spinning" the samples around a fixed point) and scaling the configurations to the same orientation and size (Rohlf and Slice 1990). Scaling the object to the same size is accomplished by dividing the coordinates of each form by its centroid size, which is defined as the square root of the sum of the squared distances between the geometric centre of the form and the landmarks (Bookstein 1991). The remaining differences in landmark positions after Procrustes superimposition are called "Procrustes residuals" and represent the shape differences between samples.

The shape of gastropod shells has been a popular area of study for morphometric analysis since the 19th century. As snails grow, new shell material is deposited on the existing aperture, resulting in accretionary growth at the margins of the aperture (Ackerly, 1989). This accretionary growth provides biologists with a detailed growth history throughout a snail's developmental history (ontogeny; (Johnston *et al*., 1991)). The form of an individual is a direct product of its ontogeny and can be used to investigate the factors affecting development, growth and overall phenotype of individuals or populations (Roth and Mercer 2000). Quantification of shell form can be challenging, as many shells lack homologous structures and have a spiral form that can be difficult to effectively capture with linear measurements (Liew and Schilthuizen, 2016). With juvenile snails there is also the issue of allometric growth, where parts of the shell grow and develop at different rates than others. Past the juvenile stage snails undergo isometric growth, where all aspects of the shell grow at the same rate.

Segmentina nitida has a history of being reclassified, with 43 name changes between 1774 and 1884 as the perceptions of families, genera and species have changed (Kennard and Woodward, 1926). It has often been conflated with the highly similar shaped species, *Hippeutis complanatus*. The present study will be the first where shell shape has been quantitatively investigated in *S. nitida.* Previous

studies considered only qualitative differences (Hill-Cottingham 2004). Geometric morphometrics may reveal statistical differences and areas of intra- and interspecific variation to be identified to enhance the distinction between and identification of the two species. Comparing *H. complanatus* and *S. nitida* will also provide statistical evidence of differences between the two species, (as the two species have been synonymised multiple times (Reeve 1863)) and identify features on the shell to better differentiate the two.

Geometric morphometrics has been used as an effective tool in phylogenetic studies on gastropods, either by itself (Cruz *et al.*, 2012) or complementing and contributing evidence for building phylogenetic hypotheses using molecular and morphological data (e.g. Smith and Hendricks, 2013). Perez (2011) used geometric morphometrics in combination with the cytochrome c oxidase subunit I (COI) and the 16S ribosomal markers to describe a new species in the *Practicolella* genus, as well as revise several species within the genus, which contains many morphologically similar species (Perez, 2011). This study showed differences between the species using landmarks across the shell in the apertural view, which showed distinct groupings of the various species. Interspecific analysis of the shell morphology of *Panopea generosa* (Gould 1850) and *Panopea globosa* (Dall 1898) by Leyva-Valencia *et al.* (2012) allowed quantifiable differences between the two species to be identified and confirmed the original species descriptions (Leyva-Valencia *et al.*, 2012).

In addition to aiding delimitation of species across a genera or higher order (Albrecht *et al.*, 2004) geometric morphometrics can be used to analyse intraspecific variation in gastropods. Many studies have analysed the effects of geographic distance and isolation (Sobrepeña and Demayo, 2014), environmental conditions (Kotsakiozi *et al.*, 2013) and other evolutionary pressures such as predator presence (Preston and Roberts, 2007) on shell morphology. Chiu *et al*. 2002, discovered two morphotypes of the viviparid Snail, *Cipangopaludina chinensis* in Taiwan, with distinct differences in spire shape between the morphotypes, most likely influenced by environmental factors such as water hardness and conductivity (Chiu *et al.*, 2002). In a laboratory study looking at the impact of

environmental factors in a laboratory experiment on different wild morphotypes of *Physa* species, it was found that changing environmental conditions could alter the shape of a population of snails within a single generation (Gustafson *et al.*, 2014), highlighting the influence of local conditions on shell morphology. Minton *et al.* (2008) used geometric morphometrics analyses on the genus *Lithasia*, to quantify phenotypic gradients in a freshwater environment, looking at the effect of upstream and downstream environments on freshwater snail shell morphology, through landmark-based comparison of populations (Minton *et al.,* 2008).

This chapter aims to assess and quantify the morphological differences between *Segmentina nitida* and the closely related species *Hippeutis complanatus* and identify aspects of the shell that are the most different between the two, through 2D landmark-based geometric morphometrics. Additionally, intraspecific variation of European populations of *S. nitida* was also analysed, using shells from snails inform the field and from museum collections to determine whether European populations differ in shape and therefore may be genetically isolated and distinct from each other, which impacts on the importance of conserving UK populations and the potential genetic ramifications of potential reintroductions into the UK from more abundant European populations. This chapter also aims to explore shape differences which might indicate differences in phenotype due to habitat and/or differences in genotype due to isolation in refugia during the last ice age, or strong evolutionary pressures in current populations of *S. nitida*.

4.2. Materials and Methods

4.2.1. Acquisition of *Segmentina nitida* and *Hippeutis complanatus* shell samples for geometric morphometric analysis

Twelve *Hippeutis complanatus* and twelve *Segmentina nitida* samples (Figure 4.2) were collected from a site in the Ash Level in the UK in the summer of 2016 (Figure 4.3). Samples were placed in absolute ethanol in 2mL sample tubes for transport to the laboratory. Species were distinguished by presence

(*S. nitida*) and absence (*H. complanatus*) of internal shell thickenings by hand lens in the field, and identification was confirmed by light microscopy in the laboratory.

Figure 4.2- *Segmentina nitida* individual (left) and *Hippeutis complanatus* individual (right)

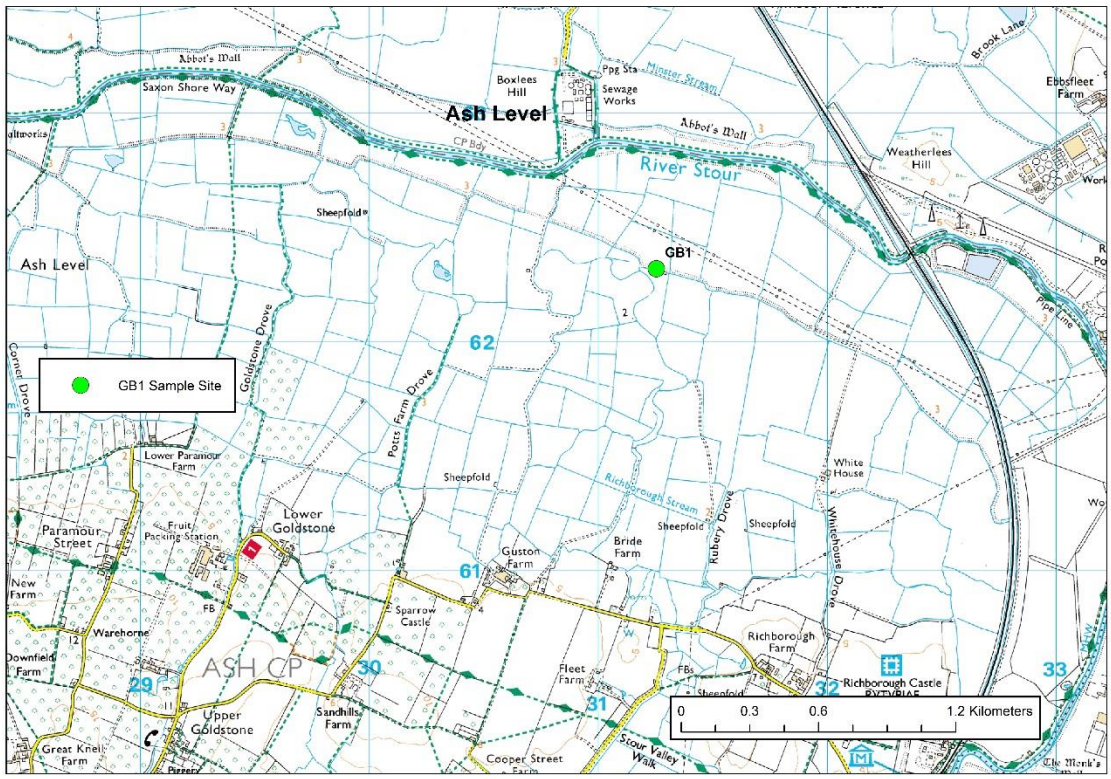


Figure 4.3. Map showing sample location in Ash Level for *Hippeutis complanatus* and *Segmentina nitida* individuals.

4.2.2. Acquisition of *Segmentina nitida* samples for intraspecific geometric morphometric analysis

All samples were obtained from the field in the summer of 2016. Samples were collected from locations in Germany, Poland, Sweden, and the UK, (Figure 4.3). Samples were stored in absolute ethanol or 95% isopropanol in 2ml sample tubes for transport from the field, and then transferred to

individual tubes in absolute ethanol upon arrival in the UK. Locations with GPS coordinates for each sample and sample ID numbers are included in Appendix B1. Samples were also obtained in 2017 from the collections of the Natural History Museum, London, and populations from the Czech Republic were sent to the author by Prof. Michal Horsák, Masaryk University in 2017 for inclusion in the analysis (Appendix B1). The number of shells from each country are shown in Table 4.1.

Table 4.1. Number of *Segmentina nitida* individuals from each European country used for intraspecific geometric morphometric shell shape analysis

Country	**Number of *Segmentina nitida***
Czech Republic	76
Germany	77
UK	100
Poland	113
Sweden	227
Total	593

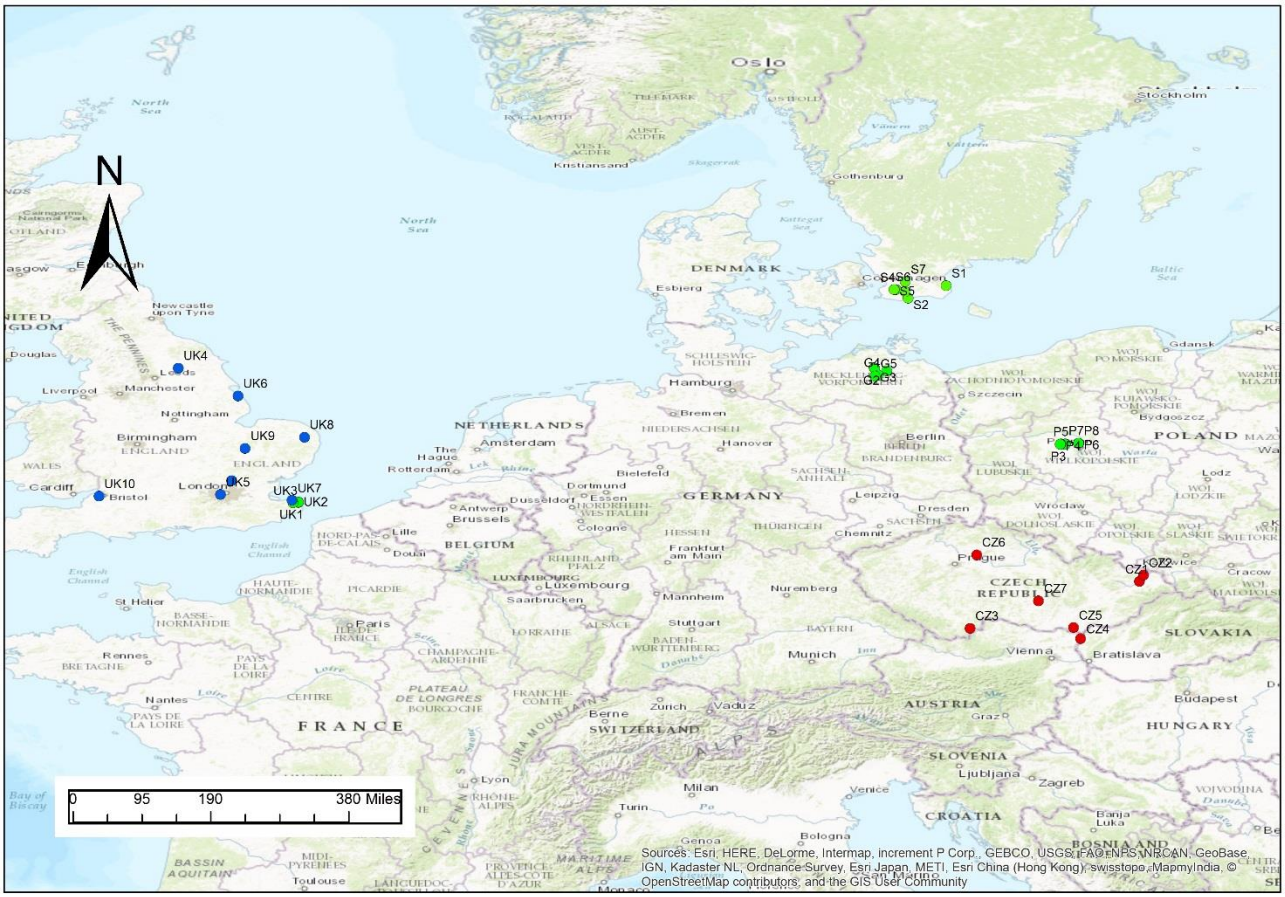


Figure 4.4**.** Map showing sample locations throughout Europe and source location of samples from the Natural History Museum London Mollusc collection, and Masaryk University (Czech Republic samples). Key: Green: Populations from 2016 fieldwork. Blue: Samples from Natural History Museum collection (London). Red: Samples donated by Prof. Michal Horsák (Masaryk University).

4.2.3. Photography of shells for two-dimensional geometric morphometric analysis

A photography rig was designed and built to ensure consistency in camera position, focal length and shell orientation between photographs. This rig consisted of a stage for the sample to be placed upon, with a reference line in the centre for the shells to be consistently oriented and lined up against. The camera was slotted into a set of rails to ensure that it could not tip, with a block of wood for the lens to rest upon. This rig setup is shown in Figures 4.4 and 4.5.

Figure 4.5. Photograph of rig setup for photography of individuals

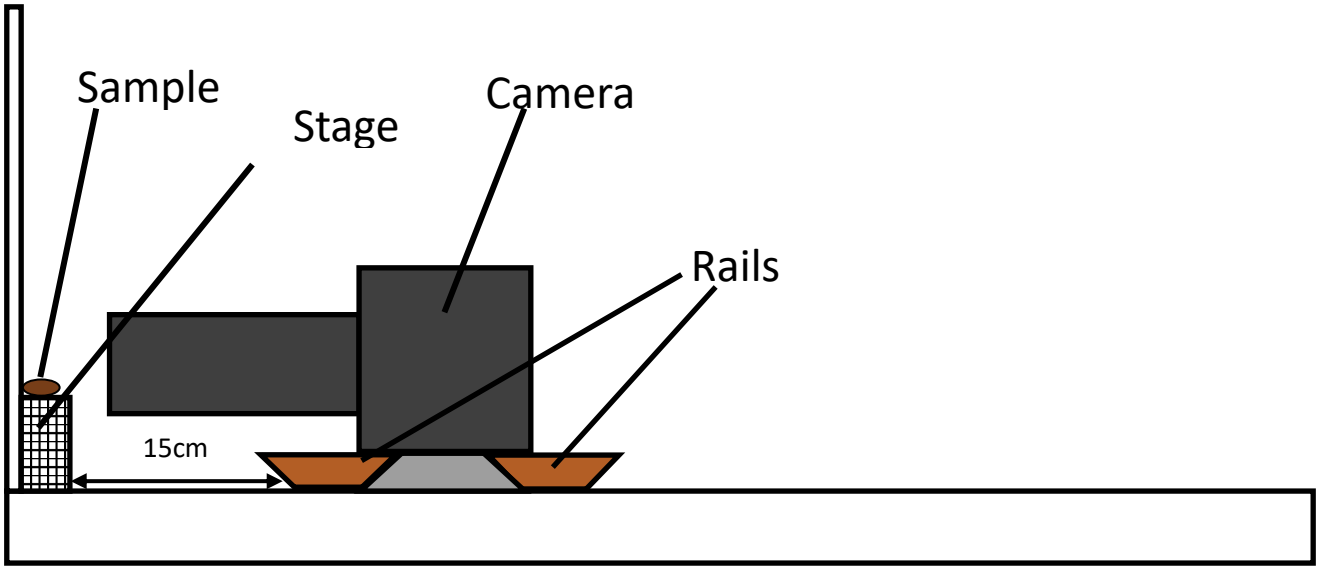


Figure 4.6. Diagram of rig setup

The camera used for the experiments was a Canon EOS 1100D, fitted with an extension tube to get the required magnification of the samples placed on the stage. Camera settings: ISO: AUTO, shutter speed: 0”8, aperture setting: F32. Samples were placed on the stage with the umbilicus facing upward and the aperture facing the camera and focussed by eye (Figure 4.6).

Figure 4.7. Example photograph of *Segmentina nitida* shell, with aperture on the left, lined up with vertical reference line on left of the shell.

For scale calibration, a photograph of a set of callipers was taken, and the 1mm distance on the callipers used as the scale for all subsequent photographs. This allows accurate measurements of diameter to be incorporated in the analysis of the images.

For the interspecific geometric morphometric analysis comparing the shape of *Hippeutis complanatus* and *Segmentina nitida,* twelve *H. complanatus* individuals and twelve *S. nitida* individuals from the UK were photographed (see 4.2.1). Shell shape in snails has been shown to change during ontogeny (Raup, 1961), therefore only adult individuals (>2mm) were used for this study. Specimens with damaged or broken shells, or obscured landmark positions were not included in the analysis. All shells imaged were taken from the same field location in the UK (GB1). This ensured that shape differences between the two species were not due to habitat differences.

For the intraspecific geometric morphometric analysis of variation in the shape of *S. nitida* across Europe, a total of 593 *Segmentina nitida* individuals from 5 countries were photographed. Again, only adult snails (>2mm) were photographed to avoid issues with allometry, and damaged shells were not included in analysis.

4.2.4. Digitisation and landmark placement for all geometric morphometric analysis

The photographs of shells were loaded into tpsUtil, version 1.65 to build a file for landmark placement. Landmark digitisation was performed in tpsDig2 version 2.22 (Rohlf, 2015). Six landmarks were used (Figure 4.6). Based on initial observations of samples in the field, it was anticipated that the shell structure with the most morphological variation was the aperture. There also seemed to be some variation in the positioning of the keel, a ridge around the edge of the shell (Figure 4.7).

Consequently, two landmarks were placed to capture the position and orientation of the keel of shells (Figure 4.7). In the literature, differentiation between *S. nitida* and *H. complanatus* has been based on the location of the keel, with the keel of *S. nitida* being dorsal, whilst the keel of *H. complanatus* is described as being located more median to the shell (Hill-Cottingham, 2004, 2008).

Landmarks 1, 4, and 6 are Type II landmarks (landmarks which show the tip of a structure) and they capture the widest part of the shell at each edge and on the inner body whorl in the aperture. These three landmarks represent the positioning of the keel on the shell. Landmarks 2, 3, and 5 represent Type I landmarks, and all show the joining of two structures at a definitive point. Landmark 2 represents the joining of the edge of the aperture and the outer body whorl. Landmark 3 represents the joining of the bottom of the aperture with the body whorl. Landmark 5 represents the joining of the top end of the aperture with the body whorl of the shell.

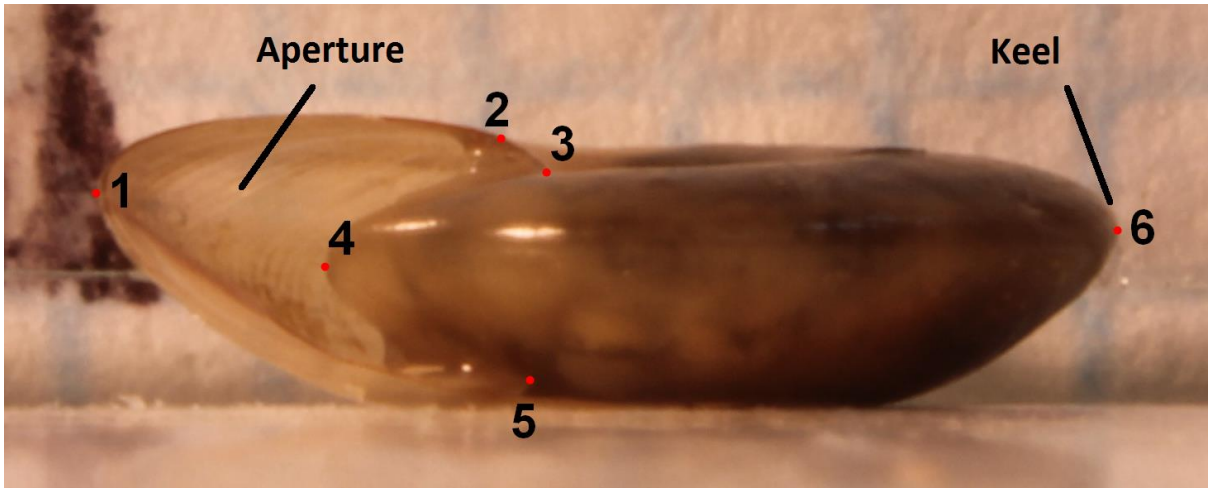


Figure 4.8. Landmark placement scheme for geometric morphometric analysis of snail shells. Red dots represent landmarks, next to corresponding landmark number.

4.2.5. Data analysis and statistics comparing shell morphology of *Segmentina nitida* and *Hippeutis complanatus*

Scale, orientation, and position were removed from landmark data using Procrustes superimposition with the R package 'geomorph' (Adams *et al.,* 2019), giving the Procrustes coordinates and centroid size for all individuals. A grouped Procrustes superimposition and a grouped Procrustes superimposition of the mean landmark coordinates for each population were performed using PCAGen (Sheets, 2014b), with shells grouped according to their country of origin. An analysis of jackknife variance was performed in CoordGen (Sheets, 2014a). This shows the overall shape variation in the sample set when each landmark is removed from the analysis in turn. If a low score (low variation) is produced when a landmark is removed, this landmark contributes more to the shape variation, relative to overall variation for all landmarks combined, and if a high score (high variation) is produced when a landmark is removed, it contributes less to the overall variation observed. This shows which landmarks vary the most across a dataset, highlighting where morphological differences occur.

The landmark coordinate dataset was then split by the two species (*Hippeutis complanatus* and *Segmentina nitida*) and the mean shape for each species calculated using 'geomorph'. These mean

shapes were then compared to each other using thin plate splines (deformation grids) in tpsSplin (Rohlf, 2016b). Deformation grids are graphical representations of two-dimensional shape variance, showing deformation of a square grid based on the difference in position of individual landmarks between one shape and another (Mitteroecker and Gunz, 2009). Expansion or contraction of the deformation grid shows where the positioning of a certain landmarks differs between the subjects of analysis. The greater the distortion in the grid, the more different that area of the organism is in comparison to the reference (Webster and Sheets, 2010).

A set of Procrustes shape coordinates was obtained from the Procrustes superimposition of all samples. These values represent the *x*- and *y*-coordinates of each of the landmarks used in the analysis, so for the six landmarks there are twelve Procrustes shape coordinates for each sample ([*X1, Y1]; [X2,Y2],* etc.). A principal component analysis (PCA) of the Procrustes shape coordinates was performed in 'geomorph' to determine the linear combinations of the coordinates that maximise the variation in the data. The loadings of each of the principal components were extracted to determine which areas of the shell contributed to the grouping and shape differences along each axis. Warp grids of the shape at the minimum and maximum value for each principal component visualised in relation to the mean shape were plotted with the PCA. A Mann-Whitney U test was run in Statistics Package for the Social Sciences (SPSS) version 24.0.0.2 (IBM 2018) on the principal components that described over 80% of the total shape variation in the sample, with samples grouped by species. The principal components had equal variance (Levene's test, $\alpha = 0.05$), and passed Box's M test ($\alpha = 0.05$) but were not normally distributed (Shapiro-Wilk test, $\alpha = 0.05$).

The Procrustes shape coordinates were used for a discriminant analysis in Past version 3.21 (Hammer *et al.,* 2001) to show groupings of the different species based on shape, and whether individuals from the two species could be distinguished from each other and assigned to the correct species by shape alone.

4.2.6. Data analysis and statistics comparing intraspecific shell morphology of European *Segmentina nitida* populations

Scale, orientation, and position were removed from landmark data using Procrustes superimposition with 'geomorph' (Adams *et al.,* 2019), giving Procrustes coordinates and centroid size for each sample. Grouped Procrustes superimpositions of the mean landmark coordinates for each population were performed using PCAGen (Sheets, 2014b), with shells grouped according to their country of origin. An analysis of jackknife variance was performed in CoordGen (Sheets, 2014a)

Centroid size is the square root of the sum of squared distances of all landmarks to the centroid (centre point) of the landmark configuration (Bookstein, 1991) and is often used as an estimator of size in geometric morphometrics studies (Bookstein, 1996; Frost *et al.*, 2003). The data did not have equal variance (Levine's test (α= 0.05)), so a Kruskal-Wallace test with pairwise differences was performed to compare median landmark centroid sizes between snails from different countries in SPSS. Shell size was calculated using the Traditional Morphometrics Generator tool in CoordGen (Sheets, 2014a) and regressed against centroid size to assess the relationship between the two.

The dataset was then split by country (GB, CZ, D, S, and P) and a consensus configuration was saved for each country (showing the mean shell shape for each country). These means were then compared to the overall mean shape for all individuals using thin plate splines to produce deformation grids in tpsSplin (Rohlf, 2016b).

A PCA of the Procrustes shape coordinates obtained from the Procrustes superimposition was performed in 'geomorph' to determine the linear combinations of the coordinates that maximise the variation in the data. The loadings of each of the principal components were extracted to determine which areas of the shell contributed to the grouping and shape differences along each axis. Warp grids showing the deviation of landmarks from the mean shape of the dataset at the minimum and maximum of each of the PCA axes were displayed.

The principal components failed both Levene's test of equal variance and the Shapiro-Wilk test of normality ($\alpha = 0.05$), therefore pairwise Kruskall-Wallis tests were performed in SPSS on the principal components that described over 80% of the total shape variation, with shells grouped by country. The Procrustes shape coordinates were then used for a discriminant analysis in Past version 3.21 (Hammer *et al*., 2001) to show groupings of the different populations by shape, and whether shells from different countries could be discriminated from each other and assigned to country of origin by shape alone.

4.3. Results

4.3.1. Geometric morphometric comparison of external shell shape of *Segmentina nitida and Hippeutis complanatus*

A grouped Procrustes superimposition of *H. complanatus* and *S. nitida* shows landmark locations for each species after size, rotation and sample placement on stage are removed (Figure 4.8). Tighter grouped points show lower spread in landmark (LM) position. The greatest difference in landmark position between *S. nitida* and *H. complanatus* were found at LMs 5, 6, and 4, with almost no overlap between the points. There is overlap at LM2 and the points at this location are comparatively widely spread, especially for *S. nitida* individuals. There is some overlap between the two species at LM3, with *S. nitida* being tightly grouped, and *H. complanatus* more loosely grouped.

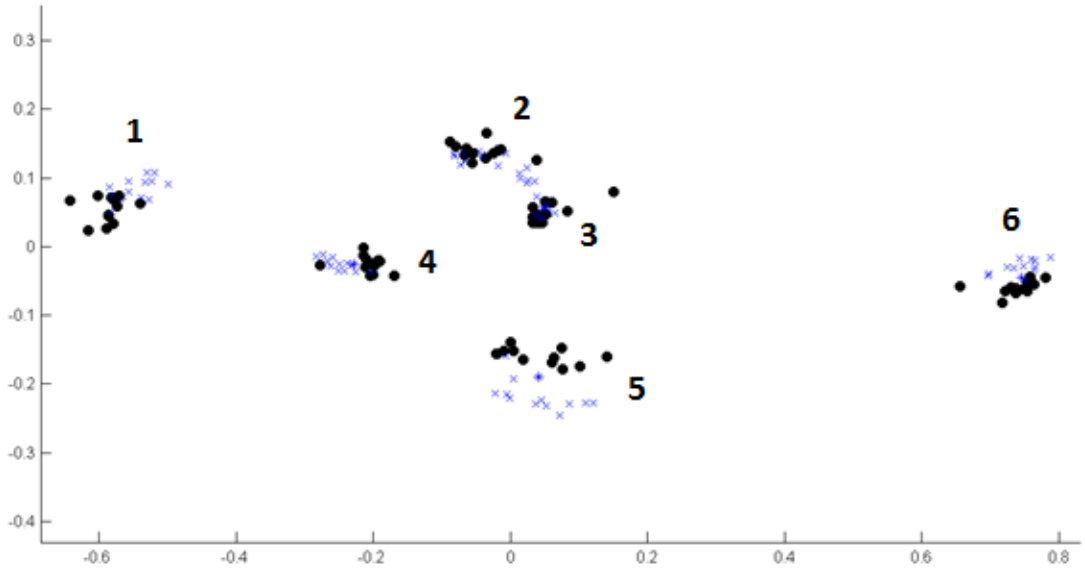


Figure 4.9. Grouped Procrustes superimposition of digitised *Segmentina nitida* and *Hippeutis complanatus* landmarks on shells. Symbols show the position of landmarks for each sample, grouped by country. Blue crosses= *S. nitida,* black circles= *H. complanatus*. Numbers indicate landmark positions.

The mean Procrustes superimposition of the mean landmark positions for the six landmarks, grouped by species (Figure 4.9) shows differences in landmark position between *H. complanatus* and *S. nitida*. The average placement of LM 2 and 3 for both species overlap, whilst LM 1, 4, 5 and 6 show clear distinction between the species. This shows *H. complanatus* with the keel higher on the shell (shells were photographed upside down) shown by LM1 and 6 and a narrower shell than *S. nitida*.

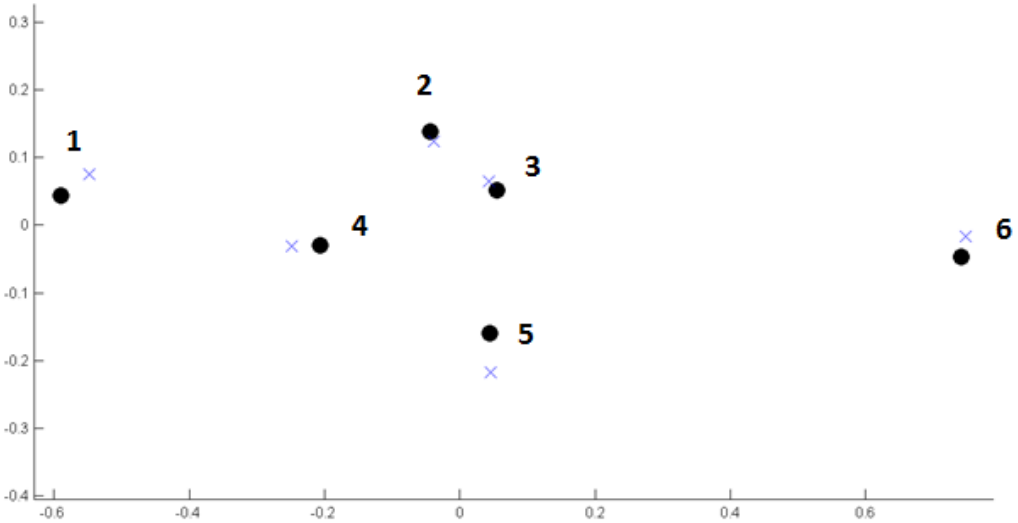


Figure 4.10. Procrustes superimposition of mean landmark positions of *Segmentina nitida* and *Hippeutis complanatus* samples. Blue crosses= *S. nitida,* black circles= *H. complanatus*. Numbers indicate landmark positions.

Jackknife variance is the variance in shape across all samples remaining as each landmark is omitted sequentially in order of contribution to shape variance (Table 4.2). When the remaining variance is low when a landmark is omitted, that indicates that landmark contributes more to the total variance.

Table 4.2. Remaining variance of all superimposed landmark points for *Segmentina nitida* and *Hippeutis complanatus* when each landmark is removed. Ranked by decreasing contribution to overall variance.

Landmark	Total remaining variance when landmark is omitted
5	0.0058992
2	0.0084803
3	0.0090875
4	0.0093627
1	0.011425
6	0.017929

LM 5 contributed the most to the overall variance in shape for all samples of *Hippeutis complanatus* and *Segmentina nitida* analysed, followed by LM 2, LM3, and LM 4, with LM 1 and 6 contributing the least to the variance. LMs 2-5 describe the shape of the aperture on the shells, whilst LMs 1 and 6 describe the keel of the shell.

Deformation grids comparing the consensus of *S. nitida* to the consensus of *H. complanatus* and *vice versa* (Figure 4.10) show strong bending of the grid lines around LM2 and reveal differences in the keel positioning (LM 1 and 6), causing bending of the overall grid. Deformation grid A, where *H. complanatus* is mapped to the reference of *S. nitida*, shows that *H. complanatus* has a much narrower shell than that of *S. nitida*.

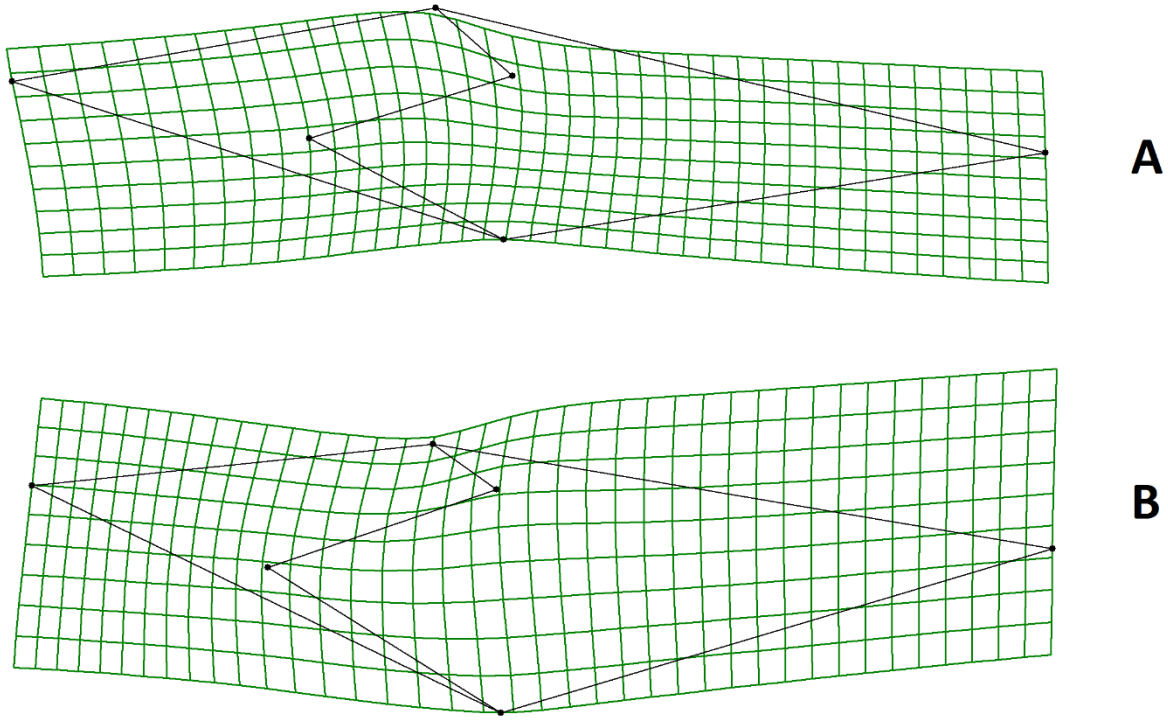


Figure 4.11. Deformation grids produced using tpsSpline. A) *Segmentina nitida* consensus used as reference shape, compared to *Hippeutis complanatus* consensus. B) *H. complanatus* consensus used as reference shape, compared to *S. nitida* consensus.

A principal component analysis of the 12 Procrustes shape coordinates of the six landmarks placed on *H. complanatus* and *S. nitida* shells shows distinct groupings for the two species when the first two principal components are plotted (Figure 4.11). The first three principal components (PC1, PC2, and PC3) account for 45.1%. 35.3%, and 12.2% of the variance in shape observed, respectively, and 88.6% of variance when combined. One of the *S. nitida* shells (GB1.10, Figure 4.11) grouped with the *H. complanatus* shells, and was likely misidentified prior to analysis. This misidentification has implications for the diagnostic characters that were used to differentiate between the two species, namely the white internal thickenings in the shell typically characteristic of *S. nitida* and absent in *H. complanatus* (Hill-Cottingham 2004). Repaired shell fractures in *H. complanatus* shells may appear as white lines like those representing the internal thickenings in *S. nitida*. When the sequence for the COI marker for GB1.10 was subjected to a BLAST analysis (Basic Local Alignment Search Tool: https://blast.ncbi.nlm.nih.gov/Blast.cgi) it showed 98.9% identity with the partial COI sequence for

Hippeutis complanatus (NCBI Accession Number EF012170 (Albrecht *et al.,* 2007), the highest identity score reported (98.67%). For statistical analyses, this specimen was therefore classified as *Hippeutis complanatus*.

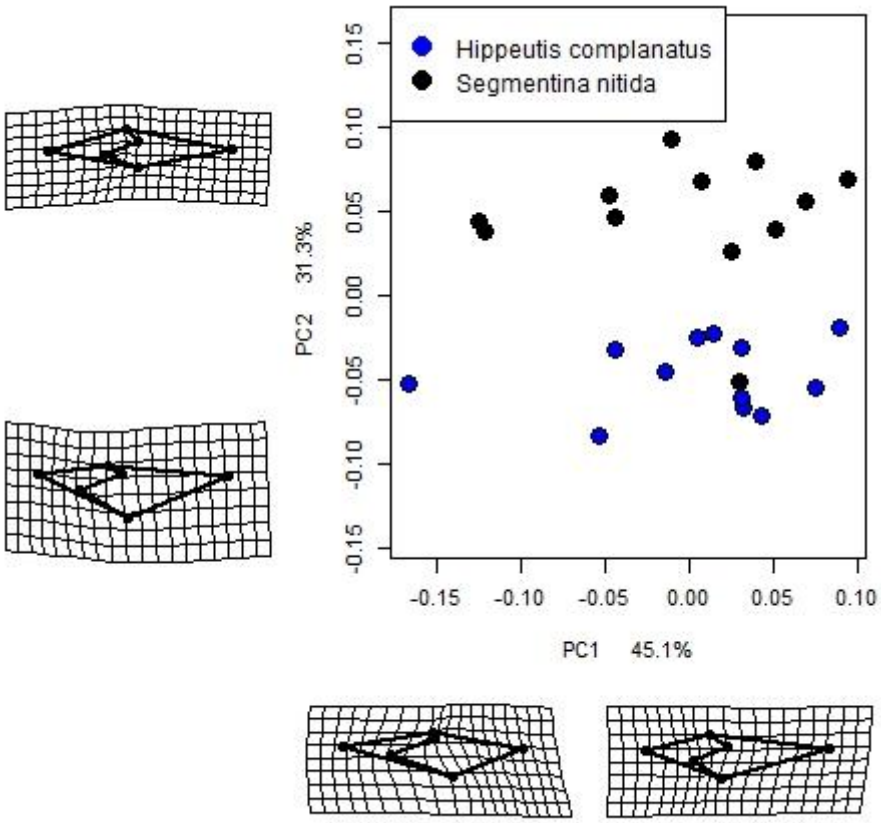


Figure 4.12. Ordination of the principal components of the 12 coordinates for 6 landmarks on *Hippeutis complanatus* and *Segmentina nitida* shells. Warp grids indicate position of landmarks positions at the minimum and maximum of each principal component in relation to mean shape of all individuals.

There was a significant difference in shell shape between *S. nitida* and *H. complanatus* based on PC2 (explaining 31.3% of the total shape variance) of the Procrustes shape coordinates, (Mann-Whitney U test, U=143, *P*=0.00). PC1 and PC3 (describing 45.1 and 12.2% of shape variation respectively) showed no significant difference between species (PC1: Mann-Whitney U test, U= 67, *P*=0.820; PC3: U= 54, *P*=0.331).

The discriminant analysis of the Procrustes shape coordinates of *S. nitida* and *H. complanatus* resulted in one discriminant function (DF) which accounted for 100% of shape variation. The X and Y coordinates for LM1 and the *y* coordinate for LM6 had the highest positive loadings for this DF, which represent the keel of the shell. The *y* coordinate for LM5 and the *x* coordinate for LM4 had the highest negative loadings for this DF, which represent the aperture (Table 4.3).

Table 4.3. Loadings for the discriminant function of the discriminant analysis of *Segmentina nitida* and *Hippeutis complanatus*, based on Procrustes shape coordinates for the six digitised landmarks.

	X1	**Y1**	**X2**	**Y2**	**X3**	**Y3**	**X4**	**Y4**	**X5**	**Y5**	**X6**	**Y6**
Loading	0.004	0.004	0.000	-0.002	-0.001	0.001	-0.005	-0.000	0.001	-0.006	0.000	0.003

This discriminant function assigned individuals to the correct species based on the Procrustes shape coordinates 95.83% of the time (jack-knifed), with one *H. complanatus* individual being classified as *S. nitida* (Table 4.4).

Table 4.4. Confusion matrix for discriminant analysis of *Segmentina nitida* and *Hippeutis complanatus*. Rows: given species from dataset. Columns: predicted groups based on discriminant function of Procrustes shape coordinates. Numbers indicate number of individuals classified as a particular species, with percentage of total number of individuals in parentheses. Blue cells indicate number and percent of individuals classified as the correct species

	Species assigned by discriminant function			
Actual species		***Hippeutis complanatus***	***Segmentina nitida***	**Total**
	Hippeutis complanatus	12 (92.3)	1 (7.7)	13
	Segmentina nitida	0 (0)	11 (100)	11
	Total	12	12	24

4.3.2. Geometric morphometrics analyses of shell shape variation in *Segmentina nitida* across Europe.

The grouped Procrustes superimposition (Figure 4.13 and 4.14) shows the spread of the landmark positions, grouped by country. There is a wide spread of points at LM 2 and LM5 (the top and bottom of the aperture), and the lowest spread was around at LM 1 and 6 (the keel).

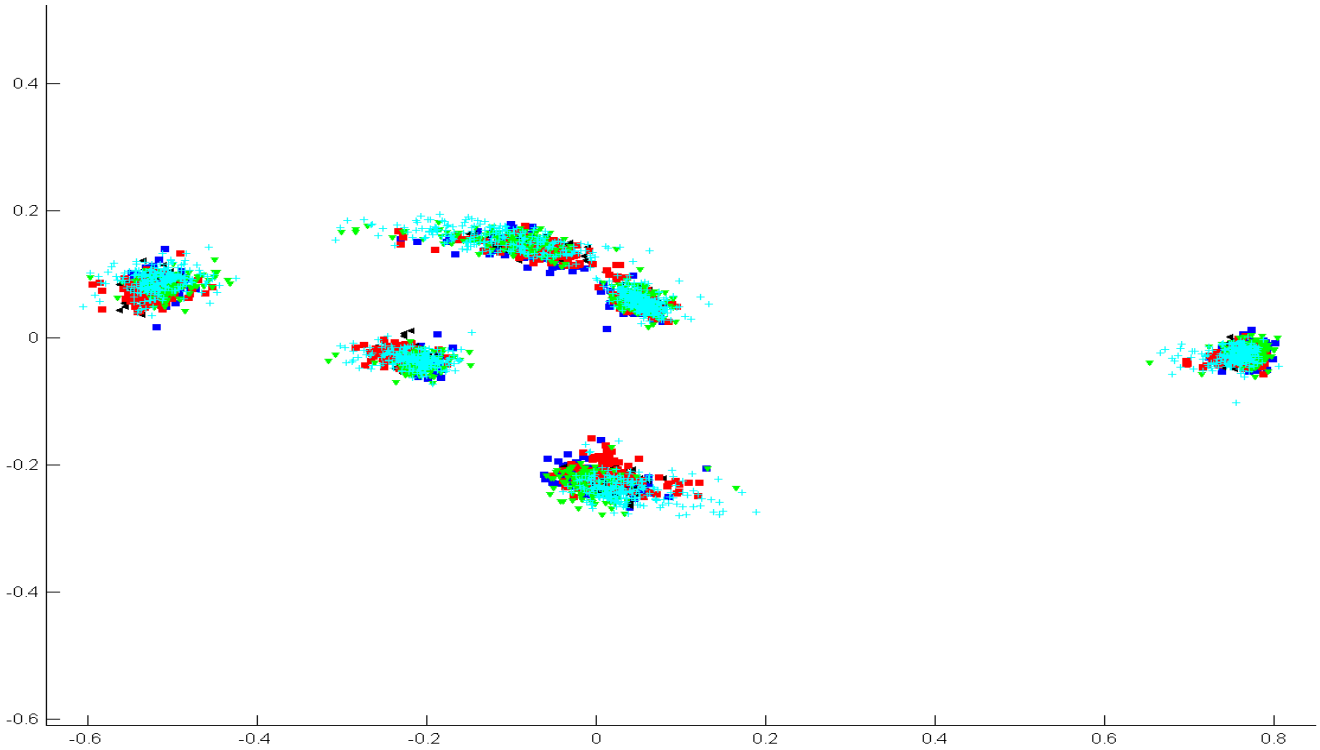


Figure 4.13. Procrustes superimposition of landmarks from all digitised European *Segmentina nitida* shells, grouped by country of origin. Red squares: UK. Green triangles: Poland. Blue squares: Czech Republic. Light blue crosses: Sweden. Black triangles: Germany.

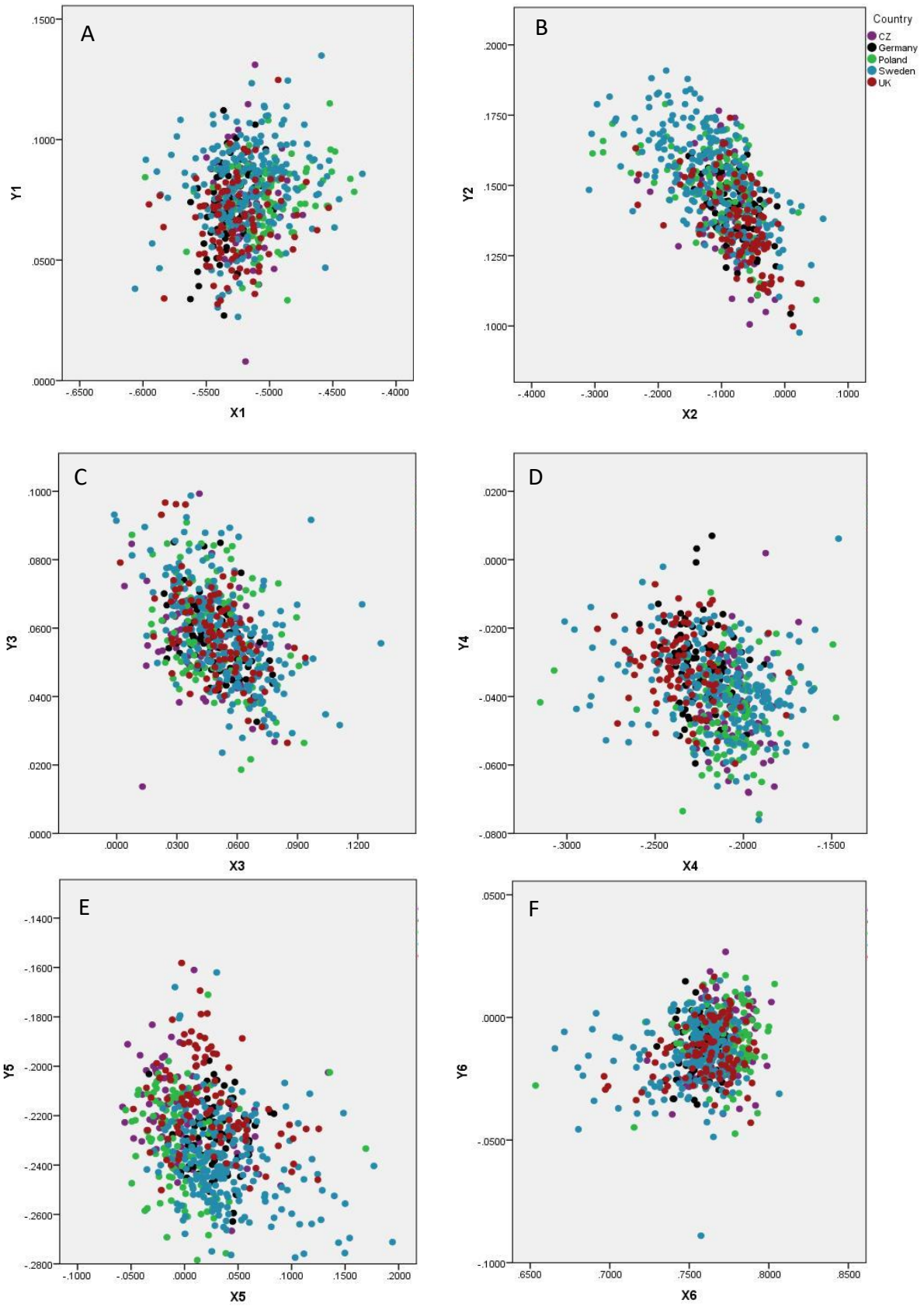


Figure 4.14. Procrustes coordinates for each shell at each landmark (LM). A) LM1; B) LM2; C) LM3; D) LM4; E) LM5; F) LM6. Key applies to all graphs. Purple: CZ; Black: Germany; Green: Poland; Red: UK; Blue: Sweden

LM 3 showed little variation between the mean landmark positions for the different countries, whilst LM 2, LM4, and LM5 show a relatively large spread of landmark locations within and between the countries. These landmarks describe the shape of the aperture.

LM 2 contribute most to the total variance in shape for all samples of *Segmentina nitida* analysed, followed by LM 5, LM3, and LM 4, with LM 1 and 6 contributing the least to the variance (Table 4.5). LMs 2 to 5 describe the shape of the aperture on the shells, whilst LMs 1 and 6 describe the keel of the shell.

Table 4.5. Total variance of all superimposed landmark points for *Segmentina nitida* when each landmark is removed in sequence. Organised by decreasing contribution to overall variance.

Landmark	Total variance when landmark is omitted
2	0.0039348
5	0.0064211
3	0.007917
4	0.0079643
1	0.01003
6	0.020527

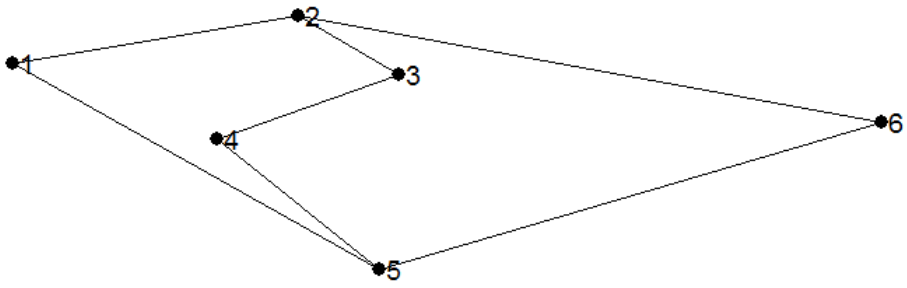


Figure 4.15. Consensus image of the average shaped *Segmentina nitida* shell (across all European populations) of the six landmarks placed in tpsDig

The average shape configuration of the six landmarks across all individuals digitised is shown in Figure 4.15. This consensus image was used as the reference for all thin-plate spline and deformation grid analysis of European populations. Thin-plate splines (deformation grids) show the difference between

S. nitida individuals from each country in relation to the mean shape (Figure 4.16). Key areas of variation from the average shape were LM2 (especially for the UK, which showed the most deformation at this location) showing the top of the aperture, and LM5, showing the bottom of the aperture. There was little variation in the positions of LM1 and 6 (the keel).

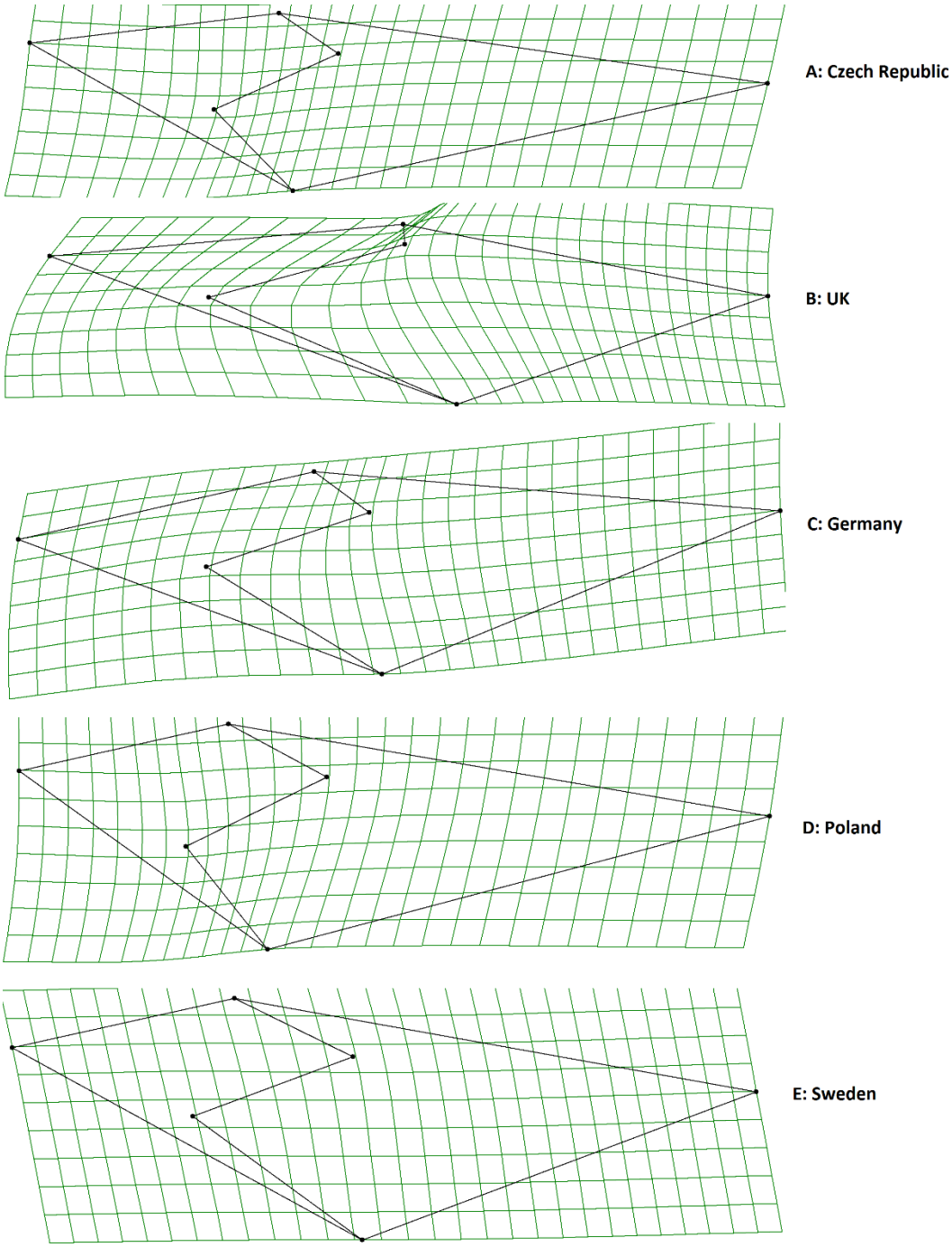


Figure 4.16. Thin-plate spline grids showing the deformation of the average shaped *Segmentina nitida* individual from each country in relation to the average shape of all *S. nitida* individuals.

There was a significant difference in centroid size between the individuals from different countries (Kruskall-Wallace test; H= 146.04 *P*= <0.0001).

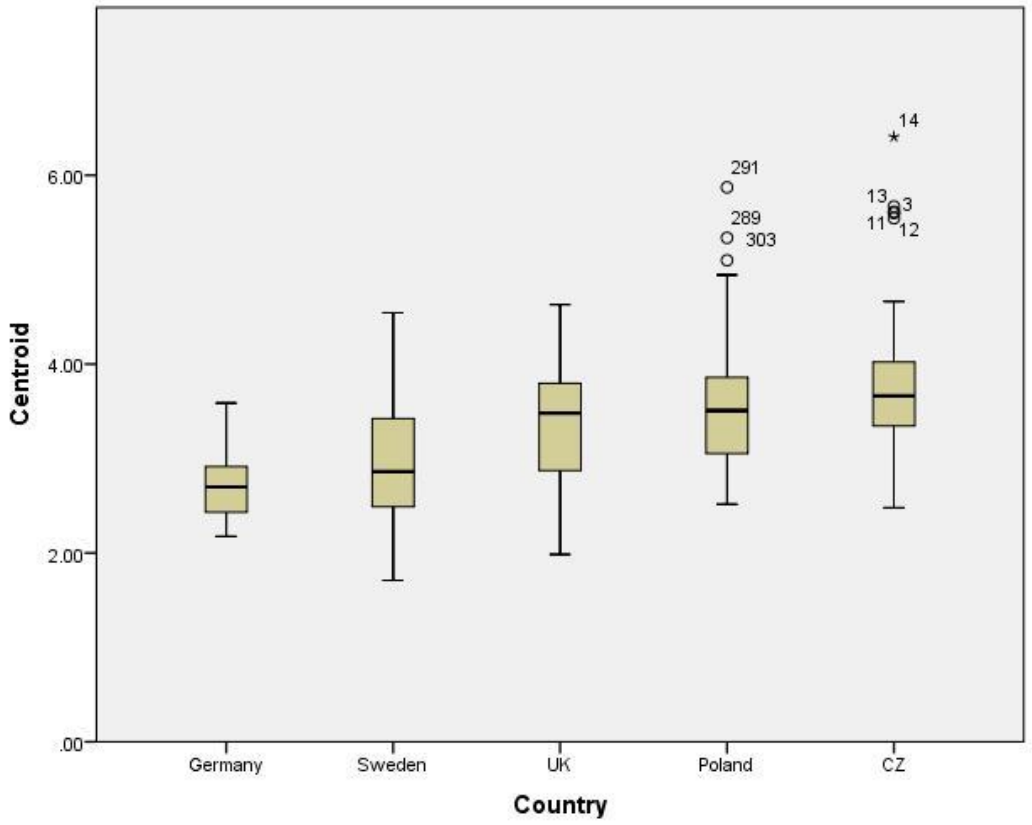


Figure 4.17. Boxplots of shell centroid size of *Segmentina nitida* individuals from five European countries. Groups are arranged by increasing centroid size. Lines inside boxes show median, boxes around median show upper and lower quartile and whiskers show outer quartiles. Sample sizes- Germany: 77; Sweden: 277; UK: 100; Poland: 113; CZ: 76.

German snails had the smallest centroid size, followed by Swedish, UK, Polish, and Czech snails (Figure 4.17). All outliers in the Polish and Czech Republic datasets were checked for digitising errors and contained none. The outliers were all very large shells (7.2-7.4mm in diameter) and may have been older snails. Of the Czech outliers, shell 12 (CZ1.12) appeared more concave in shape than other shells, whilst all the other outliers were unusually large. For the Polish outliers, shell 304 (P6.1) had a keel on the apertural (left) side of the shell that appeared lower than in other Polish samples, which affected the landmark positioning and resulted in a greater centroid size, while the other two outliers were larger overall than other samples.

Pairwise comparisons showed significant differences in centroid size between all countries except the UK and Poland, and Poland and the Czech Republic, based on Bonferroni-corrected *P*-values (Table 4.6).

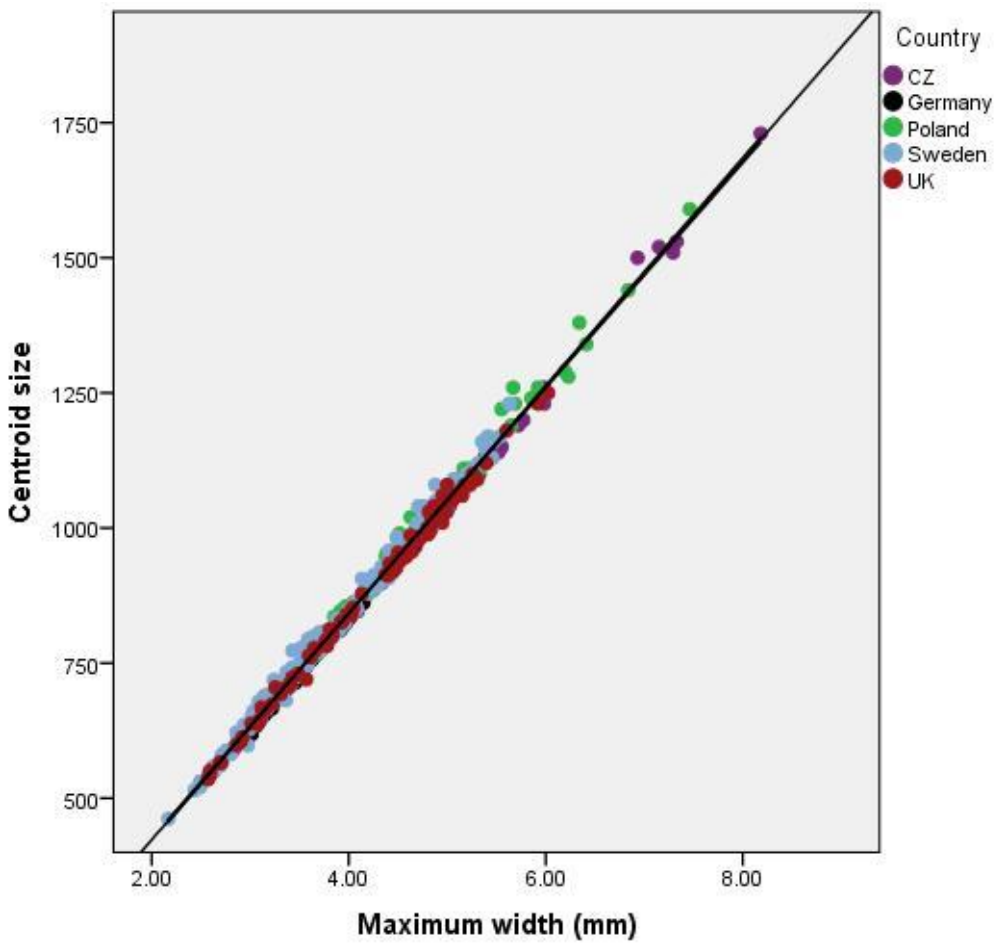


Figure 4.18. Graph showing the linear regression of the centroid size against maximum width of shell for each *Segmentina nitida* sample (mm), grouped by country.

Table 4.6. Pairwise Kruskal-Wallace tests of centroid size between *Segmentina nitida* individuals from each country. Values indicate Bonferroni-corrected *P* values. All significant values are indicated with *

Country	UK	Germany	Czech Rep.	Poland	Sweden
UK		0.00*	0.01*	0.30	0.00*
Germany	0.00*		0.00*	0.00*	0.01*
Czech Rep.	0.01*	0.00*		1.00	0.00*
Poland	0.30	0.00*	1.00		0.00*
Sweden	0.00*	0.01*	0.00*	0.00*	

There was a significant positive correlation between centroid size and shell width (R^2= 0.998, ANOVA *P*= <0.0001, F= 119813.885), showing that centroid size is a good measurement to use when comparing different populations of *S. nitida* (Figure 4.18).

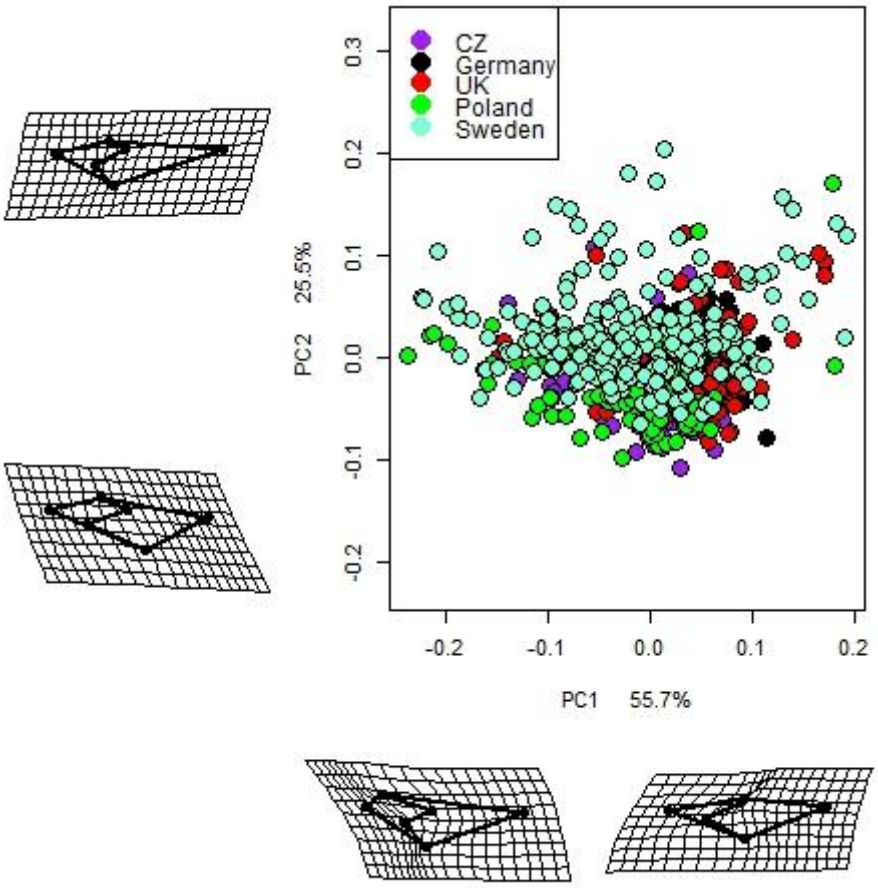


Figure 4.19. Principal component plot of *Segmentina nitida* individuals grouped by country of origin. Deformation grids indicate the shape of landmarks at the extremes of each Principal Component, in relation to the mean shape of all individuals.

A PCA of the 12 Procrustes shape coordinates of the six landmarks placed on *Segmentina nitida* individuals across Europe shows that for some populations the variation in shape within a population is almost as great as the variation between populations (Figure 4.19). The shells from Sweden and the UK seem to have the greatest variation in shape, whilst shells from Germany, the Czech Republic and Poland group more closely. PC1 and PC2 account for 81.2% of variation in shape (55.7% and 25.5% respectively).

There was a significant difference in both PC1 score and PC2 score between the individuals from different countries (Kruskall-Wallace test; PC1: H= 62.45 *P*= <0.0001; PC2: H= 148.93 *P*=<0.0001). Pairwise differences between countries for PC1 (which accounts for 55.7% of total shape variation) showed significant differences between individuals from the UK and individuals from Sweden, Poland, and the Czech Republic, and significant differences between individuals from Germany and individuals from Sweden and Poland (Table 4.7). For PC2 (accounting for 25.5% of variation) there were significant differences between all pairs of countries except the UK and Germany, Germany and Sweden, and Poland and the Czech Republic (Table 4.8).

Table 4.7. Pairwise Kruskal-Wallace tests of PC1 score between *Segmentina nitida* individuals from each country. Values indicate Bonferroni-corrected *P* values. All significant values are indicated with *

Country	UK	Germany	Czech Rep.	Poland	Sweden
UK		1.00	0.01*	0.00*	0.00*
Germany	1.00		0.11	0.00*	0.00*
Czech Rep.	0.01*	0.11		0.69	1.00
Poland	0.00*	0.00*	0.69		1.00
Sweden	0.00*	1.00	1.00	1.00	

Table 4.8 Pairwise Kruskal-Wallace tests of PC2 score between *Segmentina nitida* individuals from each country. Values indicate Bonferroni-corrected *P* values. All significant values are indicated with *

Country	UK	Germany	Czech Rep.	Poland	Sweden
UK		0.07	0.00*	0.00*	0.00*
Germany	0.07		0.12	0.00*	1.00
Czech Rep.	0.00*	0.12		1.00	0.00*
Poland	0.00*	0.00*	1.00		0.00*
Sweden	0.00*	1.00	0.00*	0.00*	

The first two discriminant functions (DF), DF1 and DF2 of the discriminant analysis of the 12 Procrustes components accounted for 48.47% and 33.05% of shape variation (81.52% total). For DF1 the *x*

coordinate for LM4 had the highest positive loading and the *x* coordinate for LM2 and the *y* coordinate for LM5 had the highest negative loading (Table 4.9). For DF2 the *x* coordinate for LM5 had the highest positive loading, and *x* coordinates for LM1 and LM6 had the highest negative loadings (Table 4.9).

Table 4.9. Loadings for the discriminant functions of the discriminant analysis of the European populations of *Segmentina nitida,* based on Procrustes shape coordinates for the six digitised landmarks.

Coordinate	**Axis 1**	**Axis 2**
X1	0.0074395	-0.010475
Y1	0.0076104	0.0024417
X2	-0.023657	-0.0020483
Y2	0.0068605	0.0031804
X3	0.0020259	0.0041985
Y3	0.00047018	-0.00014654
X4	0.011996	-0.0081781
Y4	-0.0041147	0.005522
X5	0.0026011	0.02964
Y5	-0.010992	-0.0053858
X6	-0.00040595	-0.013137
Y6	0.00016559	-0.0056118

These two DFs correctly assigned 48.8% of the shells in the analysis to their country of origin. The percentage of correct assignment across all countries ranged from 39% (Germany) to 58.6% (the UK). The highest levels of incorrect assignment were obtained for individuals from the Czech Republic that were classified as Polish (22.1%), and German individuals that were classified as from the UK (20.8%), indicating similarities in shape between these populations (Table 4.10).

Table 4.10. Matrix of classifications of European *Segmentina nitida* shells in discriminant analysis model of Procrustes shape coordinates. Rows: country shell originated from. Columns: Predicted country based on shape. Numbers indicate number of individuals assigned to each country, with percentage of total number of individuals collected from that country in parentheses. Blue cells indicate number and percent of individuals assigned to the correct country.

		Country assigned by analysis					
Actual country of origin		CZ	Germany	UK	Poland	Sweden	Total
	CZ	36 (47.4)	10 (13.2)	7 (9.2)	12 (15.7)	11 (14.5)	76
	Germany	10 (13.0)	30 (39.0)	16 (20.8)	6 (7.8)	15 (19.4)	77
	UK	8 (8.1)	13 (13.1)	58 (58.6)	12 (12.1)	8 (8.1)	99
	Poland	25 (22.1)	7 (6.2)	5 (4.4)	55 (48.7)	21 (18.6)	113
	Sweden	23 (10.1)	33 (14.5)	25 (11.0)	36 (15.9)	110 (48.5)	227
	Total	102	93	111	121	165	592

4.4. Discussion

4.4.1. Differences in shape between *Hippeutis complanatus* and *Segmentina nitida*

Traditionally, species descriptions have been based on qualitative, often subjective visual comparisons of morphology (Mutanen and Pretorius, 2007). Geometric morphometrics has been shown to be a powerful tool for solving complex species-level identification problems (Matias *et al.*, 2001; Gumiel *et al.*, 2003; Shipunov and Bateman, 2005; Mutanen and Pretorius, 2007). Geometric morphometrics, and the statistical analysis of shape provides a much more accurate identification of individuals than visual differentiation alone, especially when dealing with cryptic and species of similar morphology (Mutanen and Pretorius, 2007). This is especially important for *S. nitida* and *H. complanatus* as they have been classified as the same species at several points in their history (reviewed in Hill-Cottingham (2004)). The geometric morphometrics analyses of the shell morphology of *Segmentina nitida* and *Hippeutis complanatus* revealed distinct shell shapes that can be separated into two groups based on the results of both principal components analysis and discriminant analysis. The main differences in shape between the two species are shown in the location of the keel, as well as the aperture shape. The areas of greatest variation in shape were the top of the aperture, where it meets the body whorl,

as well as the position of the keel at the edges of the shell and across the aperture. The keel for *H. complanatus* is much higher on the shell than in *S. nitida*, and is much less pronounced in the aperture, resulting in a much wider aperture, with an overall narrower shell shape than *S. nitida*. This agrees with qualitative descriptions of the two species as given by Hill-Cottingham (2004). A discriminant analysis based on the Procrustes coordinates could assign individuals to the correct species based on shape alone over 95% of the time, with only one individual assigned to the incorrect species, indicating that the six chosen landmarks can be reliably used to differentiate between the species if required.

An interesting outlier in the analysis was the placement of sample 21 (a sample classified as *S. nitida* based on the apparent presence of shell thickenings) in the PCA, which grouped with *H. complanatus* shells. This could be a due to misidentification of the snail when collected from the field. This will be explored in Chapter 5, focussing on the genetics of *S. nitida* across Europe, where genetic differences between this samples and other *S. nitida* may confirm this hypothesis. This would also confirm the misclassification and support the evidence that the six landmarks and the PCA analysis can highlight incorrectly classified samples based on overall shape.

4.4.2. Shape variation of *Segmentina nitida* between populations in different countries across Europe

4.4.2.1. Overall shape variation in European populations of *S. nitida*

Shape variation in molluscs is often attributed to 'phenotypic plasticity', which results in issues with classification and differentiation between species (Minton, 2002; Perez and Minton, 2008). Phenotypic plasticity refers to the expression of alternative phenotypes by the same genotype, usually in response to environmental conditions (Stearns, 1989). Plasticity in snail shell form has been attributed to factors such as thermal stress (Hazel and Johnson, 1990), population density (Kemp and Bertness, 1984), and predation (Appleton and Palmer, 1988; DeWitt, 1998). This change in shell morphology can occur suddenly, sometimes within the growth phase of a single generation (Johnson and Black, 1999; Urabe, 2000; Minton and Gunderson, 2001). Phenotypic plasticity in conjunction with

evolutionary pressures may drive variation in shell morphology (Kistner and Dybdahl, 2013). Jackknife variance and deformation grid analysis of the shell morphology of *S. nitida* individuals from populations across Europe showed that variation was greatest in aperture shape, with little variation in the positioning of the keel. Shell shape and aperture shape are not phylogenetically constrained and have been found relate to divergence of populations (Kotsakiozi *et al.*, 2013). Apertural shape variation has been observed *Elimia livescens* (Pleuroceridae), with variation between snails occupying different habitats (flowing and non-flowing), latitude and longitude, temperature, conductivity, substrate type, presence of woody debris and drainage area (Dunithan *et al.,* 2012). The golden apple snail *Pomacea canaliculata*, a freshwater gastropod species, has shown variation in shape in the shell apex, aperture and operculum, with shape potentially affected by geographical location, water flow and substrates (Torres *et al.*, 2013). Whilst the morphology of gastropods can vary with multiple environmental and biotic variables (Covich, 2010) the majority of studies on spatial variation in morphology that have investigated potential biotic or abiotic influences on shape provide only correlative analyses. They are therefore unable to verify mechanistic causes of morphological variation, and can be influenced by additional, un-quantified variables (Dunithan *et al.,* 2012). Genetic analyses may contribute toward confirming whether the observed morphological variation in *Segmentina nitida* results from directional local selection, phenotypic plasticity, or both.

4.4.2.2. Shape similarity in *S. nitida* populations with similar habitats

The principal components analysis of Procrustes shape coordinates, and statistical analysis of generated principal components showed similarities in shell shape between *S. nitida* populations in the Czech Republic and Poland, as well as between snails from the UK and from Germany. From observations in the field, *S. nitida* individuals from Germany and the UK share similar habitats, both inhabiting permanent water bodies, at advanced stages of hydroseral succession (Kerney, 1991b; Watson, 2002; Watson and Ormerod, 2004a; Zettler *et al.*, 2006; Clark, 2011). UK habitats appeared to be in later stages of succession, with much less open water than those in Germany (pers. obs.).

Morphological variation between populations of a species may be attributed to either genetic differences or to environmental stresses acting on the genotype to influence phenotypic expression of characters (Kemp and Bertness, 1984; Preston and Roberts, 2007). Freshwater habitats tend to have high spatial and temporal variability of attributes such as water chemistry, water temperature, and drying of habitats (Cole, 1994). This physical variation results in high potential shape variation among populations of freshwater gastropods (Dunithan *et al.,* 2012). German and UK habitats showing similar but not identical shell morphology could be a result of the similarity of their habitats, but likely some differences in other variables, such as water chemistry, temperature or substrate, specifically *S. nitida* being described as a snail that does well in acidic water in Germany (Zettler *et al.*, 2006), very different from the UK. Adaptive evolution in response to local regimes of natural selection leads to genotypes specialised for different local environments, and also facilitates population spread across an environmental gradient (Lee, 2002; Lee and Gelembiuk, 2008). Specific shell morphologies are favoured under different environmental conditions such as flow-rate in lotic habitats, temperature, and predator abundance (Struhsaker, 1968; Janson and Sundberg, 1983; Vermeij, 1995; Rolan-Alvarez *et al.,* 1997; Bourdeau, 2009). However, it is unlikely that flow rate is affecting the shape differences in *S. nitida*, as they live in stagnant habitats. One of the unexplored pressures in lentic freshwater habitats could be permanence of the habitats. When habitats dry out, snails will enter aestivation (Russell-Hunter and Eversole, 1976; Jokinen, 1978). In Poland, *S. nitida* is found in semi-permanent kettle ponds (Książkiewicz and Gołdyn, 2008), whilst in the UK and Germany, *S. nitida* inhabits permanent drainage ditches surrounding grazing and farmland (Kerney, 1991b; Watson and Ormerod, 2004a; Clark, 2011). The species is found in mostly permanent shallow glacial ponds in Sweden (pers. obv.). The need to enter aestivation upon drying out of the water body may be a strong pressure on the shape variation in the aperture, which is partly responsible for protection from desiccation (McNair *et al.*, 1981) and can reduce the energy needed for forming the seal across the aperture to enter aestivation or increase its effectivity at preventing desiccation, which may help explain the difference in aperture shape between Polish and UK snails, for example.

4.4.2.3. Shape variation in relation to dispersal patterns

Shape can be determined by the genotype of individuals, and genetic isolation of populations would lead to distinct genotypes across Europe, possibly represented phenotypically. The pattern of variation in shell morphology of *S. nitida* across Europe may be influenced by the history, vectors, and ability for dispersal of *S. nitida*. The dispersal of less-mobile aquatic organisms over land via waterbirds was hypothesised by Darwin (1859), due to the high diversity found in relatively isolated freshwater habitats. Wetlands are often regarded as a 'relict' habitat, one which was much more widespread historically, and due to human influence and habitat change is now becoming increasingly fragmented (Van Strien *et al.*, 1991; Armitage *et al.*, 2003; Herzon and Helenius, 2008). *S. nitida* have limited dispersal ability (Niggebrugge *et al.*, 2007), and anecdotal evidence from Sadler (pers. comm.) has highlighted the poor dispersal of *S. nitida* between adjacent ditches even after prolonged flooding of a site. Therefore, it seems likely that any movement of the species would be through means of a vector, such as waterbirds. The frequent, directed movements of waterbirds between ecologically similar wetlands make them particularly suitable transport vectors (Figuerola and Green, 2002; Green *et al.* 2002; Bohonak and Jenkins, 2003; Nathan *et al.*, 2008; van Leeuwen, *et al.*, 2012). Snails or their propagules (egg masses) can be transported via avian means in one of two ways: endozoochory (internal transport via gut) or ectozoochory (external dispersal caught on feathers or on feet) (Frisch *et al.,* 2007). In other planorbids, such as the genera *Planorbarius* (a fairly closely related genus to *S. nitida*), fossil and actual distribution patterns have indicated that dispersal via birds played an important part in dispersal in the geological past (Wesselingh *et al.,* 1999). With the similarity between German and UK habitats (Kerney, 1991b; Watson, 2002; Watson and Ormerod, 2004a; Clark, 2011) not only would the snails be potentially under the same environmental evolutionary pressures on phenotype, but transport of propagules in the gut of birds, or of whole snails on feathers or feet, would increase connectivity of the populations. If these populations are much more closely related through this bird-mediated dispersal, with phenotypic similarities this may also be expressed genotypically, which is explored further in Chapter 5. This may also explain the greater difference in

shape between UK individuals and those in Poland, Sweden and the Czech Republic, which are all further away, and are very different habitats (at least for Sweden and Poland) from those found in the UK, that may not be frequented by the same birds.

4.4.2.4. Shape variation in relation to geographic history and glacial refugia

Population structure is affected not only by current habitat and environmental conditions, but also historical conditions (Hewitt 1999). In Central and Northern Europe, a major aspect of the historical geography of species have been the periods of glaciation during ice ages (Hewitt, 2000, Stewart *et al.,* 2009). The advancing and retreating of the ice sheets in glacial cycles during the most recent glacial maximum (LGM), around 23-18ka before present (BP), had a great impact on the current distribution of species throughout Europe (Martinez *et al.*, 2004; Provan and Bennett, 2008; Normand *et al.*, 2011). During the glacial maximum of the last ice age, most of Scandinavia and the UK was covered by an ice sheet, with much of northern Europe covered in permafrost (Provan and Bennett, 2008). Responses of species to glacial periods include range contractions and local extinctions (Dalén *et al.,* 2007; Schmitt 2007; Stewart *et al.,* 2009) as well as failure to recolonise previously glaciated areas due to dispersal limitation and biotic interactions (Normand *et al,*. 2011). Molecular data has confirmed that the southern peninsulas of Europe acted (Iberia, Italy, the Balkans, Turkey and Greece) as refugia in the last ice age (Hewitt, 1999; Sommer and Nadachowski 2006, Kühne *et al.,* 2017). Glacial refugia are locations further north where local climatic conditions were less extreme and therefore allowed the persistence of some of the biota throughout the glacial period, which have been identified as the source of many mammalian species across Europe (Vega *et al.*, 2010; McDevitt *et al.,* 2012; Herman *et al.,* 2016). Glacial refugia could have served as sources for biotic dispersal upon regional deglaciation after the glacial maximum (Carrara *et al.*, 2007). During the ice age many species which now range across Europe would have had their refuges in the south of Europe (Hewitt*,* 1999*,* Petit *et al.,* 2003). When the climate warmed these species expanded northward from these refugia. Some species would colonise from just one refugium, others from several (Hewitt, 1999, Herman *et al.,*

2016). This may also have an impact in the shape similarities and differences observed in populations of *S. nitida* across Europe. German and UK snails may have originated from the same refugium, possibly from the south Iberian or Calabrian refugia, whilst Polish and Czech Republic populations may have colonised from identified refugia in the Carpathian Mountains, known to harbour snail assemblages during the Holocene (Horsák *et al*,. 2019). The genetic structure of these populations, and their divergence from each other, may help to identify potential origins for the populations, and be able to relate them to geographic subdivision and their divergence, and see if these patterns are similar to those shown in the shell morphology. An alternative scenario is that possibly some populations of *S. nitida* in the UK or elsewhere persisted through the ice age in previously undiscovered local refugia, rather than through an expansion from the southern refugia. However, being able to prove this with the current samples would be impossible. This study looks at relative shape differences from current-day specimens, without access to glacial or pre-glacial era samples it is impossible to test for historical trends in shape, and infer which scenario is more likely. Due to the small size of *S. nitida*, their thin, fragile shell, and the temporary nature of their habitats, finding these samples would prove to be very difficult.

4.4.2.5. Differences in centroid size between populations of *S. nitida*

In the analysis of centroid size, Polish and Czech Republic snails had much bigger centroids than the other countries, whilst Germany had the smallest. This is unlikely to be caused by temporal differences in the sampling of the snails, as samples were collected from Germany first, then Poland, followed by Sweden, and collected in the space of a week. This appears too short a time frame for populations to show appreciable differences in growth due to timing, though this may be due to climate differences between the countries, with Swedish samples growing more slowly due to a lower temperature than other countries. The difference in centroid sizes may be related again to environmental conditions, dispersal or historical biogeography of the species, or a combination thereof. Larger land snails have been found to be more attractive as prey than smaller snails, increasing their chances of passive

dispersal (Pfenninger, 2004). With the semi-permanence of the habitats found in Poland (Książkiewicz and Gołdyn, 2008), this tendency towards a larger sized shell may be an evolved trait to enable the snails to disperse more readily to other viable habitats, maximising survival. Additionally, there is evidence of tissue degrowth in snails when they are in aestivation for a long time (Russell-Hunter and Eversole, 1976). With Polish *S. nitida* having to be in aestivation for a long time, sometimes for several years (Książkiewicz and Gołdyn 2008; Gołdyn pers. comm.), being larger and able to suffer from this de-growth for a longer period of time would be an important environmental adaption. This would also benefit long-range ectozoochory dispersal of individuals, as they are at higher risk of desiccation (Van Leeuwen, 2012), therefore entering aestivation for long periods would be beneficial. For the much smaller German snails, in bird-mediated endozoochory of macro-invertebrates, smaller propagules are less affected by digestion than larger ones (Van Leeuwen *et al.*, 2012). Therefore, being smaller may be of benefit to these snails to produce smaller eggs, aiding in dispersal of the species in the gut of wading birds visiting wetlands, potentially explaining their small size, when combined with the more permanent nature of their habitat.

4.5. Conclusion

The geometric morphometrics analyses presented in this chapter highlight shape and size differences between European *Segmentina nitida* populations. These shape differences seem to have a pattern, with UK and German samples clustering together, and Sweden, Poland and the Czech Republic clustering together based on principal components analysis and pairwise comparison of the generated principle components, as well as discriminant analysis. These differences may be the result of phenotypic plasticity and adaption of populations to local habitats, or may reflect genetic differences between populations, genetic isolation, and potentially patterns pre- or post-glacial dispersal and biogeography.

Chapter 5: Population genetics of European populations of *Segmentina nitida*

5.1. Introduction

Population genetics is the study of genetic diversity and variation within and between natural populations of a species. The primary goals of researching population genetics are to understand the factors determining evolutionary change and stasis, as well as describe the distribution of genetic variation within and between populations (Mavárez *et al.*, 2002a; Hedrick, 2005; Hartl and Clark, 2007). Genetic diversity within a population or species is determined by multiple interconnected factors, including mutation, hybridisation, migration, selection, and genetic drift (Harrison, 1991; Vellend and Geber, 2005; Gu *et al.*, 2015). The relative role and impact that each of these forces play in generating or reducing genetic variation and diversity depends on life-history traits (Bowen *et al.*, 2014), the mating system (Samadi *et al.*, 1999) and the dispersal ability of a species (Zickovich and Bohonak, 2007). Typically, genetic drift (the change of frequency of alleles over time due to random chance) and inbreeding reduce the amount of genetic variation in a population, and mutation tends to increase genetic variation (Hartl and Clark, 2007). Inbreeding is more likely when populations are small and geographically or ecologically isolated. The geographic history of an area and anthropogenic impacts on it, such as habitat fragmentation influence the exchange of genes between populations and thereby can affect the genetic diversity of natural populations (Husemann *et al.*, 2012; Fernández-García *et al.*, 2014; Eberhart-Phillips *et al.*, 2015).

Due to their usually low-mobility lifestyle (Nekola, 2012), freshwater gastropods, such as *Segmentina nitida*, represent interesting models for the study of extrinsic factors on population genetics. Low mobility reduces dispersal and therefore gene flow between populations which is why many snail species show strong genetic differences between populations, even within small geographic ranges (Hurtrez-Boussès *et al.*, 2010; Tian-Bi *et al.*, 2013). However, large population sizes can reduce the amount of genetic drift experienced by a population, thereby counteracting genetic differentiation between populations (Tibbets and Dowling, 1996). Passive dispersal via larvae or adults may facilitate gene flow between populations and also reduce genetic differentiation, especially in and along river habitats (e.g. Gu *et al.* (2015). Long distance dispersal, however, normally only occurs via attachment

to animals (zoochory) or by the action of humans (anthropogenic translocation), either by accident or on purpose (Gittenberger *et al.*, 2006; Gittenberger, 2012).

Despite the existence of multiple modes of passive dispersal, strong genetic divergence of populations is often observed between local snail populations (Sinclair-Winters, 2014), either as a result of natural geographic isolation or anthropogenic habitat fragmentation. The genetic divergence is often accompanied by greater effects of genetic drift (González-Astorga and Núñez-Farfán, 2001) and a reduction in genetic diversity and effective population size (N_e) (Zuberogoitia *et al.*, 2013). Effective population size translates the observed size of a sampled population into the size of an 'ideal' population showing the same rate of genetic diversity loss as the sampled population (Husemann *et al.*, 2016). Reduced genetic diversity following habitat fragmentation (Dixo *et al.*, 2009), combined with low dispersal (Schtickzelle *et al.,* 2006), can also limit the ability of populations to adapt to environmental change (Bijlsma *et al.*, 2000; Reed and Frankham, 2003; Willi *et al.*, 2006). Therefore, estimating genetic diversity and differentiation can provide important information and evidence for the threat status of a species or one of its populations (Toro and Caballero, 2005). Additionally, analyses of genetic structure and demographic analyses using multiple genetic markers with different evolutionary speeds may help to understand the historic and current factors that have been and continue to be most relevant in shaping a population's genetic structure (Waples and Gaggiotti, 2006; Roberts *et al.*, 2013; Gu *et al.*, 2015).

Extinction and recolonization events in species have attracted increasing interest in the field of population genetics because they influence both within-population diversity and between-population differentiation (Wade and McCauley, 1988; McCauley, 1991; Rousset, 2001). The colonisation of new habitats by a small number of founder individuals usually leads to a genetic bottleneck, as does the extinction of the majority of individuals in a population. In organisms with clonal reproduction, extreme bottlenecks are possible, as a single individual and therefore a single genotype can originate a new population. Thus, more generally, a reduced number of genotypes is expected in recently

founded populations (Samadi *et al.*, 1999). Bottlenecks and founder effects in populations at the leading edge of an expansion of a species can also lead to reduced genetic diversity in these regions (Ray *et al.*, 2015). Moreover, reintroduction of a species into a habitat using only a small number of individuals can lead to an artificial genetic bottleneck (Kautenburger and Sander, 2008). Understanding the genetics of populations is therefore imperative when contemplating reintroduction of a species to a location.

The effects of freshwater habitat fragmentation on the genetic structure of populations are well documented (Frankham *et al.,* 2010). Disconnected habitat fragments contain small, isolated populations (Janečka *et al.*, 2008), which typically lose genetic diversity and experience inbreeding depression and reduced levels of population-wide fitness (Slate *et al.*, 2000; Újvári *et al.*, 2002; Liberg *et al.*, 2005), all of which can increase the risk of species extinction (Bijlsma *et al.,* 2000). The configuration of a habitat can greatly influence the dispersal of a species, which will then affect its population genetic structure. For example, fragmented habitats or those with physical barriers separating them (such as mountains or oceans) can limit the seasonal movements and migrations of species (Epps *et al.*, 2005; Roffler *et al.*, 2014).

The currently observed population structure of a species is therefore the result of a complex interplay of current and past processes which are difficult to disentangle. Detailed knowledge of how genetic diversity of a species is structured across fragmented landscapes, such as the once widespread wetland habitat of *S. nitida*, and the extent of genetic differentiation, connectivity and effective population sizes (N_e), are key to formulating a conservation strategy that maintains genetic variability and promotes the evolutionary potential of threatened species and prevent the loss of distinct populations (Da Silva *et al.*, 2006; Peery *et al.*, 2012; Williams *et al.*, 2015)

To support conservation management strategies for *S. nitida*, it is necessary to analyse the genetic structure of its natural populations as it is potentially relevant to their long-term viability (JNCC, 2010b). Understanding patterns of population genetic structure frequently poses a challenge to

conservation managers. It is important to determine whether the observed genetic structure is a consequence of recent population fragmentation or a remnant of historical localised adaption (Buckland *et al.*, 2014). Misdiagnosing the former when the latter is true risks disrupting patterns of local adaption and can lead to outbreeding depression if incompatible populations are mixed (Buckland *et al.*, 2014). Conversely, interpreting structured patterns to be signatures of local adaption when they are a consequence of isolation and drift risks inappropriate management to maintain existing genetic patters, when maximising gene flow between populations might reduce the loss of genetic diversity and thereby risk of extinction. Identifying the origins of genetic structure is particularly important when deciding whether to use translocation to reinforce existing populations and/or establish new populations, and to determine how many founding individuals are required to retain the existing genetic diversity. It is also important to interpret genetic patterns alongside ecological factors such as habitat loss when deciding the most appropriate management option (Buckland *et al.*, 2014).

The eukaryotic ribosomal RNA locus is an attractive target for phylogenetic studies because of its high gene copy number, universality and diverse rates of evolution within and between component subunits and spacers. The two Internal Transcribed Spacer (ITS) regions (ITS1 and ITS2), are routinely used for studies on phylogenetic reconstruction, genetic variability and species divergence for a wide range of organisms including molluscs (Chilton *et al.*, 2001; Subbotin *et al.*, 2001; Weekers *et al.*, 2001; De Rojas *et al.*, 2002), particularly to resolve taxonomy within controversial or poorly resolved groups (Stothard *et al.,* 1996; Remigio and Blair, 1997; Bargues *et al.*, 2001).

Another commonly used genetic marker in population genetic analysis is the mitochondrial gene Cytochrome C Oxidase subunit 1 (COI). COI has a faster evolutionary rate than nuclear DNA, with more sequence polymorphisms and mutations originating over a much shorter time (Li, 1997). Mitochondrial DNA allows relationships among populations and species via direct comparison of sequence data, rather than allele-based datasets (Bowen *et al.*, 2014) therefore, complementing COI

with nuclear markers allows for stronger conclusions on phylogeny and population structure, providing resolution at different levels of genetic variation (Meudt and Clarke, 2007). Zhou *et al.* (2017) used COI and the 16S region in the mitochondrial DNA in conjunction with ITS2 sequences to analyse population genetic structure of the land snail *Camaena cicatricose* (Müller 1774 (Stylommatophora: Camaenidae)), an important parasite vector in China. This study found across all markers that climatic changes, as well as geographical and human barriers did not substantially affect the population structure and distribution of the species (Zhou *et al.*, 2017). Gu *et al.* (2015) used COI as well as seven microsatellite loci to assess the population genetic structure of *Bellamya aeruginosa* (Reeve 1863 (Gastropoda: Viviparidae)). This study found low levels of genetic differentiation between populations, even those isolated by large geographic distances, probably facilitated by flooding, zoochoric dispersal and anthropogenic translocations (Gu *et al.*, 2015).

Simple sequence repeats (SSR) or 'microsatellites' are DNA tandem repeats composed of short repeat motifs (1-6 bp) (Richard *et al.*, 2008; Techen *et al.*, 2010) and show high levels of polymorphism as they have high mutation rates due by their instability (Gemayel *et al.*, 2012). Microsatellites are usually considered to be neutral markers (the genes detected do not have any direct effect on fitness, as opposed to adaptive markers) and mainly occur in non-coding DNA (Ellegren, 2004). Mutations in simple repeats lead to the insertion or deletion of one or a few repeat units, rather than point mutations. They are present in prokaryotes and eukaryotes and even in the smallest bacterial genomes (Field and Wills, 1996; Hancock, 1996). Microsatellites are widely used in population genetics as they are highly informative, codominant, and multi-allelic markers (Mason, 2015), as well as being able to reveal fine-scale population structure on small temporal scales (Estoup *et al.*, 1998; Palo *et al.*, 2004; Neumann *et al.*, 2005). Using single locus markers such as ITS2 and COI by themselves however, may provide a limited view of the population history of a species, and may even be misleading (Pamilo and Nei, 1988; Wu, 1991; Palumbi and Baker, 1994; Hare and Avise, 1998; Alves *et al.*, 2006). Reliable inferences of population structure and evolutionary history should therefore be drawn from analysis of several independent loci (Slatkin and Maddison, 1990; Karl and Avise, 1992; Hare, 2001).

Microsatellites have been used in genetic studies on a number of molluscan species. Djuikwo-Teukeng *et al*. (2014) used six microsatellite loci to analyse the population genetic structure of populations of the freshwater snail *Bulinus globosus* (Morelet 1866) (Gastropoda: Planorbidae). This study suggested that genetic drift and gene flow were the main factors shaping the population genetic structure, as well as allowing inferences about mating preferences of the species (Djuikwo-Teukeng *et al.*, 2014). Samadi *et al.* (1999) used three microsatellite loci in combination with morphological analysis to investigate clonal variability in the freshwater snail *Melanoides tuberculata* and explored the effect of flooding events and geography on the distribution of genetic variability (Samadi *et al.*, 1999). Seven microsatellite loci were used for genetic differentiation and analysis of dispersal and the mating system of *Biomphalaria glabrata* (Mavárez *et al.*, 2002a), revealing a significant isolation-by-distance pattern in populations of the snail in South America, and differentiation between populations with reference to colonisation events and radiation (Mavárez *et al,.* 2002a).

To date, there has only been one investigation of the population genetics of *S. nitida* (Mensch, 2009). This study used COI, 12S and amplified fragment length polymorphism (AFLP) data for genetic analysis to elucidate genetic differences between populations of *S. nitida* in Germany, Poland, and the UK. The mitochondrial marker (COI) *S. nitida* showed low diversity in the UK and it was estimated this loss of diversity had occurred in the last 40 years, based on comparisons with DNA samples collected in the UK in 1969, though no specific locality information was provided (Mensch 2009). Polish and UK samples were markedly different from each other based on mtDNA sequence data, implying the possible existence of a cryptic species or of misclassification of some samples (Mensch 2009). The AFLP analysis however presented contradictory results with regard to the genetic diversity between populations depending on which AFLP analysis method was used. The study concluded by highlighting the need for further investigation using multiple markers to assess the extent of genetic variation in *S. nitida* (Mensch, 2009).

This chapter aims to expand on this study, using newly collected contemporary samples from European populations already included in the Mensch (2009) study (Germany, UK, Poland), but also expanding the geographic range of sampling to include Sweden. Novel *Segmentina nitida* microsatellites were developed and used for genetic analyses in addition to using established ITS and COI markers. The aims of this study were to:

i) Assess the genetic structure and genetic diversity of each European population of *Segmentina nitida* sampled.

ii) Assess the overall genetic structure and genetic diversity of *S. nitida* in Europe.

iii) Identify lineages and relationships between lineages in European populations of *S. nitida*.

Understanding the genetic structure of *S. nitida* in Europe will inform potential reintroduction or translocation programmes for the species, by identifying potential breeding stock populations, as part of the translocation action point detailed in the Biodiversity Action Plan for *S. nitida* (JNCC, 2010b).

5.2. Materials and Methods

5.2.1. Origin of samples for genetic analysis

During the summer of 2016 481 *Segmentina nitida* individuals were collected from locations in Germany, Poland, Sweden, and the UK (Figure 5.1), and five additional samples collected from the macrocosm breeding experiment (originally from UK population GB1) (Section 3.3.4) in the winter of 2017. Samples were stored in absolute ethanol or 70% isopropyl alcohol (for German samples) in 2mL sample tubes for transport from the field, with between one and three tubes per population, and then transferred to individual tubes in absolute ethanol upon arrival in the UK. GPS coordinates for sampling location and sample ID numbers are shown in Appendix C1.

The number of snails collected per population, and the number of snails from that initial number that were successfully sequenced for ITS2, COI and microsatellite markers is shown in Table 5.1. Samples sequenced for COI and ITS2, with ID, country, and population information are shown in Appendices

C2 and C3, respectively. Whilst some of the populations are represented by small samples (e.g. n<10), differences between the genetic structure of populations with as few as 11 individuals have been detected (Sekino and Hara, 2001; Casu *et al.*, 2002; De Wolf *et al.*, 2004; McDevitt *et al.*, 2011).

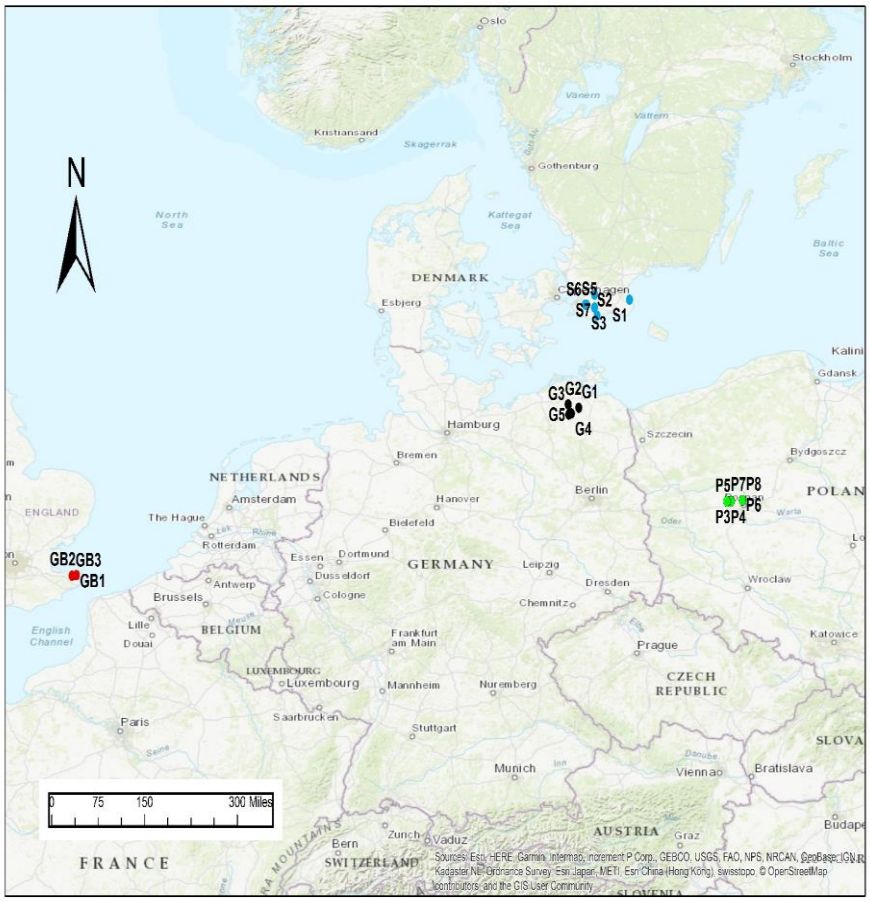


Figure 5.1. Map showing sample locations throughout Europe. GB= UK, G=Germany, S=Sweden, P=Poland.

Table 5.1. Summary of *Segmentina nitida* individuals collected from European countries in 2016, and the number of individuals successfully sequenced/ genotyped for each genetic marker used.

Population	Individuals Collected	No. of individuals successfully sequenced for COI	No. of individuals successfully sequenced for ITS2	No. of individuals successfully genotyped with microsatellites
G1	15	4	5	14
G2	34	5	5	30
G3	16	4	5	15
G4	15	4	5	15
G5	3	2	3	3
GB1	20	5	5	16
GB2	9	5	5	9
GB3	6	5	4	6
P2	18	4	5	15
P3	12	5	5	12
P5	22	4	5	17
P6	17	6	5	14
P7	19	5	6	11
P8	29	5	5	14
S1	37	5	5	28
S2	23	5	5	16
S3	46	6	3	29
S4	46	4	5	26
S5	33	5	4	25
S6	26	5	5	24
S7	40	5	5	28
Total	486	98	100	367

5.2.2. DNA extraction

DNA was isolated from individual snails either using a QIAGEN DNeasy kit or a Thermo Scientific GeneJET genomic DNA purification kit, following the manufacturer's instructions. Whole snails with shells were ground up in extraction buffer prior to DNA extraction with sterile grinding rods (one rod per snail, autoclaved after each use). Preliminary experiments indicated that not removing the shell prior to DNA extraction did not affect quantity or quality of genomic DNA extracted (personal observation by the author based on ten extractions using each protocol). Of the 483 individual snails from which DNA was extracted, 367 yielded DNA for molecular analysis, determined by a Nanodrop fluorometer (Thermo Fisher).

5.2.3. Polymerase chain reaction (PCR) of mitochondrial and ribosomal markers, clean-up, and sequencing

A subset of 100 specimens were selected for amplification of MT-COI and ITS2. COI sequences were amplified using the modified COI marker from Albrecht *et al.,* (2007). ITS2 sequences were amplified using the primer set in Vidigal *et al*. (2002). Reaction mixtures for both primer sets consisted of QIAGEN Multiplex PCR Master Mix, 5uM forward primer, 5uM reverse primer, ddH_2O and sample DNA with a final reaction volume of 10µL. COI primers: forward- LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'); reverse- HCO2198 (5'- TAAACTTCAGGGTGACAAAAAATCA-3'). ITS2 primers- forward- ITS2F (5'- CGTCCGTCTGAGGGTCGGTTTGC-3'); reverse- ETTS2 (5'-TGCTTAAGTTCAGCGGGT-3'). For COI markers, reactions were cycled in a DNA Engine Peltier Thermo Cycler (BioRad) with the following conditions: 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 48°C for 1 minute, and 72°C for 1 minute, followed a final extension of 72°C for 5 minutes (modified from Albrecht *et al.* (2007)). For ITS2 markers, reactions were cycled with the following conditions: 95°C for 15 minutes, followed by 32 cycles of 95°C for 45 seconds, 54°C for 1 minute, and 72°C for 2 minutes, followed a final extension of 72°C for 5 minutes (modified from Vidigal *et al.* (1998)).

After initial PCRs for both markers, 4µL of product were run on a 1% agarose gel with ethidium bromide to visualise products and determine concentration, with 6x Orange G as a loading dye. PCR clean-up was undertaken with EXO-Sap (ThermoFisher Scientific) a PCR product clean-up reagent which hydrolyses primers and nucleotides (Dugan *et al.*, 2002). After PCR clean-up samples were cycle sequenced, using BigDye V3.1 cycle sequencing kit (Applied Biosystems) (Rosenblum *et al.*, 1997). The BigDye sequencer PCR was run according to manufacturer's instructions, with BigDye at 1/8th volume (0.5µL per reaction). Automated sequencing was performed by capillary electrophoresis on an ABI3730 sequencer (Applied Biosystems). Three specimens failed to amplify for COI and were not included in analysis.

5.2.4. Microsatellite marker PCR running and genotyping

All samples (*N* = 367) were genotyped at seven microsatellite loci, which were generated specifically for this project at NBAF node Sheffield (Table 5.2).Primers P5943, P25580, and P12998 were developed from a Polish individual from population P5. Primers UK21826, UK15523, UK16382, and UK19417 were developed from a UK individual from population UK1. PCR reactions were performed in a total volume of 2 μL (2-10 ng genomic DNA, 0.2 uM F and R primer, Qiagen MasterMix) (Kenta *et al.*, 2008). Thermal cycling was performed with a DNA Engine Peltier Thermo Cycler (BioRad) under the following conditions: 94°C for 15 min, 44 cycles at 94°C for 30 sec, annealing at 56°C for 1 min 30 seconds, 72°C for 1 min 30 seconds, and a final extension at 72°C for 30 min. Forward primers were 5′-labeled with HEX or 6FAM fluorescent dyes (Table 5.2). Amplified products of different sizes and contrasting fluorescent labels were multiplexed with the sizes of the fluorescently labelled PCR products estimated according to an internal size marker (ROX-500) and designed using Multiplex Manager v. 1.2. (Holleley and Geerts, 2009) and run on an ABI3730 capillary electrophoresis sequencer (Applied Biosystems).

Table 5.2. Microsatellite markers designed for *Segmentina nitida* with fluorescent dyes attached to 5' forward primer. Shows the multiplex each marker was used in for the experiment, and sequence for both forward (F) and reverse (R) primers.

Marker Name	Fluorescent Dye	Multiplex	Sequence
P5943	6FAM	1	F: GACCGCGTCTCACACAATC R: TTGATCACTGGCACCCAAC
P25580	6FAM	1	F: AACTTTCTCCGCATGACGAC R: TGGCCCAATCCTACCTTTC
UK21826	6FAM	2	F: ATCAACACGAATGCTTGAAGG R: TTCCGTAACATTGACGTAGAAATG
UK15523	HEX	2	F: TACGTTGGTGCTAACAGTGC R: TCTGTATTAGCAATCTTCAATACCC
UK16382	6FAM	3	F: TTGGTTTCCAAACACATACCG R: GACCCTAACAGGGGTGAAGG
P12998	6FAM	4	F: TTTCATTAAAGAGCACTAGATACAAATC R: TGTGGTCTGGAGTGTGTTTG
UK19417	6FAM	4	F: TCCCTGTGTATCCAGCTTATCG R: TGGCTTTCGAGAACCACATC

Microsatellite alleles were scored using GeneMapper version 3.7 software (Applied Biosystems). Randomized, resampled genotyping (n=72) of different *Segmentina nitida* populations from different European countries consistently showed the same allele profile for each of these seven loci. 95.8% (n=69) of the samples produced the same allele genotype in the repeat PCRs of the samples. Five of the PCR products from three individual snails across all samples and all loci produced different profiles between runs (three individual snails). These samples were excluded from the analysis.

5.2.5. Data analysis of COI and ITS2 amplified sequences

Sequences were checked for errors and edited by eye with CodonCode Aligner v. 8.0.2. (CodonCode Corporation, http:www.codoncode.com). Sequence products were aligned with MUSCLE using MEGAX (Kumar *et al.*, 2016). The number of polymorphisms, haplotypes, nucleotide diversity, average number of nucleotide differences between samples, and phylogenetically informative sites for all European populations, as well as within each country were calculated in DnaSP v6 (Rozas *et al.*, 2017). Mismatch analysis was then used to identify lineages within the dataset and identify population size changes in DnaSP (Rozas *et al.*, 2017). Mismatch distributions summarize information about genetic differences between pairs of subjects in a sample. They are built by counting the number of nucleotide

site differences between each pair of subjects and display the relative frequencies of pairs that differ by zero sites, by one site, by two sites, etc. (Rogers *et al.*, 1994).

The original aligned sequences were compared and reduced to unique haplotypes in DnaSP. Maximum likelihood (ML) phylogenies were inferred for the haplotypes using MEGAX. To select appropriate models for the ML analyses, 24 ML nucleotide substitution models were evaluated using BIC and Akaike Information Criterion (AIC) in MEGAX. Strength of support of tree nodes was assessed via bootstrapping (n=500). For COI, a partial COI sequence for *Hippeutis complanatus* that provided complete coverage for the *S. nitida* marker was used to root the tree as an outgroup, found on NCBI (https://www.ncbi.nlm.nih.gov/) (accession number: EF012170, individual from Germany (Albrecht *et al.,* 2007). For ITS2, a unrooted phylogenetic tree was produced as there no ITS sequences exist for *H. complanatus*. Any distinct lineages found in the ML trees and mismatch analysis for both markers were separated and then run through polymorphism analysis to find recurring polymorphisms in markers and mismatch analysis again in DnaSP.

5.2.6. Data analysis of amplified microsatellite PCR products

Standard population genetics parameters were used to determine the genetic diversity within each population (country): number of alleles per locus per population, observed and expected heterozygosity (H_O and H_E, respectively) at each microsatellite locus. Genotypic linkage disequilibrium, inbreeding and departures from Hardy-Weinburg equilibrium (HWE) at the seven microsatellite loci were assessed using Fisher's exact test (Sokal and Rohlf, 1995), pairwise genotypic distances (F_{ST}), inbreeding coefficients (F_{IS}) and gene flow estimates were obtained using ARLEQUIN V.3.5.2.2 (Excoffier and Lischer, 2010). Possibility of null alleles (alleles where only one of the two allele pairs amplified) in the data was calculated using Cervus v3.0 software (Kalinowski *et al.*, 2007).

STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) was used to compute the possible number of genetic lineages (K) to which individuals belong across the entire dataset. Prior locality information was included in the analysis, treating each sampling location in each country as a distinct population. A

range of 1-26 potential lineages was used in the analysis. A Markov chain Monte Carlo (MCMC) analysis was done with a burnin period of 10000 iterations and 50000 iterations after burnin. The analysis was done using the admixture model and with allele frequencies correlated model. 15 runs for each K value were executed with the software. Structure Harvester (Earl and VonHoldt, 2012) was used to generate an output, and the five least likely iterations for each value of K (those with the lowest estimated log likelihood of data in the Structure Harvester output) were removed. The ten remaining iterations for each value of K were then re-run through Structure Harvester, and a new output generated. The Evanno table and plot (Evanno *et al.*, 2005) obtained in this output were then used to choose the most likely number of distinct populations (K) using the highest Delta K value. The output for the most likely K value was then visualised using Lineage Matching and Permutation Program (CLUMPP) (Jakobsson and Rosenberg, 2007) and distruct (Rosenberg, 2003). Any lineages found with the initial structure analysis were then re-run through the software separately under the same MCMC conditions with the number of potential lineages equal to $n + 3$, where n is the number of populations within that lineage. Evanno results were visualised with CLUMPP and distruct.

5.2.7. Geometric morphometrics of identified genetic lineages

Of the 367 individuals with microsatellite data, geometric morphometric data from the six landmarks detailed in Chapter 4 were available for 339 shells. The microsatellite data for these 339 individuals were grouped by the lineages identified in the structure analysis in 5.2.6 and analysed to see if morphological differences corresponded with genetic differences between lineages. Scale, orientation, and position were removed from landmark data using Procrustes superimposition with the R package 'geomorph' (Adams *et al.,* 2019), to obtain Procrustes coordinates and centroid size for each sample.

Centroid sizes were compared between genetic lineages. The data did not have equal variance (Levine's test ($\alpha = 0.05$)), so a Mann Whitney-U was performed to compare median landmark centroid

sizes between snails from the different lineages in Statistics Package for the Social Sciences (SPSS) (2018).

The dataset was then split by the identified lineages and a consensus configuration was generated for each lineage, reflecting the mean shell shape. The specimens that most closely represented the mean shape for each lineage were identified using 'geomorph'. The mean shapes were then compared to each other using thin plate splines to produce deformation grids in 'geomorph' to determine key areas of difference in shell shape between lineages.

A PCA of the Procrustes shape coordinates obtained from the Procrustes superimposition was performed in 'geomorph' to determine the linear combinations of the coordinates that maximise the variation in the data. The loadings of each of the principal components were extracted to determine which areas of the shell contributed to the grouping and shape differences along each axis. Warp grids that showed the deviation of landmarks from the mean shape of all samples at the minimum and maximum of each principal component were displayed.

Pairwise Kruskall-Wallis tests were performed in SPSS on the principal components that described over 80% of the total shape variation in the sample, with samples grouped by lineage. The principal components failed both a Levene's test of equal variance and a Shapiro-Wilk test of normality (α = 0.05). The Procrustes shape coordinates were then used for a discriminant analysis in Past version 3.21 (Hammer *et al.,* 2001) to show groupings of the different lineage by shape, and individuals from lineages could be distinguished from each other and assigned to the correct genetic lineage by shell shape alone. Any further potential genetic lineages identified from the COI and ITS2 analysis were also tested for morphological differences through PCA and discriminant analysis as described above.

5.3. Results

5.3.1. Nuclear and Mitochondrial Markers

97 COI sequences were generated with 90 sequences with a consensus length of 513 bp, and seven individuals with a single 9 bp block deletion which did not affect the reading frame, resulting in a consensus length of 504 bp. The individuals that this deletion occurred in were: P3.2, P3.3, P3.4, P7.3, P7.8, P7.15, and G4.14. The alignment of all sequences without the 9 bp deletion contained 106 polymorphic sites (20.1%), of which 88 were phylogenetically informative, representing 31 unique haplotypes.

Nucleotide diversity across all populations was 0.07. The UK had the lowest nucleotide diversity of all countries (0.001), with Poland with the next lowest in comparison to the overall diversity of all populations and diversity of other countries (0.02), and Germany had the highest nucleotide diversity (0.08) of the countries represented. The seven individuals containing the 9 bp deletion contained 70 polymorphic sites (13.9%), none phylogenetically informative, with two unique haplotypes. All six Polish individuals with the 9 bp deletion represented one haplotype and the German individual with this deletion represented the other haplotype.

Table 5.3. Summary of COI sequence polymorphisms in *Segmentina nitida* across sampled European countries. All samples column and breakdown of polymorphisms by county does not include the seven snails with the 9 bp deletion in their COI sequences

COI	All Samples	Germany	UK	Poland	Sweden	All Indel Samples	Poland Indel
Sequences	90	18	14	23	35	7	6
Length of sequences (bp)	513	513	513	513	513	504	504
Polymorphic sites	106	91	2	27	88	70	0
No. of individuals containing 9 bp deletion	7	1	0	6	0	7	6
Haplotypes	31	11	2	10	9	2	1
Haplotype diversity	0.93	0.92	0.36	0.85	0.84	0.286	0
Nucleotide diversity	0.07	0.08	0.001	0.02	0.05	0.04	0
Av. No. of nucleotide diffs.	35.74	39.88	0.73	8.96	24.61	20	0
Phylogenetically informative sites	88	75	2	21	86	0	0

100 sequences were generated for ITS2, with a consensus sequence length of 412 bp for 66 individuals, a different consensus sequence 412 bp in length for 17 individuals, and a consensus sequence length of 413 bp for 17 individuals. Seven polymorphic sites were present in the dataset, producing three haplotypes. Four were sequence differences, and three were insertion/deletion (indel) polymorphisms, with insertions/deletions of 1 bp at three locations in the sequences. As ITS2 is a non-coding region these polymorphisms were regarded as correct and were present in multiple. A 1 bp deletion was present in 36 individuals at both sequence position 373 and a 1 bp insertion at sequence position 407 in the same 36 individuals. A 1 bp insertion was also present at sequence position 408 in 17 individuals, all of which included the other two indel polymorphisms. . Indel Haplotype A represented all individuals in Poland, eight individuals from Germany, and all Swedish individuals except population S2. Indel Haplotype B represented 12 individuals from Germany and population S2. Indel Haplotype C represented all UK individuals and three individuals from Germany.. Germany and

Sweden had the highest nucleotide diversity (0.005 and 0.003, respectively), and Poland and UK had no haplotypic diversity (no polymorphisms present) (Table 5.4).

Table 5.4. Summary of ITS2 sequence polymorphisms in *Segmentina nitida* across sampled European countries. [1]Number of base pairs varies in countries that contain individuals containing any of the three 1bp deletions in the sequence which were excluded from polymorphic site analysis.

ITS2	Germany	UK	Poland	Sweden
Sequences	23	14	31	32
Sites[1]	411	413	412	411
Polymorphic sites (non-indel)	4	0	0	4
Haplotypes	2	1	1	2
Haplotype (gene) diversity	0.474	0	0	0.272
Nucleotide diversity	0.005	0	0	0.003
Av. No. of nucleotide diffs.	1.9	0	0	1.1
Phylogenetically informative sites	4	0	0	4
Number of individuals representing Indel Haplotype A	8	0	31	27
Number of individuals representing Indel Haplotype B	12	0	0	5
Number of individuals representing Indel Haplotype C	3	14	0	0

Two well supported lineages were detected with the Maximum Likelihood tree for COI, using the Tamura Nei model of nucleotide substitution, with gamma distribution rate=5 (Figure 5.2). COI data from the type locality in Denmark could not be used in the construction of the tree as the fragment of COI used in the previous study for an individual from Denmark did not overlap with the sequence used here (Jørgensen *et al.* 2004). When aligned to the complete COI gene for *Biomphalaria glabrata* (accession number: AY380531, bp 10474-12000 in mitochondrial genome), the COI fragment by Jørgensen *et al.* (2004) extended from 750-1091 bp in the sequence. The COI fragment amplified here extends from 105-617 bp. Six of the individuals with the 9 bp indel fell within in the East lineage in the phylogenetic tree, with G4.14 as the only individual in the West lineage with this sequence

polymorphism. The six individuals in the East lineage are clustered closely with other individuals in this lineage without the deletion (Figure 5.2).

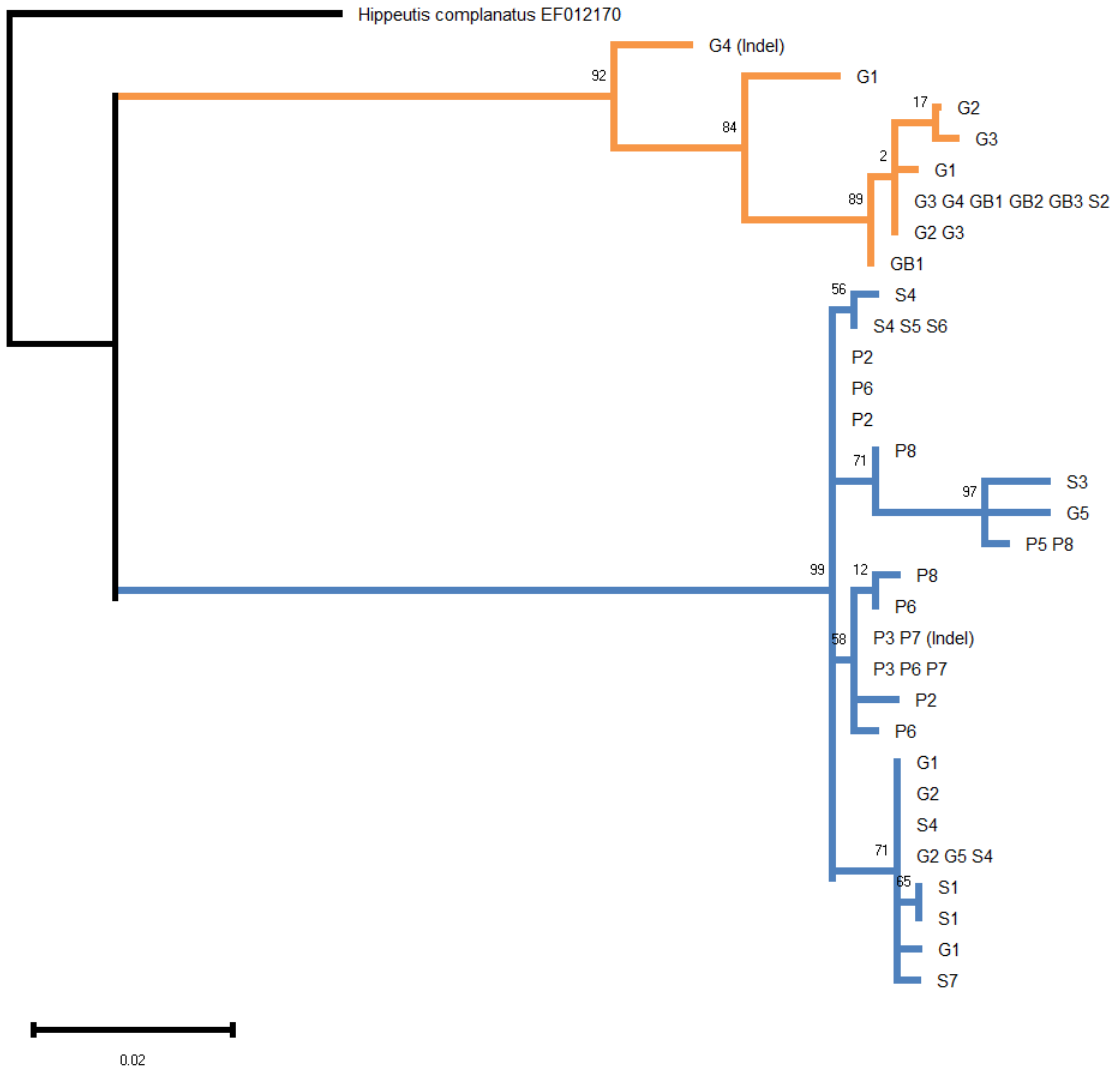


Figure 5.2. Maximum Likelihood tree for COI, with *Hippeutis complanatus* as the outgroup. Nodes show bootstrapping values based on 500 repeats and show the percentage of bootstrapping repeats that resulted in each node. G: Germany; GB: United Kingdom; P: Poland; S: Sweden with number of the population the individuals that represent that haplotype originated from.

When the COI dataset was split into these two lineages, lineage 1 (hereby referred to as the East linage) (Polish, Swedish and some German individuals, *N*= 72) showed 45 polymorphic sites (18 of which were phylogenetically informative), 23 unique haplotypes and a nucleotide diversity of 0.02. Lineage 2 (hereby referred to as the West lineage) (German, UK, and population S2 individuals, *n= 31*)

showed 39 polymorphic sites (ten phylogenetically informative), eight unique haplotypes and a nucleotide diversity of 0.007 (Table 5.5).

When all individuals were run through the Basic Local Alignment Search Tool (BLAST), individuals in the West lineage aligned with a partial COI sequence of *S. nitida* from a German individual reported by Albrecht *et al.* (2007) with identity scores of 94-100% (accession number: EF012178; mean ID: 99.5%). The West lineage also showed 94-100% identity to a sequence from Lodz, central Poland (accession number: LC429369, Saito *et al.,* 2018). Individuals in the East lineage only showed between 85 and 86% identity to the same sequences (mean ID: 85.6%). All samples had 99% coverage with the cited reference sequences.

Table 5.5. Summary of COI sequence polymorphisms in *Segmentina nitida* for the two lineages detected in the Maximum Likelihood tree analysis. [1]Number of base pairs is lower than full sequence (513 bp) as the 9 bp deletion in some individuals was excluded from polymorphic site analysis.

COI	**East Lineage**	**West Lineage**
Sequences	66	31
Length of sequences (bp)[1]	504	504
Polymorphic sites	45	39
Haplotypes	23	8
Haplotype (gene) diversity	1.00	0.58
Nucleotide diversity	0.02	0.01
Av. No. of nucleotide diffs.	8.06	3.51
Phylogenetically informative sites	18	10

For phylogenetic analysis each of these haplotypes was split into the constituent countries within them, with haplotype A split into Germany A, Sweden A, and Poland, haplotype B split into Sweden B and Germany B, and haplotype C named as UK (Figure 5.3). When the ITS2 dataset was split into the two lineages represented in the phylogenetic tree (Figure 5.3), the East lineage (Polish, Swedish, and some German individuals, *n= 66*) showed no polymorphic sites, with two fixed gaps in the sequences.

The West lineage (UK, some German, and population S2 individuals, *n= 34*) showed one polymorphic insertion-deletion (indel) site and one fixed gap, with two unique haplotypes (Table 5.6).

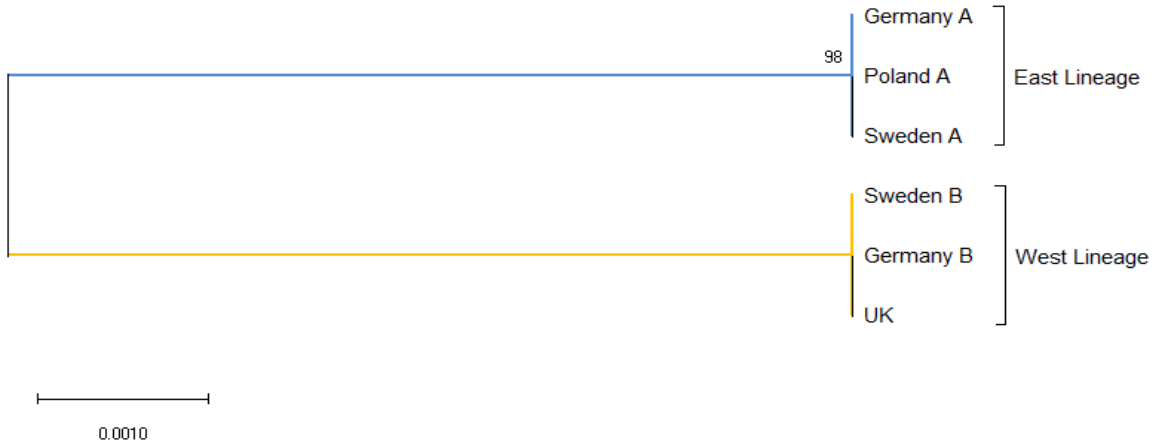


Figure 5.3. Maximum Likelihood Tree for ITS2 sequences of *Segmentina nitida*. Node values based on 500 bootstrapping results.

Table 5.6. Summary of ITS2 insertion-deletion (indel) sequence polymorphisms of *Segmentina nitida* for the two lineages detected in the Maximum Likelihood tree analysis

ITS2	East Lineage	West Lineage
Sequences	66	34
Sites	412	413
Polymorphic sites	0	1
Haplotypes	1	2
Indel haplotype diversity	0	0.515

The mismatch analysis of all samples in the mitochondrial and nuclear DNA also showed two lineages of individuals (Figure 5.4A and 5.5A), with two distinct peaks in the mismatch graphs, at around 0-20 bp differences and 65-75 bp differences for COI, and 0-1 bp and 4-6 bp differences for ITS2. When split into the two lineages identified in the ML trees, the mismatch plots for the West lineage for COI showed data more similar to the expected pairwise difference values of a constant population size whilst the East lineage showed values deviating from the expected frequencies of differences.

Mismatch analysis could not be performed on each separate lineage within the ITS2 dataset as there were no non-indel polymorphisms in either lineage.

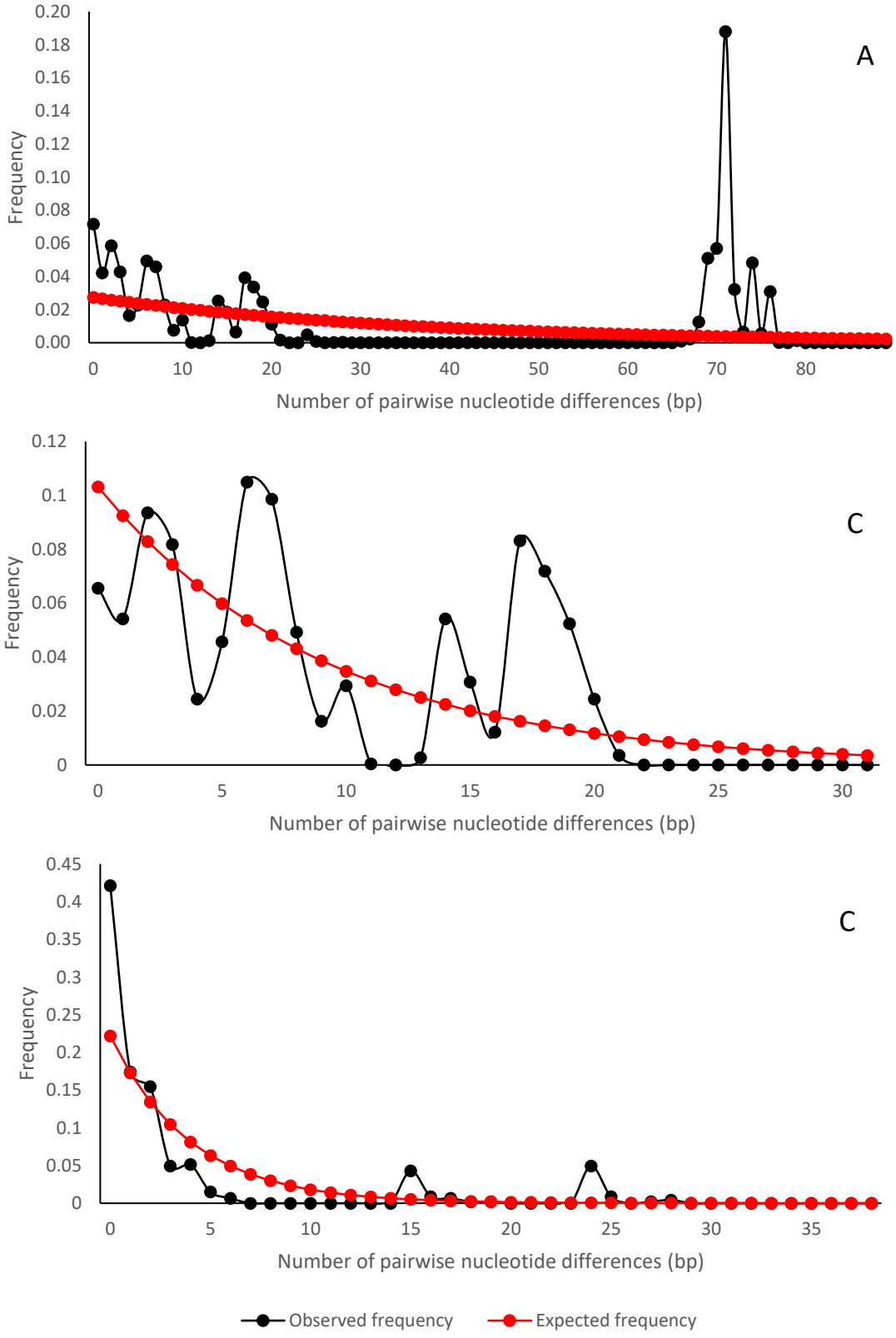


Figure 5.4. Mismatch analysis plots for COI sequences. A: All samples. B: East Lineage. C: West lineage. Expected frequency line indicates expected pairwise differences in constant population size.

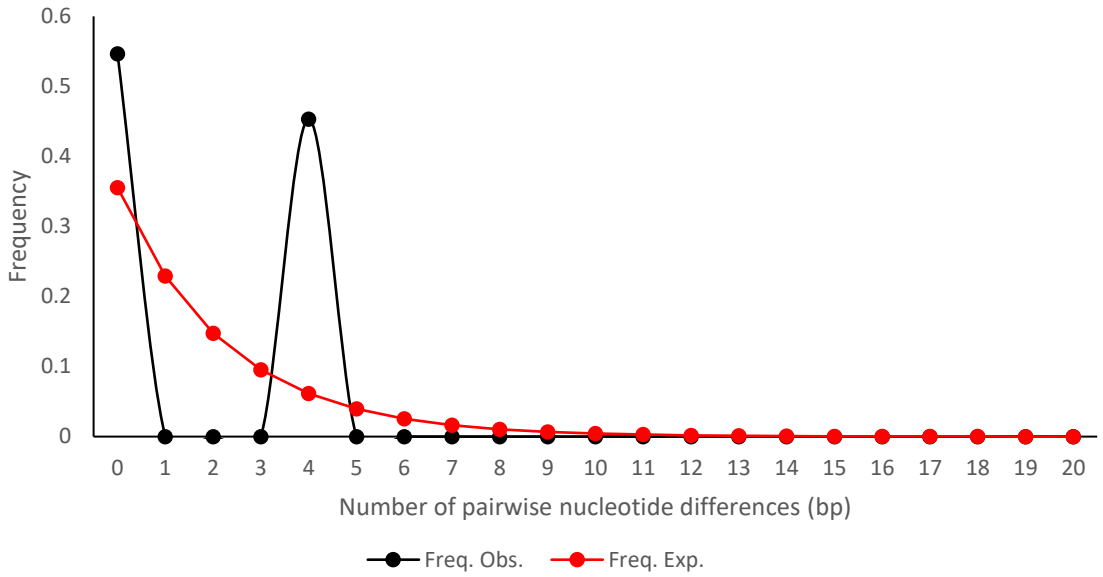


Figure 5.5. Mismatch analysis plot for all ITS2 sequences. Expected frequency line indicates expected pairwise differences in constant population size.

5.3.2. Genetic diversity and genetic structure of European populations of *Segmentina nitida* using microsatellite markers

There were 56 genotypes across the entire sample (Table 5.7). No more than two alleles were found at any locus for any individual snail except for snail GB1.10, which had a tetraploid profile. The mean number of alleles per population across all loci ranged from 1.43 for the UK and 6.14 for Germany. The separate loci across all populations displayed between 4 (P12988, P25580, UK19417) and 13 alleles (UK21826) (Table 5.7). All markers had lower than expected heterozygosity when viewed as an entire dataset including all countries. When viewed independently, the UK, Poland, and Sweden has similar observed and expected heterozygosity (generally within 0.1 of each other) whilst Germany only showed this for one locus (UK15523). All other loci for German individuals showed differences between H_O and H_E similar to the entire European dataset. However, when the mean H_O and H_E was calculated for each country (Table 5.8), they were all within 0.1 of each other for all populations.

Table 5.7. Genetic diversity of all samples at each microsatellite locus. N_{ALL}= number of alleles present. H_O= Observed heterozygosity. H_E= Expected heterozygosity. *P (HW)* = *p*-value of Hardy-Weinberg equilibrium test. ***P<0.001. *F(Null) = estimated null allele frequency*

Locus	Sample Size	N_{ALL}	H_O	H_E	HW	F(Null)
P12988	366	4	0.25	0.65	***	0.44
P25580	366	4	0.07	0.43	***	0.74
P5943	366	8	0.22	0.53	***	0.43
UK15523	366	11	0.48	0.81	***	0.25
UK16382	341	4	0.11	0.70	***	0.73
UK19417	359	5	0.28	0.65	***	0.39
UK21826	342	13	0.34	0.67	***	0.33
Mean	358	7	0.25	0.63		0.47

Table 5.8. Mean number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), F_{IS} significance, number of usable loci, and number of polymorphic loci across all microsatellite markers for each European *Segmentina nitida* population. F_{IS} significance: Probability that random F_{IS} > observed F_{IS}. Usable loci: number of loci with fewer than 5% missing data. Number of polymorphic loci: number of usable loci with polymorphisms present in the population.

Population	N_{ALL}	H_O	H_E	F_{IS}	F_{IS} Significance	Number of Usable Loci	Number of Polymorphic Loci
Germany	6.143	0.64	0.57	0.53	***	5	5
UK	1.429	0.14	0.11	-0.17	0.955	7	3
Poland	3.429	0.35	0.31	0.08	0.07	7	6
Sweden	4.857	0.50	0.45	0.42	***	5	5

Null alleles were found at all loci with an estimated frequency range of between 0.248 (UK15523) and 0.742 (P25580). P25580 only had a maximum of three alleles found within any one population (Germany and Sweden) but no populations showed monomorphic profiles. The F_{IS} values (Table 5.8) show the highest levels of inbreeding in Germany and Sweden, with low levels in the UK and Poland. The negative value of F_{IS} for the UK population indicates a higher level of heterozygosity then expected in a randomly mating population. Overall, all loci were found to be in pairwise linkage disequilibrium (*p*=<0.0001). However, when split into separate countries, all loci were in pairwise linkage disequilibrium for German and Swedish individuals whilst all loci in UK individuals were in linkage equilibrium, and five of 21 potential pairs of loci were in linkage disequilibrium for Polish individuals (Table 5.9).

Table 5.9. Matrices for pairwise linkage disequilibrium tests of all microsatellite loci for *Segmentina nitida*, split by country. ***<0.0001, **<0.01, *<0.05, NS= Not significant

Germany

	Loci						
	P12988	P25580	P5943	UK15523	UK16382	UK19417	UK21826
P12988		***	***	***	***	***	***
P25580	***		***	***	***	***	***
P5943	***	***		***	***	***	***
UK15523	***	***	***		***	***	***
UK16382	***	***	***	***		***	***
UK19417	***	***	***	***	***		***
UK21826	***	***	***	***	***	***	

UK

	Loci						
	P12988	P25580	P5943	UK15523	UK16382	UK19417	UK21826
P12988		NS	NS	NS	NS	NS	NS
P25580	NS		NS	NS	NS	NS	NS
P5943	NS	NS		NS	NS	NS	NS
UK15523	NS	NS	NS		NS	NS	NS
UK16382	NS	NS	NS	NS		NS	NS
UK19417	NS	NS	NS	NS	NS		NS
UK21826	NS	NS	NS	NS	NS	NS	

Poland

	Loci						
	P12988	P25580	P5943	UK15523	UK16382	UK19417	UK21826
P12988		NS	NS	NS	NS	NS	NS
P25580	NS		NS	NS	NS	NS	NS
P5943	NS	NS		NS	NS	NS	NS
UK15523	NS	NS	NS		***	*	***
UK16382	NS	NS	NS	***		NS	*
UK19417	NS	NS	NS	*	NS		*
UK21826	NS	NS	NS	***	*	*	

Sweden

	Loci						
	P12988	P25580	P5943	UK15523	UK16382	UK19417	UK21826
P12988		***	***	***	***	***	***
P25580	***		***	***	***	***	***
P5943	***	***		***	***	***	***
UK15523	***	***	***		***	***	***
UK16382	***	***	***	***		***	***
UK19417	***	***	***	***	***		***
UK21826	***	***	***	***	***	***	

Of the 28 possible population/locus combinations, five were monomorphic, however no loci were monomorphic across all populations (Table 5.10). Four loci were monomorphic in the UK (P12988, P5943, UK15523, and UK16382) and one in Poland (P12988). Significant deviation from Hardy-Weinberg equilibrium was detected at 17 of the 28 possible population/locus combinations, all due to a deficit of heterozygotes (Table 5.10). The highest observed heterozygosity was found in locus UK15523 in Germany (0.610). This locus had high observed heterozygosity in all countries except the UK, which was monomorphic. UK21826 also had high observed heterozygosity (0.3-0.5) across all countries, with the highest (0.5) in the UK. The lowest observed heterozygosity was in locus P25580, with a mean H_O of 0.07, and a range of 0.10 across all populations. No monomorphic population contained more than one allele. Overall the four countries were partially inbred, with an overall F_{IS} value of 0.3880.

Table 5.10. Genetic diversity of *Segmentina nitida* broken down by country. *n*= Number of individuals genotyped. N_{ALL}= number of alleles present. H_O= Observed heterozygosity. H_E= Expected heterozygosity. *P (HW)* = *p*-value of Hardy-Weinberg Equilibrium test. **= <0.05, ***<0.001 NS=Not significant. ND (mono) = not done due to monomorphic locus

Locus	Parameters	Germany	UK	Poland	Sweden
P12988	*n*	77	31	83	175
	N_{ALL}	4	1	1	4
	H_O	0.156	0	0	0.451
	H_E	0.515	0	0	0.629
	P(HW)	***	ND (Mono)	ND (Mono)	***
P25580	*n*	77	31	83	175
	N_{ALL}	3	2	2	3
	H_O	0.013	0	0.060	0.103
	H_E	0.463	0	0.059	0.246
	P(HW)	***	ND (Mono)	NS	***

P5943	*n*	77	31	83	175
	N_{ALL}	8	1	4	6
	H_O	0.351	0.484	0.241	0.114
	H_E	0.664	0.405	0.261	0.258
	P(HW)	***	NS	NS	***
UK15523	*n*	77	31	81	177
	N_{ALL}	9	1	5	8
	H_O	0.610	0	0.568	0.469
	H_E	0.793	0	0.696	0.677
	P(HW)	***	ND (Mono)	**	***
UK16382	*n*	75	31	79	156
	N_{ALL}	4	1	3	4
	H_O	0.080	0	0.228	0.077
	H_E	0.693	0	0.469	0.601
	P(HW)	***	ND (Mono)	***	***
UK19417	*n*	72	31	83	173
	N_{ALL}	4	2	2	3
	H_O	0.292	0.097	0.464	0.202
	H_E	0.600	0.094	0.355	0.531
	P(HW)	***	NS	NS	***
UK21826	*n*	64	30	81	167
	N_{ALL}	11	2	7	6
	H_O	0.313	0.500	0.383	0.305
	H_E	0.718	0.463	0.494	0.589
	P(HW)	***	NS	**	***

All F_{ST} *p*-values were significant (<0.001) over 110 permutations. The F_{ST} across all loci gave a value of 0.40. Pairwise F_{ST} values ranged between 0.15 and 0.77 (Table 5.11), with the UK population in comparison to both Polish and Swedish populations showing values greater than 0.50.

Table 5.11. Pairwise genetic distance (F_{ST}) estimates with significance levels for the four European populations of *Segmentina nitida*.. ***p<0.001.

Fst All Samples

	Germany	**UK**	**Poland**	**Sweden**
Germany				
UK	0.31***			
Poland	0.45***	0.77***		
Sweden	0.30***	0.62***	0.15***	

The partitioning of the genetic variation in the dataset using the AMOVA showed that the amount of genetic variation between populations (39.39%) was similar to that between all individuals (36.96%). Differences between individuals within populations accounted for 23.64% of variation (Table 5.12).

Table 5.12. Analysis of molecular variance (AMOVA) of microsatellite data for different groups of *Segmentina nitida*. ***p<0.001

Source of variation	**Df**	**Sum of squares**	**Variance components**	**Percentage of variation**	***P***
Among populations	3	340.26	0.69	39.39	***
Among individuals within populations	363	532.04	0.41	23.64	***
Within individuals	367	236	0.64	36.96	***
Total	733	1108.30	1.74		

The Evanno plot generated by STRUCTURE HARVESTER (Figure 5.6) shows the likelihood of each of the potential values of K (distinct lineages) run in the STRUCTURE software. K=2 is the most likely number of lineages for the dataset with a Delta K value of 1249.2, whilst all other values of K have relatively low Delta K values (0.33-73.8) (Appendix C4). K=2 was then chosen as the value to derive the STRUCTURE graphical output with Distruct for (Figure 5.7).

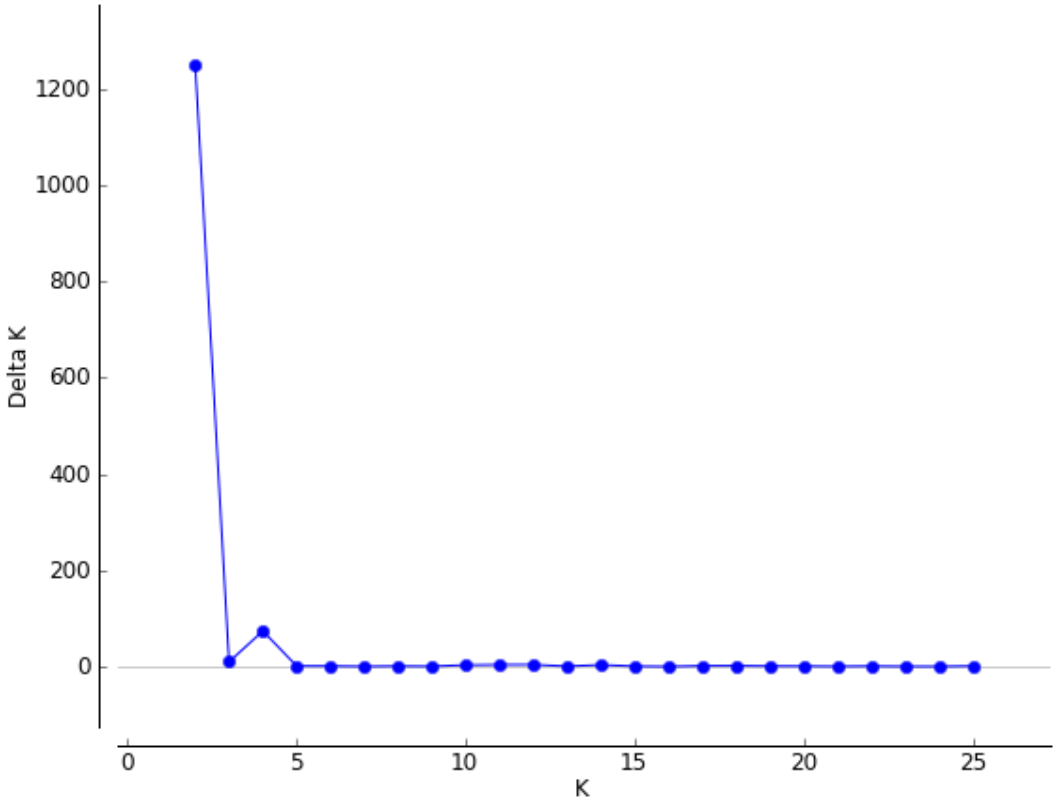


Figure 5.6 Evanno's plot generated by STRUCTURE HARVESTER for the detection of the most likely number of lineages in the microsatellite dataset (K) by the value of Delta K (the rate of change in the log probability of data between successive K values (Evanno *et al.,* 2005)).

STRUCTURE assigns an individual to a lineage based on proportional similarity, with individuals falling into more than one lineage indicating an admixture. Only one individual in population S1 (S.1.16) showed evidence of any admixture, with a probability of being assigned to one lineage of 83.3% (Figure 5.7). All others fall entirely into either one lineage or the other, with over 95% probability. Lineage one (blue, *n*=270) comprised of all individuals in Poland, all individuals in Sweden (apart those at site S2) and some individuals in the German sites G1, G2 and G3, as well as all three individuals in G5. Lineage two (orange, *n*=97) included all UK individuals, some of the German individuals (although this lineage is the only lineage found in population G4) as well as the entire S2 population in Sweden. When F_{ST} analysis was performed in Arlequin there was an F_{ST} value of 0.64, and a significant difference between the two lineages (*P*= <0.005) over 110 permutations. Both lineages had significant F_{IS} index scores (*P*=<0.005, 1023 permutations) with an F_{IS} value of 0.26 for the east lineage, and 0.35 for the west lineage.

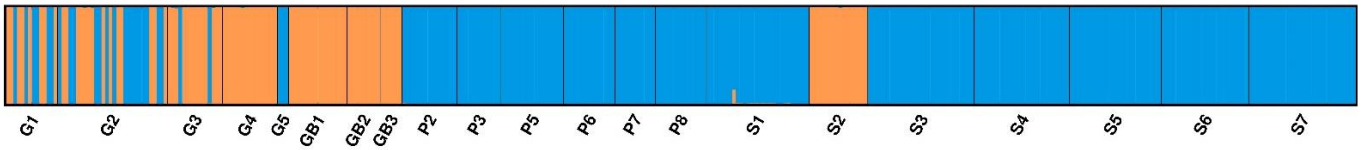


Figure 5.7. *Segmentina nitida* lineages inferred from the posterior probability of individual assignment to a lineage based on genotypes. Each vertical line represents one individual, and each colour represents a single lineage. The height of the line indicates the probability of the individual being assigned to that lineage. Plotted for K= 2. G=Germany, GB=UK, P=Poland, S=Sweden

5.3.3. Genetic diversity and genetic structure of genetic lineages of *Segmentina nitida* in Europe using microsatellite markers

When split into the two separate lineages (East and West) and rerun through Structure analysis the east lineage was tested for 19 sub-lineages and the west lineage tested for 11 sub-lineages.

The Evanno plot generated by STRUCTURE HARVESTER for the East lineage (Figure 5.8) shows K= 3 is the most likely number of sub-lineages for the dataset with a Delta K value of 442.8, with K=7 being the next likely (Delta K= 178.8) and then K= 5 (Delta K=78.1) (Appendix C5). A graphical output was generated with Distruct for K=3, K=5, and K=7 (Figure 5.9). The much higher DeltaK value for K=3 than the other values suggests that this number of natural sub-lineages is the most adequate to explain the data. When K=5 or 7, the sub-lineages became less distinct, with individuals being assigned to groups with probabilities of less than 80% (percentage of total samples in East lineage assigned to a sub-lineage with a probability of less than 80%- K=5: 32.6%; K=7: 42.2%).

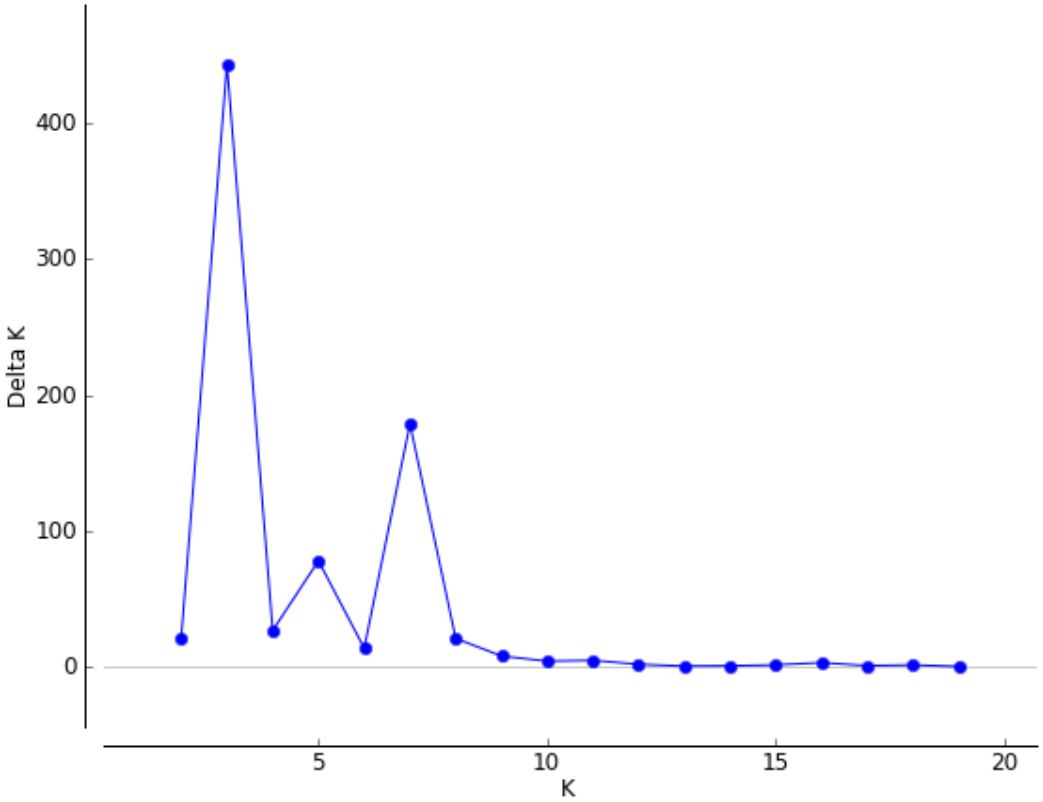


Figure 5.8 Evanno's plot generated by STRUCTURE HARVESTER for the East lineage of *Segmentina nitida* for the detection of the most likely number of sub-lineages within the microsatellite dataset (K) by the value of Delta K.

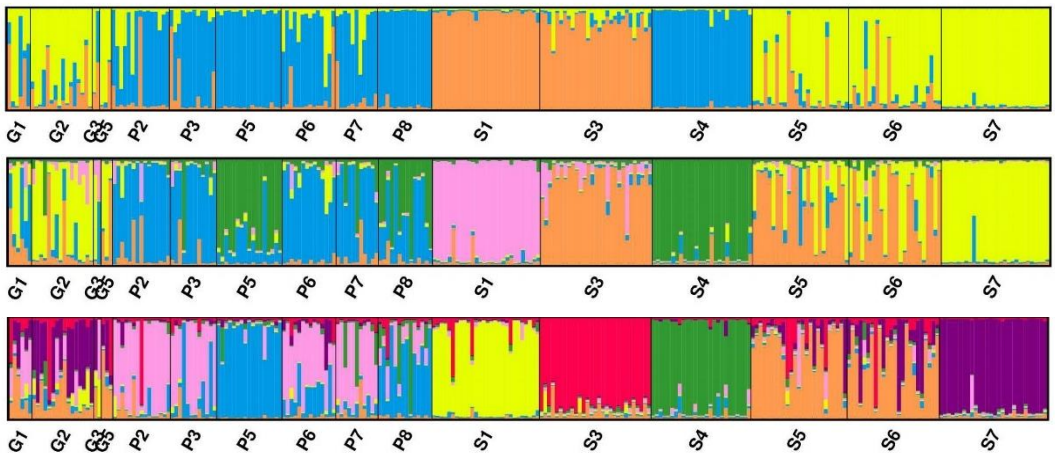


Figure 5.9. Genetic sub-lineages within the East lineage of *S. nitida* inferred from the posterior probability of individual assignment to a sub-lineage based on genotypes. Each vertical line represents one individual, and each colour represents a single sub-lineage. The height of the line indicates the probability of the individual being assigned to that sub-lineage. Plotted for K= 3 (A), K=5 (B), and K=7 (C). G=Germany, P=Poland, S=Sweden

For K=3, all three distinct genetic sub-lineages are present in Sweden. Populations S1, S4, and S7 have very low levels of genetic admixture, with population S3 having some levels of genetic admixture with

the ‘yellow’ sub-lineage, and populations S5 and S5 having high levels of genetic admixture in some individuals with the ‘orange’ sub-lineages (Figure 5.9). Germany had high levels of genetic admixture in all populations between all three sub-lineages in many individuals. In Poland, there were low levels of genetic admixture present in populations P5 and P8; high levels of genetic admixture in populations P2, P6, and P7; and moderate levels in some individuals in population P3.

The partitioning of the genetic variation in the east lineage dataset using an AMOVA, when grouped by country of origin, showed that the majority of genetic variation occurred within the individuals of the entire dataset (67.08%). Genetic differences within countries and between countries accounted for 18.80 and 14.12% of observed variation respectively (Table 5.13.).

Table 5.13. Analysis of molecular variance (AMOVA) of microsatellite data for the East lineage of *Segmentina nitida*. ***p<0.001. Samples grouped by country of origin.

Source of variation	**Df**	**Sum of squares**	**Variance components**	**Percentage of variation**	***P***
Among populations	2	57.53	0.19	14.12	***
Among individuals within populations	267	368.82	0.25	18.80	***
Within individuals	270	239	0.89	67.08	***
Total	539	665.35	1.33		

All F_{ST} p-values comparing individuals in the three countries in the East lineage were significant (<0.001) over 110 permutations. F_{ST} across all loci gave a value of 0.14, indicating relatively low genetic differentiation. Pairwise F_{ST} values ranged between 0.05 and 0.25 (Table 5.14), with Germany in comparison to Poland showing the highest F_{ST} value (0.25), and Sweden in comparison to Germany showing the lowest (0.05).

Table 5.14. Pairwise genetic distance (F_{ST}) estimates with significance levels for the three European countries of *Segmentina nitida* within the East lineage. ***p<0.001.

	Germany	**Poland**	**Sweden**
Germany			
Poland	0.25***		
Sweden	0.05***	0.15***	

The West lineage Evanno output showed K= 2 as the most likely number of sub-lineages (Delta K= 531.4), closely followed by K=3 (Delta K= 524). All other K values had low Delta K (ranging from 0.1-117) (Figure 5.10). Graphical outputs were generated with Distruct for K=2 and K=3 (Figure 5.9).

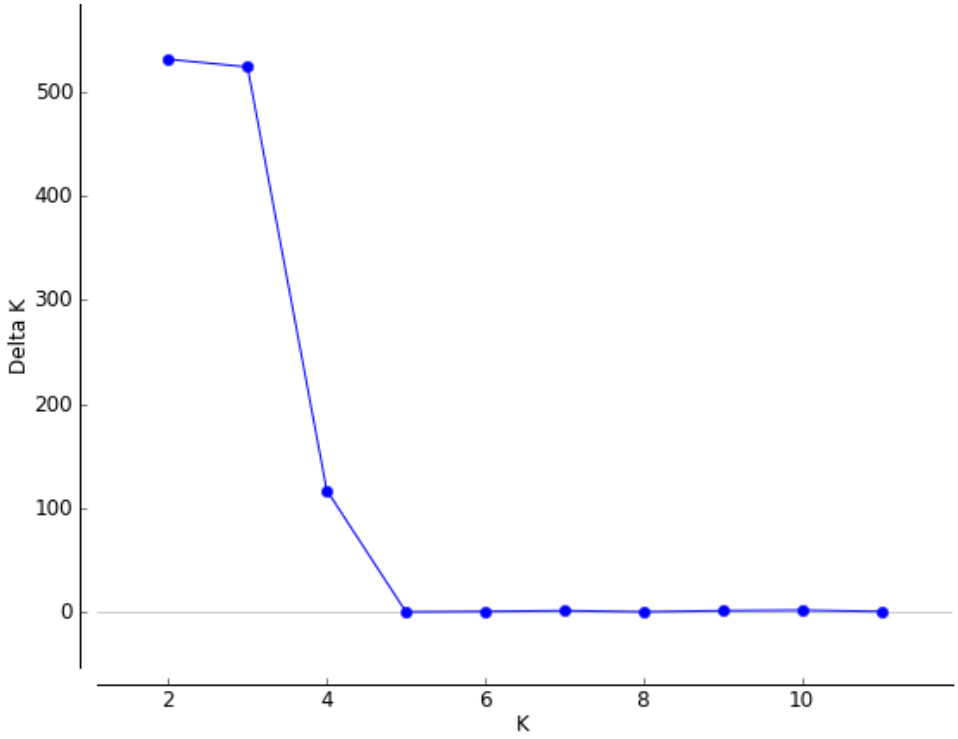


Figure 5.10. Evanno's plot generated by STRUCTURE HARVESTER for the West lineage of *Segmentina nitida* for the detection of the most likely number of sub-lineages within the microsatellite dataset (K) by the value of Delta K.

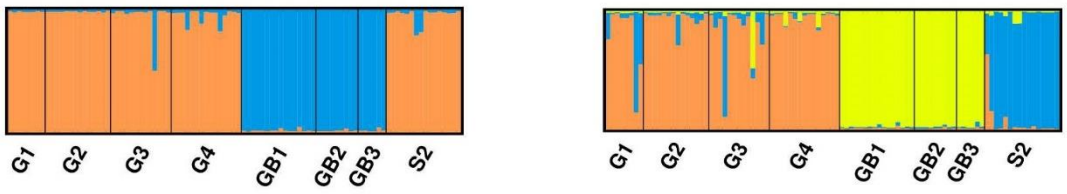


Figure 5.11. Sub-lineages within the West lineage of *S. nitida* inferred from the posterior probability of individual assignment to a sub-lineage based on genotypes. Each vertical line represents one individual, and each colour represents a single sub-lineage. The height of the line indicates the probability of the individual being assigned to that lineage. Plotted for K=2 (A) and K=3 (B). G=Germany, GB=UK, S=Sweden

In both the K=2 and K=3 models the UK is shown as a distinct sub-lineage isolated from all other populations. There is some indication of admixture in the German and Swedish populations from the UK sub-lineage using both K values. In K=2 there is evidence of admixture in German populations G3 and G4 (one and three individuals, respectively), and two individuals in population S2 showing admixture with the UK sub-lineage. For K=3, there is admixture of Swedish genotypes in populations G1, G2, and G3 (three, two, and four individuals, respectively), and admixture of UK genotypes in G3 (one individual) and G4 (three individuals). Sweden shows admixture from both sub-lineages in K=3 (four individuals).

The AMOVA for the West lineage, when grouped by country of origin, showed that the most genetic variation occurred between countries (48.84%) followed by genetic variation within individuals accounted for (48.73%). Genetic differences within countries only accounted for 1.42% of the observed variation (Table 5.15.), indicating little genetic diversity within each country.

Table 5.15. Analysis of molecular variance (AMOVA) of microsatellite data for the West lineage of *Segmentina nitida*. ***p<0.001

Source of variation	**Df**	**Sum of squares**	**Variance components**	**Percentage of variation**	***P***
Among populations	2	44.71	0.37	49.84	***
Among individuals within populations	94	36.41	0.01	1.42	0.38
Within individuals	97	35.50	0.37	48.73	***
Total	193	116.62	0.76		

F_{ST} p-values comparing individuals in the three countries in the west lineage were significant (<0.001) over 110 permutations. F_{ST} across all loci gave a value of 0.50. Pairwise F_{ST} values ranged between 0.45 and 0.69, indicating high genetic differentiation between the countries (Table 5.16). Germany in

comparison to the UK and Sweden showed an F_{ST} value of 0.45, while the comparison between Sweden and the UK showed an F_{ST} value of 0.69.

Table 5.16. Pairwise genetic distance (F_{ST}) estimates with significance levels for the three European countries within the West lineage of *Segmentina nitida*. ***p<0.001.

	Germany	UK	Sweden
Germany			
UK	0.45***		
Sweden	0.45***	0.69***	

5.3.4. Geometric morphometrics of genetic lineages of *Segmentina nitida* in Europe

The mean shapes of the six landmarks across all individuals from both the east and west lineages were generated for deformation grid analysis (Figure 5.12.). The specimen that most closely represented the mean shape for the East lineage was G3.12, and for West lineage, G2.15 was the closest specimen to the mean shape (Figure 5.12)

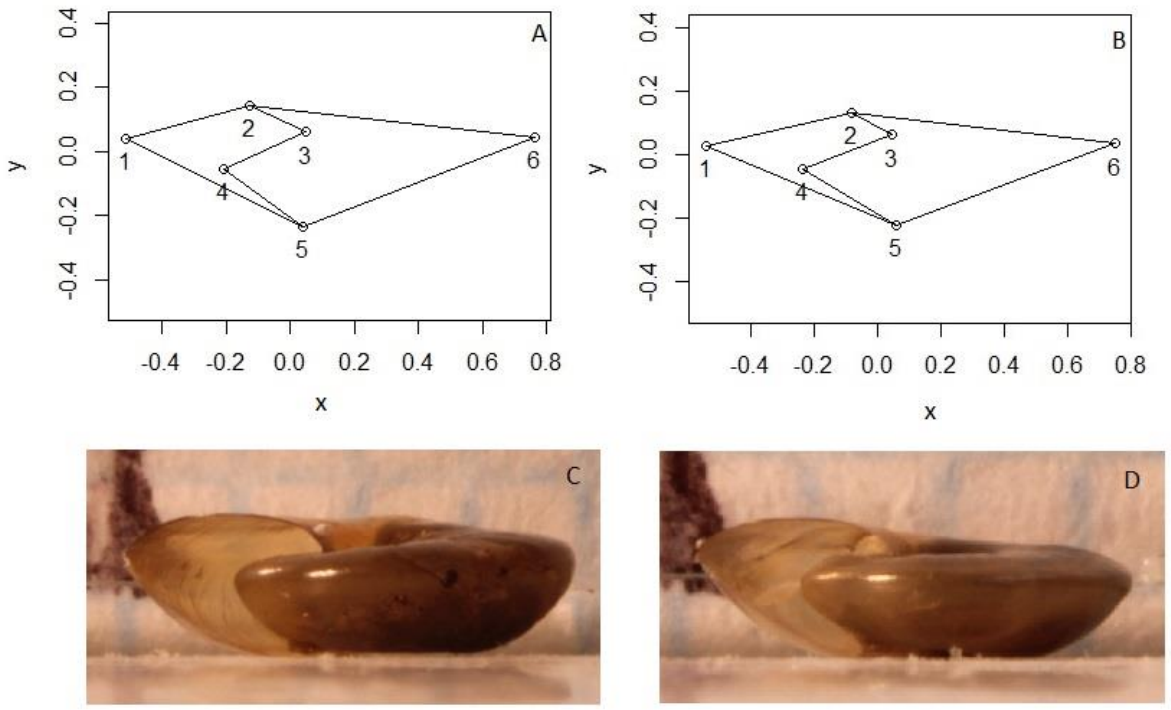


Figure 5.12. Mean shaped landmark orientations of the two genetic lineages and the two specimens most closely representing these mean shapes. A- East lineage mean shape; B-West lineage mean shape; C- G3.12, specimen most closely representing the mean shape of the East lineage; D- G2.15, specimen most closely representing the mean shape of the West lineage.

Deformation grids show the differences between the mean shape of each of the lineages to the overall mean shape, as well as between each other (Figure 5.13). Key areas of variation between the mean shapes of the two lineages (those that showed the most deformation to the grid) were landmark (LM) 2, LM3, LM4 and LM5, all detailing the shape of the aperture of the shells, with moderate deformation at LM1, the keel by the aperture. There is little deformation focussed around LM6, showing the keel on the side of the shell furthest from the aperture

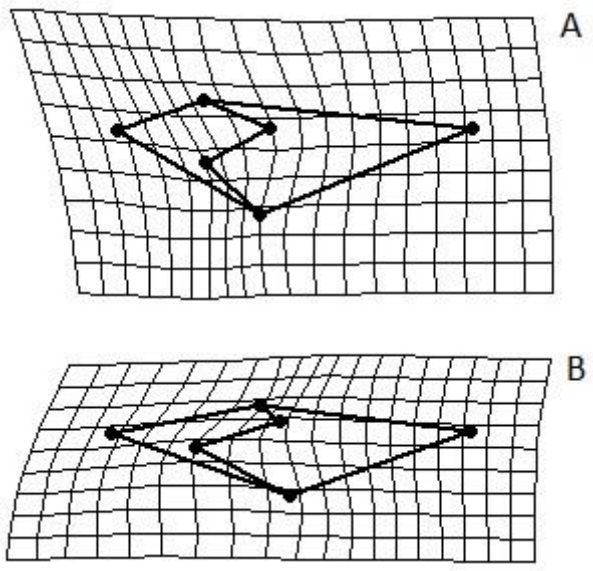


Figure 5.13. Thin-plate spline grids showing the deformation of the average shaped *Segmentina nitida* individual from each lineage to each other. A- East lineage mean shape compared to west lineage mean shape as reference; B- West lineage mean shape compared to east lineage mean shape as reference.

The centroid size of individuals in the east lineage was significantly higher than those in the west lineage (Mann-Whitney U test; U= 7420, *P*= <0.0001) (Figure 5.14).

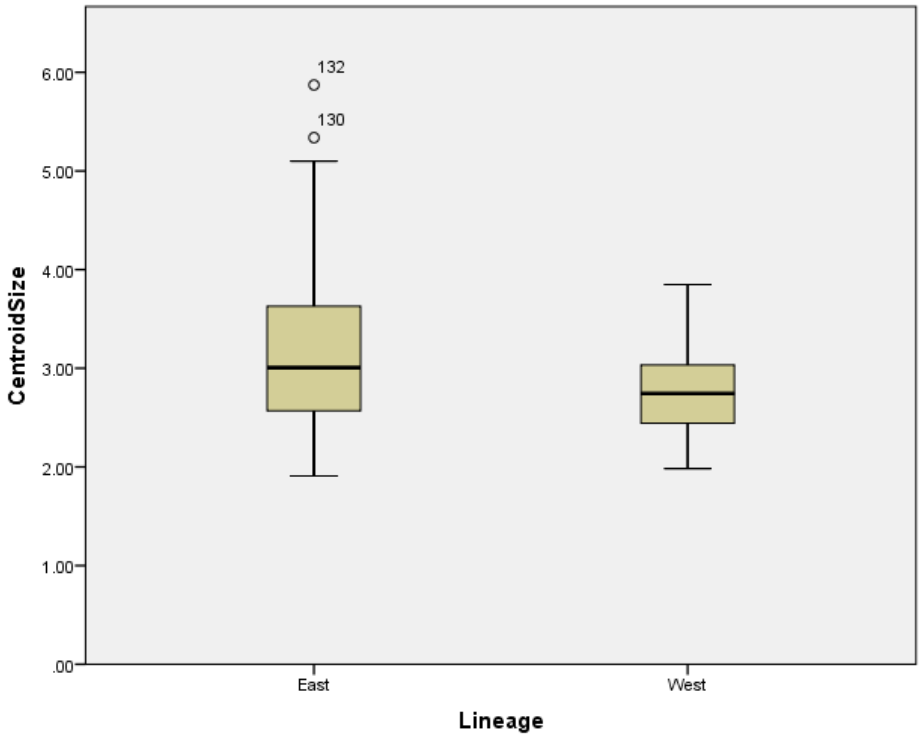


Figure 5.14. Boxplots of shell centroid size of *Segmentina nitida* individuals from the East and West genetic lineages. Lines inside boxes show median, boxes around median show upper and lower quartile and whiskers show outer quartiles.

A PCA of the 12 Procrustes shape coordinates of the six landmarks placed on *Segmentina nitida* individuals from the two genetic lineages shows that the west lineage snails cluster relatively tightly, whilst there is a much wider spread in points for the east lineage (Figure 5.15). Principal component (PC1) 1 and PC2 account for 84% of variation in shape (60.6% and 23.4% respectively). There was a significant difference in both PC1 score and PC2 score between the individuals from the two lineages (Mann-Whitney U Test; PC1: U=4583, *P*=<0.0001; PC2: U=7627, *P*=<0.0001).

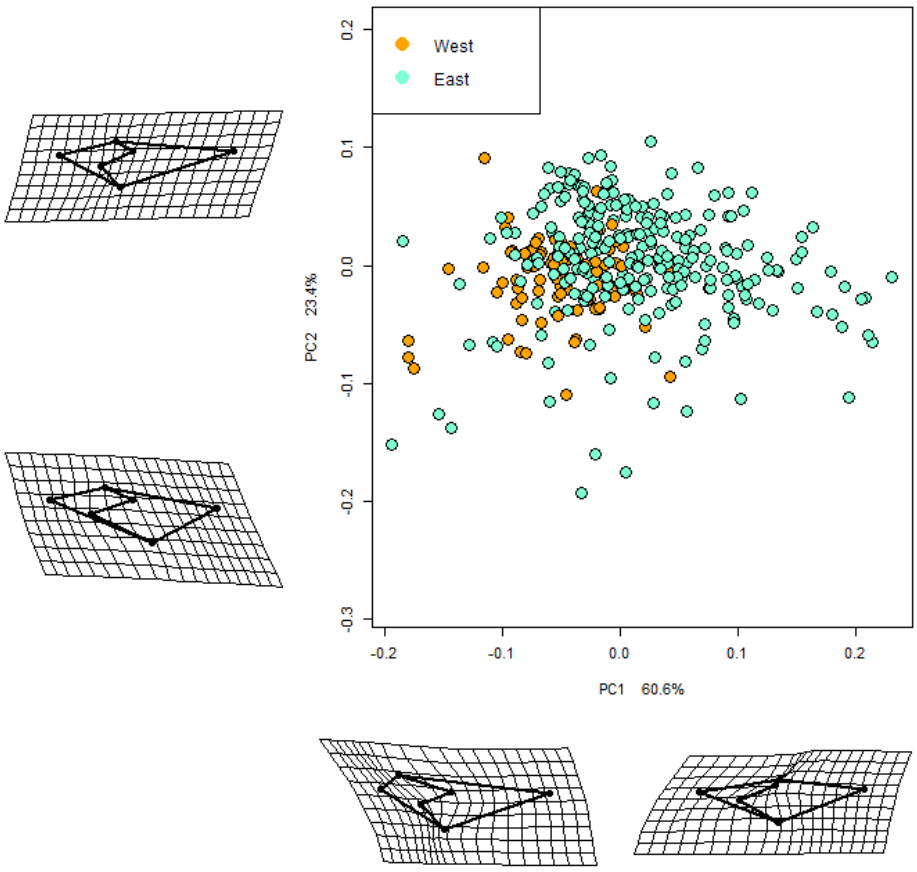


Figure 5.15. Principal component plot of *Segmentina nitida* individuals grouped by genetic lineage. Deformation grids indicate the shape of landmarks at the extremes of each Principal Component, in relation to the mean shape of all individuals.

When grouped by country, the first two discriminant functions (DFs) of a discriminant analysis of the twelve Procrustes shape coordinates accounted for 87.3% of the total shape variation in the dataset (DF1- 56.3%, DF2- 31.0%). The discriminant functions were able to correctly assign 54.87% of the shells in the analysis to their correct country of origin (jackknifed). The UK and Poland had the highest percentage of individuals assigned correctly (70.8% and 61.7%, respectively), whilst Sweden has the lowest percentage of individuals correctly assigned (48.8%) (Table 5.17).

Table 5.17. Matrix of classifications of *Segmentina nitida* shells grouped by country in discriminant analysis model of Procrustes shape coordinates. Numbers represent the number of shells classified as belonging to each country, with percentage in parentheses. Blue cells show number and percentage of shells correctly classified to country. Rows: Country of origin. Columns: Predicted country based on shape.

		Country assignment by analysis				
		Germany	**UK**	**Poland**	**Sweden**	**Total**
Actual country of origin	**Germany**	40 (55.6)	12 (16.6)	10 (13.9)	10 (13.9)	72
	UK	4 (16.7)	17 (70.8)	1 (4.2)	2 (8.3)	24
	Poland	10 (12.4)	7 (8.6)	50 (61.7)	14 (17.3)	81
	Sweden	32 (19.7)	19 (11.7)	32 (19.8)	79 (48.8)	162
	Total	86	55	93	105	339

When grouped by lineage, the discriminant analysis produced a single discriminant function, which accounted for 100% of the shape variation. The *x* coordinates for LM2 and LM5 had the highest positive loadings, showing the top and bottom points of the aperture. The *x* coordinates for LM1 and LM4 had the highest negative loadings, showing the position of the keel at the edge of the shell and in the aperture (Table 5.18).

Table 5.18. Loadings for the discriminant function of the discriminant analysis of the lineages of *Segmentina nitida,* based on Procrustes shape coordinates for the six digitised landmarks.

	X1	**Y1**	**X2**	**Y2**	**X3**	**Y3**	**X4**	**Y4**	**X5**	**Y5**	**X6**	**Y6**
DF1	-0.013	-0.006	0.023	-0.005	-0.002	0.002	-0.014	0.005	0.011	0.005	-0.004	-0.007

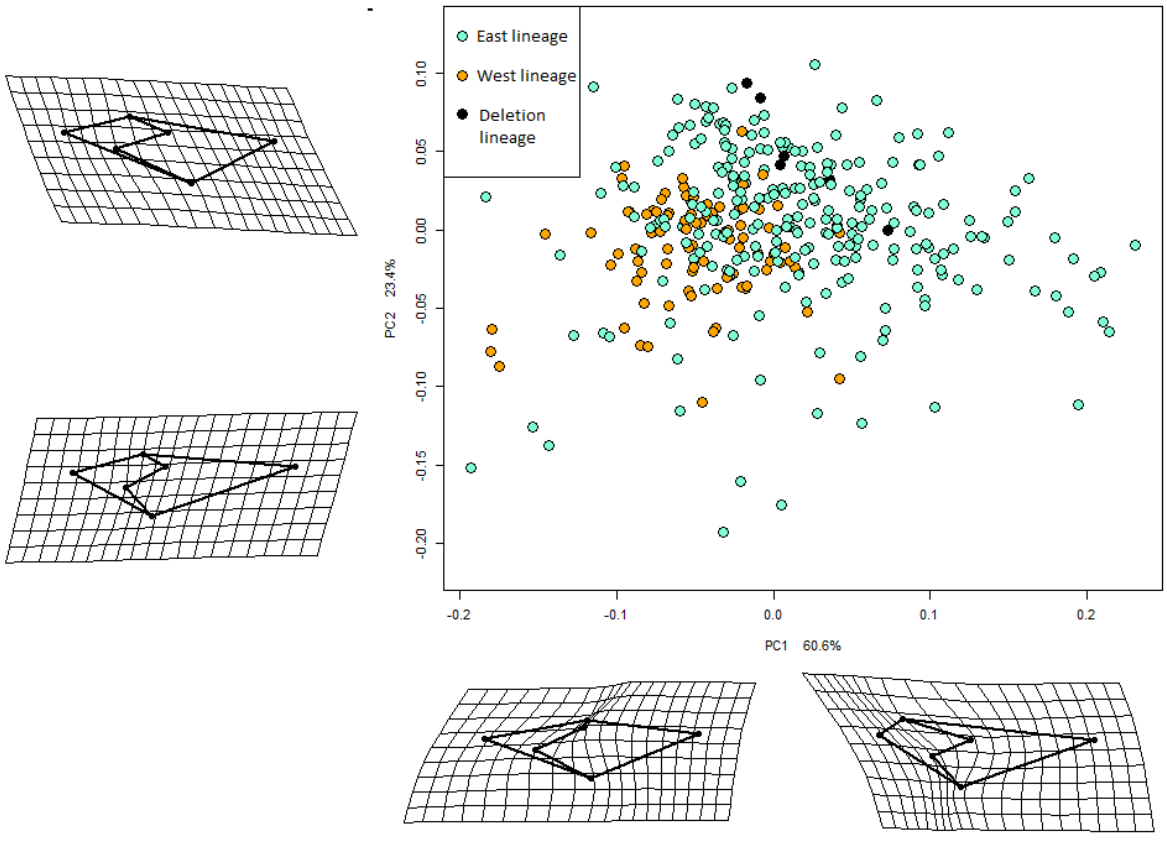
The discriminant function was able to correctly assign 85.55% of the shells in the analysis to their correct genetic lineage (jackknifed). 90.7% of the individuals assigned to the west lineage were assigned to that lineage based on the Procrustes shape coordinates, with only seven individuals being misclassified, whereas 83.8% of the east lineage were correctly classified (Table 5.19).

Table 5.19. Matrix of classifications of *Segmentina nitida* shells grouped by genetic lineage in discriminant analysis model of Procrustes shape coordinates. Numbers represent the number of shells classified as belonging to each lineage, with percentage in parentheses. Blue cells show number and percentage of shells correctly classified to genetic lineage. Rows: genetic lineage assigned by structure analysis. Columns: Predicted lineage based on shape.

		Lineage assigned by analysis		
		West Lineage	**East Lineage**	**Total**
Genetic lineage from Structure Analysis	**West Lineage**	78 (90.7)	8 (9.3)	86
	East Lineage	41 (16.2)	212 (83.8)	253
	Total	119	220	339

Of the seven individuals containing the 9 bp deletion in their COI sequence, six also had morphometrics data, all of which clustered in the East lineage. When these six individuals were classified as a unique lineage, hereby referred to as 'Deletion lineage' a PCA did not show them as a distinct group from the East Lineage (Figure 5.16).

Figure 5.16. Principal component plot of *Segmentina nitida* individuals grouped by genetic lineage, with individuals with a 9 bp deletion in their COI sequence classified as Deletion lineage. Deformation grids indicate the shape of landmarks at the extremes of each Principal Component, in relation to the mean shape of all individuals.



When grouped by these three lineages, the first two discriminant functions (DFs) of a discriminant analysis of the twelve Procrustes shape coordinates accounted for 100% of the total shape variation in the dataset (DF1- 94.7%, DF2- 5.3%). The discriminant functions were able to correctly assign 64.31% of the shells in the analysis to their correct lineage (jackknifed). Four of the individuals with the 9 bp deletion were classified as the 'deletion' lineage, with the remaining two classified as the East lineage by the discriminant analysis. The addition of the 'deletion' lineage in the discriminant analysis caused 68 individuals from the East lineage, and 13 individuals from the West lineage to be classified as the 'deletion' lineage (Table 5.20). Overall, 64.3% of individuals were assigned to the correct genetic lineage by shape when these three genetic lineages were considered.

Table 5.20. Matrix of classifications of *Segmentina nitida* shells grouped by genetic lineage in discriminant analysis model of Procrustes shape coordinates, based on three lineages, with 'deletion' lineage indicating individuals with a 9 bp deletion in their COI sequences. Numbers represent the number of shells classified as belonging to each lineage, with percentage in parentheses. Blue cells show number and percentage of shells correctly classified to genetic lineage. Rows: genetic lineage assigned by genetic analyses. Columns: Predicted lineage based on shape.

		Lineage assigned by analysis			
		West Lineage	**East Lineage**	**'Deletion' lineage**	**Total**
Genetic lineage from Genetic Analyses	**West Lineage**	66 (77)	7 (8)	13 (15)	86
	East Lineage	31 (13)	148 (60)	68 (27)	247
	'Deletion' lineage	0 (0)	2 (33)	4 (67)	6
	Total	97	157	85	339

5.4. Discussion

5.4.1. Genetic structure of European populations of *Segmentina nitida*

The mitochondrial and nuclear markers used in this study revealed two distinct lineages of European *Segmentina nitida* with high bootstrap support: one lineage represented by Polish and Swedish individuals (East), and one represented by UK individuals (West), with Germany containing a mixture of individuals from these two lineages. Groupings were consistent for COI and ITS2 markers..

Mismatch analysis for both ITS2 and COI and the seven microsatellite loci analysed also showed evidence of two distinct lineages. Whilst the evidence presented here supports the existence of two lineages of *S. nitida* in Europe, Mensch (2009) found three lineages, one comprised of UK and German individuals, one with only Polish individuals, and one comprised of German, Polish and a UK individual, using a different genome-wide genotyping method (Amplified Fragment Length Polymorphism, AFLP). Mensch (2009) also sequenced the 12S mitochondrial DNA marker, but only for UK and German individuals. The 12S marker showed German haplotypes being genetically distinct from UK haplotypes, and revealed considerable genetic variation within haplotypes found in the UK. However, it has been demonstrated that AFLP analysis suffers from relatively high genotyping error (Zhang and Hare, 2012). In a study on *Plukenetia volubilis* using AFLP genotyping, between two (K = 2) and nine unique populations (K = 9) were found, depending on genotyping errors (such as mismatches in genotype calling) as well as errors added at various experimental and analytical steps of the experiment to test the subsequent errors in analysis (Vašek *et al.*, 2017). Zhang and Hare (2012) showed that genotyping errors in AFLP can significantly affect analysis of population structure and make it impossible to determine the 'true' number of populations among a set of individuals (Zhang and Hare, 2012). In the previous population genetics study on *S. nitida* (Mensch 2009), shifting AFLP peak profiles were observed between individuals, which may cause misplacement of individuals in analyses if incorrectly genotyped. Additionally, only ten individuals from Germany were analysed by Mensch (2009), in comparison to the 77 individuals used in the microsatellite analyses presented here, which may make the analysis less robust, though these individuals were sourced from a greater geographical range within Germany than those used in the present study. It is possible that the distinct German lineage reported by Mensch (2009) was not present in my study. Additionally, different statistical methods of estimating the number of populations can yield different results, so this may also explain the difference in the number of lineages found (Vašek *et al.*, 2017). To resolve the number of lineages more conclusively, a comprehensive analysis, e.g. of genomes of *S. nitida* individuals from locations included both in Mensch (2009) and in the present study would be necessary.

Seven individuals had a 9 bp deletion in their COI sequence, one individual clustered with the West lineage, and six with the East lineage. Within the East lineage the six individuals clustered closely with other individuals without this deletion and were not supported by significant bootstrapping node values and therefore are unlikely to equate to a third lineage. The single individual with this deletion in the West lineage was supported by a strong bootstrap node value and possibly equates to a unique haplotype. With both microsatellite and ITS2 markers this individual does not appear to belong to a separate lineage to all other individuals within the West lineage. The six individuals with morphometrics data were not morphologically distinct from all other individuals, indicating that these are unlikely to be regarded as a third lineage, or evolutionarily significant unit (ESU).

A consistent pattern across all molecular markers analysed was that the German *S. nitida* populations were generally composed of a mixture of the two distinct lineages detected (East and West), with individuals from both lineages coexisting at sampling sites G1, G2, and G3. Additionally, no *S. nitida* individual from Germany contained alleles from both the East and the West lineage. There are two possible explanations for these data.

First, the two lineages may represent a species-complex in *S. nitida,* with two cryptic species (or genetically distinct sub-species) that co-exist at sites in Germany. The presence of an additional form of *Segmentina nitida* in Eastern Europe (*Segmentina nitida* f. *distinguenda*) has previously been posited in Polish literature (Piechocki, 1979; Piechocki and Wawrzyniak-Wydrowska 2016). The validity of this distinction of this form has been debated, however, with some considering this form as a distinct species (Stadnychenko 1990), *Segmentina clessini* (Westerlund 1873). 106 polymorphic sites were found among the COI sequences of Polish (West lineage) and UK (East lineage) *S. nitida* individuals in addition to seven polymorphic sites (three insertion-deletion polymorphisms, and four sequence differences) in the ITS2 sequences. Significant genetic distance between the UK and Polish populations was also observed in the microsatellite data (F_{ST}=0.77). Comparison to genetic data from *S. nitida* individuals from the species type locality (Denmark) was not possible as the only genetic data

available for this population (Jørgensen *et al.* 2004) is a fragment of the COI locus outside of the region amplified for the present study.

For the 339 individuals with both genetic and morphological data available, both shape and genotype data allowed delineation of the two lineages, more so than country of origin. When grouped by country, shape could only assign 54.87% of the snails to their correct country of origin. When grouped by lineage, this increased to 85.55%. The discriminant analysis for shells of *S. nitida* to *H. complanatus* could assign snails to the correct species 95.83% of the time (Chapter 4). Piechocki and Wawrzyniak-Wydrowska (2016) describe the shell of *Segmentina nitida* f. *distinguenda* as having a keel more displaced towards the centre of the shell perimeter, having a narrow umbilicus, more strongly developed internal thickenings, and having a lower shell height than *Segmentina nitida*. Based on the geometric morphometrics of the two genetic lineages described in this chapter, the keel of the West lineage is closer to the centre of the shell, with a lower shell height than the East lineage, and has a significantly smaller centroid size than the East lineage. Morphological comparison to shells and genetic comparison to preserved individuals already collected and identified as *S. nitida* f. *distinguenda* would be necessary to confirm whether the East lineage truly represents this morphotype. Given the genetic differences found in microsatellites, COI, and ITS2, this second form of *S. nitida* may be a different species instead of a different morphotype and would need to be described as such. Dissection and comparison of reproductive anatomy of the two lineages would be needed for species descriptions and delimitation. Additionally, sourcing individuals from the type country, Denmark (Müller 1774) and comparing genetics, shell morphology and internal anatomy to populations assigned to the East and West lineages would suggest which lineage should be described as *Segmentina nitida*, and whether one lineage should be recognised as *Segmentina clessini* (Westerlund 1873), or described as a new species. A full redescription of *Segmentina nitida* encompassing genetics, shell morphology, radula structure, and reproductive anatomy would aid in clarifying the status of and supporting consistent identification of this/ these species in Europe.

An explanation for the co-existence of two distinct lineages in Germany emerging from the present data may be that the German habitats have only very recently been colonized by the 'East' (Polish/Swedish) or West (UK) lineage of *S. nitida*, where the respective other lineage was already present, providing little time for mating and exchange of alleles between lineages. However, even assuming a very recent meeting of lineages, *S. nitida* has a relatively short reproductive cycle, with multiple breeding events throughout a year (Książkiewicz and Gołdyn, 2008). As a hermaphroditic species it is able to outcross (Mavárez *et al.*, 2002a; Lamy *et al.*, 2012), so some degree of genetic admixture between these lineages would be expected, even within a short time span. Moreover, the STRUCTURE analysis of microsatellite data revealed no evidence of admixture between lineages in any of the German samples, and in the COI and ITS2 analyses German snails fell in either the East lineage or the West lineage. The greatest genetic differentiation based on (F_{ST}) values was observed between the UK and Polish/Swedish populations (0.77 and 0.62 respectively). If the two lineages do represent separate species that cannot interbreed or are significantly more likely to mate within their lineage, using Polish or Swedish snails for reintroduction into the UK or supplementation of UK populations could amount to the introduction of a non-native species, which may unpredictably impact on the conservation of *S. nitida* and on the ecology of affected habitats in general. For example, the introduced lineage/species may out-compete native UK populations or mating may produce infertile offspring. Invasive freshwater snails can pose significant threat to native fauna and also humans, as some species can act as vectors for parasitic trematode species, which can disrupt ecological and physiological host function (Morley, 2008)

All seven microsatellite markers analysed for *S. nitida* showed significant levels of Linkage Disequilibrium (LD). LD is defined as the non-random association of alleles at two or more loci (Slatkin, 2016) where certain alleles are inherited together more often than would be expected by chance. High levels of LD can be a consequence of various factors, including natural selection, random drift in finite populations, and non-random mating within a population (Ridley, 2004), and very importantly, population subdivision and population bottlenecks (Nei and Li, 1973; Slatkin, 2016). Low LD values

usually indicate random out-crossing and the absence of strong evolutionary pressures on a population. When broken down by individual countries, all microsatellite loci in Swedish and German individuals were at pairwise LD, possibly because of the presence of the two lineages within the countries (Slatkin, 2016). All loci in UK snails were not at linkage disequilibrium, most likely due to four of the loci being monomorphic, and the remaining three loci only displaying two alleles, which indicates that the UK populations could be highly bottlenecked, or lacking in genetic diversity. Changes in population size, especially extreme reduction such as that seen in genetic bottlenecks can increase LD (Slatkin, 2016). In Polish snails some loci were in LD, and some not, and this may be due to the temporary nature of the habitats sampled there. In the survey for *S. nitida* in Poland, several populations were found in semi-permanent ponds which dry out for the summer months (Książkiewicz and Gołdyn, 2008), and can even be dry for a number of years (Gołdyn pers. comm.) which may cause extreme reductions in population size. When such a bottleneck occurs, some haplotypes may be lost, generally resulting in an increased LD. Genetic bottlenecks may also explain the overall heterozygote excess observed in the data for every country included in the analysis. In a bottleneck event many low-frequency alleles are lost from a genepool, resulting in a loss of allele diversity, and an excess of heterozygosity (Nei *et al.,* 1975). With the relatively small and fragmented populations in the UK (Killeen and Willing, 1997; Killeen, 2000; Hill-Cottingham, 2004) that show a very low number of microsatellite alleles, and the semi-permanent habitats in Poland (Książkiewicz and Gołdyn, 2008), it is possible that the increased H_O is due to genetic bottlenecks in these populations.

All microsatellite loci showed high probability of null alleles. The presence of null alleles has been known to lower the apparent genetic variability of populations, and affect population genetic analyses that rely on HWE (Chapuis and Estoup, 2007). The importance of carefully considering the presence of null alleles and the fact that the inbreeding coefficient (F_{IS}) is highly sensitive to genotyping errors (such as null alleles or short-allele dominance) have been demonstrated previously (Lamy *et al.*, 2012). Fixation index (F_{ST}) values and genetic distance have also been found to be overestimated in the presence of null alleles (Chapuis and Estoup, 2007; Chybicki and Burczyk, 2009). Therefore, the F_{IS}

values may not be a reliable indicator of inbreeding and the F_{ST} values obtained from the microsatellite data here may overestimate levels of genetic differentiation. However, the low genetic diversity and differentiation observed here for some populations was also supported by their respective ITS2 and COI markers. This illustrates the importance of using multiple markers, ideally nuclear and mitochondrial, to infer population structure and diversity (Waples and Gaggiotti, 2006; Roberts *et al.*, 2013; Gu *et al.*, 2015).

5.4.2. Genetic diversity of European populations of *S. nitida*

When data from all populations were combined, high haplotype diversity was observed in the mitochondrial DNA (COI) marker in *S. nitida*, with an overall haplotype diversity of 0.93. This is likely due to the presence of the two distinct genetic lineages across Europe. Lower haplotype diversity was found in some individual populations by country, ranging from 0.36 to 0.93. Haplotype diversity was relatively lowest in the UK (0.36), whilst relatively high in Poland and Sweden, and highest in Germany. Both Germany and Sweden were found to contain individuals and/or populations from both genetic lineages, which would explain the greater haplotype diversity. Other population genetics studies on freshwater snails have found similarly high levels of COI haplotype diversity. For example, Gu *et al.* (2015) found an overall haplotype diversity of 0.985 in populations of *Bellamya aeruginosa* in China, associated with strong gene flow facilitated by passive dispersal. Standley *et al*. (2014) found a high haplotype diversity of 0.759 in *Biomphalaria choanomphala,* which they attributed to the presence of parasitism within the species strongly influencing population structure (Standley *et al.*, 2014). When the dataset for *S. nitida* was split into the two lineages reflected in the ML tree for the COI marker, the haplotype diversity for COI dropped for both the West lineage (UK, Germany, S2) to 0.578 and the East lineage (Poland, Sweden, Germany) to 1.00. The lower diversity in the West lineage may be caused by founder effects or bottlenecks, particularly in the UK populations. When viewed by itself, the UK population of *S. nitida* has a haplotype diversity of 0.36, only two haplotypes, and a nucleotide diversity of 0.001, all of which are much lower than any other population by country. This lower diversity is also represented in the microsatellite marker data for the UK populations, with four of the

seven loci monomorphic (generated from both UK and Polish populations), with the remaining three loci only showing a maximum of two alleles each. Limited haplotypes (*N*=2) and a small number of alleles present within these populations suggests they were founded by one or very few individuals (Excoffier and Ray, 2008; Excoffier *et al.,* 2009), or experienced a severe genetic bottleneck. This founder effect in the UK *S. nitida* populations may be exacerbated by self-fertilisation, as seen in another European freshwater snail, *Radix balthica* (Pfenninger *et al.,* 2011). The ability to reproduce uniparentally has long been considered to influence colonisation success (Darwin 1876). Expansion of a species, whether ongoing or recent, generally involves founder effects at newly colonised habitats which decrease genetic variability (Excoffier and Ray, 2008; Excoffier *et al.*, 2009). The low genetic diversity in UK populations therefore indicates that the sampled habitats have only recently been colonised by a limited number of individuals or that they have undergone a genetic bottleneck. This could mean that these populations are more vulnerable to local extinction (Aguilar *et al.*, 2008), highlighting the need for larger and more interconnected habitats to help protect this UK Biodiversity Action Plan priority species.

Relatively low genetic diversity was also found in Polish *S. nitida*, with the lowest nucleotide diversity for COI (0.016) among the countries studied, and the second lowest observed heterozygosity for the microsatellite markers (0.35). Most of the sites surveyed for *S. nitida* in Poland were semi-permanent ponds in the middle of agricultural land (Książkiewicz and Gołdyn, 2008). The low genetic diversity observed in Poland may be the result of multiple successive local extinction and recolonization events in these temporary habitats. Recolonization of empty habitats is generally rapid and may be facilitated by human or animal activities (Mavárez *et al.*, 2002a). In addition to recolonization from surrounding populations via dispersal, the recolonization of these habitats may be through 'dormant' individuals aestivating in the dry ground of the pond. In *Drepanotrema depressissimum,* a tropical freshwater snail that inhabits similarly fragmented and temporary habitats, it was found that when these habitats refilled, the dormant snails would breed with each other. These populations all had low levels of selfing, even though they were initially small following drying, (Lamy *et al.*, 2012). In *D. depressissimum*

this was due to the species being an obligate outcrossing species (whilst still being a hermaphrodite) that avoids selfing. This may be the case for *S. nitida* as well, as its Polish habitats have been seen to be dry for years at a time (Gołdyn pers. comm.) and still have low levels of inbreeding (based on low F_{IS} values), potentially indicating that *S. nitida* is also an obligate outcrosser. This would need to be explored further in captive breeding experiments to provide experimental evidence.

The higher diversity in the East lineage of *S. nitida* may be due to the more permanent nature of many of the habitats its members occupy, especially in Germany and Sweden. These habitats were generally large glacial ponds in Sweden, and well-maintained drainage ditches connected to a large and periodically flooding wetland area in Germany (along the Peene river). In many freshwater habitats, the availability of water varies over time due to seasonal precipitation cycles (dry periods and wet periods), which can lead to large fluctuations in population size of gastropods and even to local extinction events (Sturrock, 1973; Brown, 1994). Rapid loss of genetic diversity can be the result of such fluctuation in population size and geographical isolation (Li *et al.*, 2011; Tian-Bi *et al.*, 2013), with populations in temporary habitats expected to exhibit less genetic diversity than those in more permanent habitats (Escobar *et al.*, 2009).

5.4.3. Genetic differentiation of European populations of Segmentina nitida

One of the important parameters used to differentiate between populations within a species is that of the fixation index (F_{ST}) (Hartl and Clark, 2007). Generally, a low level of genetic differentiation exists between populations when F_{ST} is between 0 and 0.005. When F_{ST} values are greater than 0.15 there is a high, or significant, level of genetic differentiation between populations (Hartl and Clark, 2007; Frankham *et al*., 2010). When the dataset is viewed as a whole, the F_{ST} results from the microsatellite data show the *S. nitida* populations studied here cannot be considered as being drawn from the same gametic pool. All populations appear to be genetically differentiated (overall F_{ST}= 0.40). The F_{ST} value between *S. nitida* from Sweden and Poland was 0.15, on the lower threshold of 'high genetic differentiation', likely explained by the majority of the individuals in both of these countries belonging

to the East lineage. Countries within the East lineage were not strongly differentiated from each other with an F_{ST} of 0.14, compared to the whole dataset F_{ST} value of 0.40, implying there may be a level of interconnectivity between these populations. This is also shown in the STRUCTURE analysis of the East lineage, which showed high levels of genetic admixture in some populations, especially in Germany. Anthropogenic translocations and animal-mediated dispersal via waterfowl represent a possible explanation for gene flow between distant populations or the occurrence of an unusual genotype in one locality, and these processes commonly occur in freshwater snail habitats (Green and Figuerola, 2005; Gittenberger, 2012; Kopp *et al.* 2012). Gene flow and dispersal are key processes with fundamental influences on the demography and evolution of spatially structured populations (Davis *et al.*, 2018). One of the potential avenues for gene flow between populations of the East lineage is long distance dispersal by waterbirds (Van Leeuwen *et al.*, 2012). Long distance dispersal (LDD) of aquatic invertebrates is defined as overland dispersal between wetlands separated by at least 10km that are not connected hydrologically (Green and Figuerola, 2005). LDD can be facilitated by waterbirds either internally with the ingestion of propagules (eggs) (Green and Figuerola, 2005; Van Leeuwen *et al.*, 2013; Figuerola *et al.*, 2014), or externally with individuals or eggs stuck to feathers or feet (Wesselingh *et* al., 1999; Green and Figuerola, 2005). A study by Figuerola *et al.* (2005) found that matrices of bird movement probabilities related to genetic differences between invertebrate populations and explained significant variations in the relationships between *Daphnia ambigua, Daphnia laevis,* and *Cristatella mucedo* populations for mtDNA (Figuerola *et al*., 2005), suggesting that long distance dispersal by birds is a source of ongoing gene flow in these freshwater invertebrates. If birds have travelled between Poland and Sweden this may contribute to the pattern of a metapopulation/distinct lineage for these two countries and explain the admixture of different lineages within them. Connectivity between populations is a major factor influencing gene flow as observed in other freshwater snail species (Viard *et al*., 1997; Mavárez *et al*., 2002a; Mavárez *et al.*, 2002b; Thiele *et al.,* 2013) and other freshwater invertebrates (Freeland *et al.*, 2000; Pálsson, 2000).

5.4.4. Population structure within identified genetic lineages of *Segmentina nitida* in Europe

5.4.4.1. East Lineage

The East lineage discovered in the microsatellite and COI genetic analysis most likely contains three distinct genetic sub-lineages, based on STRUCTURE analysis. There was only a single sub-lineage in Poland, with evidence of admixture in some individuals from sub-lineages found in Germany, and Swedish populations S5, S6, S7, as well as predominantly made up of genotypes of the same sub-lineage as population S4. All three sub-lineages occur in Swedish populations with populations S5 and S6 showing the highest levels of genetic admixture in Sweden, with genotypes from populations S3 (14.71km distant) and S1 (65.7km distant). Dispersal of these sub-lineages and movement between them is most likely facilitated by animal mediated dispersal, e.g. through birds. Genetic variation between the countries (only accounting for 14.12% of genetic variation) and within countries (18.80%), with the most genetic diversity within individuals. This is also represented in the high haplotype diversity found within the lineage as well (0.934). This indicates there is movement of individuals or genotypes between the different countries. Most sample sites in both Germany and Sweden were part of extensive wetlands and near large water bodies (e.g. the River Peene in Germany and coastal marsh areas in Sweden). Migratory birds favour coastal marshes as stopover sites (Figuerola *et al.*, 2003, 2014; Green and Figuerola, 2005), this may explain the high levels of admixture observed in these populations. Conversely, Polish sample sites were centred more around agricultural land in semi-permanent ponds (Książkiewicz and Gołdyn 2008), less likely to be visited by wading birds and showed much lower levels of genetic admixture.

5.4.4.2. West Lineage

The West lineage shows two to three distinct sub-lineages across all genetic markers. In both STRUCTURE models the UK appears as a distinct sub-lineage from the other countries, with some admixture of genotypes from this sub-lineage present in the other sub-lineage(s). The ITS2 marker was identical for all UK individuals, and distinct from all other European individuals through an insertion of

two base pairs at two locations. However, for the COI marker, all UK individuals except one were genetically identical to all individuals from a single population in Sweden (S2), compromising a single haplotype. For all genetic markers, individuals in population S2 grouped with the West lineage (UK/ German), unlike all other samples in Sweden, which grouped with the East lineage (Poland and Sweden), even including populations in relatively close geographic proximity (the closest population with a different lineage was 14.8km distant). If K=2 is the true number of lineages present within the East lineage then this population is likely a result of long-distance dispersal via birds as discussed in section 5.4.4.1, as this site was part of a large marsh system directly on the coast.

The origin of the two/three distinct sub-lineages of *S. nitida* in the West lineage may be connected to the glacial history of Europe and any post-glacial expansion and dispersal of *S. nitida*. As discussed in Chapter 4, in Central and Northern Europe, the historical biogeography of species is largely influenced by recurring periods of glaciation during ice ages (Hewitt, 2000; Martinez *et al.*, 2004; Provan and Bennett, 2008; Normand *et al.*, 2011). During the glacial maximum of the last ice age (23-18ka before present), most of Scandinavia and the UK was covered by an ice sheet and much of northern Europe experienced permafrost (Svenning *et al.,* 2008; Provan and Bennett, 2008). Molecular data has confirmed that the southern peninsulas of Europe (Iberia, Italy, the Balkans, Turkey and Greece) acted as a series of refugia for displaced species from northern Europe during this period (Hewitt, 1999, Gómez and Lunt, 2007; Kühne *et al.,* 2017). In Europe and elsewhere, some species spread from just one refugium, others from several (Hewitt, 1999; Elderkin *et al.*, 2007; Kühne *et al.,* 2017). Cryptic northern refugia have been hypothesised to have existed in the UK (Provan *et al.* 2005; Provan and Bennett, 2008) and in Scandinavia (Fuentes-Hurtado *et al.*, 2016). The UK and Swedish populations of the Eastern lineage of *S. nitida* may have persisted through the ice age in 'cryptic' northern refugia, whilst the German populations expanded from southern refugia (Stewart and Lister, 2001; Provan and Bennett, 2008). In the US, a similar pattern of dispersal from at least two glacial refugia was found in populations of the freshwater mussel, *Amblema plicata,* based on allozyme and mtDNA data (Elderkin *et al.*, 2007). Dépraz *et al.* (2008) found genetic evidence with COI and 16S markers of two divergent

lineages in populations the hairy land snail, *Trochulus villosus* that correlated with origins in two glacial refugia, though radiation from additional refugia may have been possible when microsatellite data was added to the study (Dépraz *et al.*, 2008). However, without calibrated molecular clock analysis of the *S. nitida* lineages, it is not possible to determine whether the two lineages observed split before the last glacial maximum, or if they have resulted from a more recent divergence following northward expansion after the last glacial maximum. Additional sampling and genetic analysis of the West lineage of *S. nitida* from countries around those sampled for the present study (e.g. France as the closest population to the UK, and the Netherlands due to the extensive wetlands there) would allow greater resolution to this hypothesis, sampling further south and east towards the Southern glacial refugia, such as the Iberian region.

5.4.5. Additional inferences from genetic data of European populations of *Segmentina nitida*

Before this study, genetic data available for *S. nitida* were very limited. *Segmentina nitida* had been included two phylogenetic studies (Jørgensen *et al.*, 2004; Albrecht *et al.,* 2007), and one population genetics study (Mensch, 2009) from which sequences for two COI, one 16S, one 18S and one small subunit RNA gene were deposited in GenBank (as of 13.09.18 (NCBI: https://www.ncbi.nlm.nih.gov/genbank)). Ploidy of the species was previously unknown, which may limit the genetic techniques available for use with *S. nitida*. For example, single nucleotide polymorphisms (SNPs) are a robust method for analysing population genetic structure of species but are challenging to use in polyploid species (Bertioli *et al.*, 2014; Clevenger *et al.*, 2015). Through the presence of a maximum of only two alleles per locus with every marker for *S. nitida*, it is most likely that individuals from both lineages are diploid.

5.4.6. Potential future genetic work for analysis of genetic structure and implications for conservation of European populations of *Segmentina nitida*.

The genetic data for *Segmentina nitida* presented here have significant implications for its conservation. If the East lineage is indeed a separate species from the West lineage, its geographical range and number of remaining populations will be greatly reduced from current assumptions. Of the 367 European individuals genetically analysed, only 26.4% (N=97) clustered with the West lineage. In the Biodiversity Action Plan for *S. nitida* the current distribution of the species is stated as "widespread but declining" and the UK populations are "unlikely to be significant in global terms" (JNCC 2010b). With the populations in the UK having the potential to represent a distinct species or a regionally distinct genotype, they may in fact be considered significant in conservation terms. The UK populations (which only belong to the West lineage) may require more attention and more extensive management and any translocation or breeding can only be supported by known West lineage populations (e.g. some German populations or a single population in Sweden). If these populations are already small and in decline, this could pose significant challenges for the conservation of the West lineage, especially with the very low levels of genetic diversity of the lineage within the UK, Sweden, and Germany.

A wider geographic range of samples would allow more effective estimation of the distribution of each lineage across Europe, particularly in areas where the proposed East and West lineage may meet. Potential informative populations could be located in Spain (coastal marshland associated with bird-mediated dispersal (Green and Figuerola, 2005; Figuerola *et al.*, 2014)), France (as the closest country to the UK), the Netherlands (containing extensively networked wetland areas) and the Czech Republic (Czech snails were found to be of similar shape to Polish individuals in Chapter 4 and are located close to the hypothesised glacial refugia in the Carpathian mountains (Horsák *et al.,* 2019). All UK snails included in this study were taken from a single marsh system in the southeast of England. Analysis of individuals from populations in Norfolk, Sussex and Yorkshire (Hill-Cottingham, 2004; Watson and

Ormerod, 2004a; Mensch, 2009) would allow comparison of populations in light of the proposed West lineage and a more in-depth analysis of local diversity and gene flow in the UK.

5.5. Conclusion

All genetic markers used in this study showed the presence of two distinct lineages of *Segmentina nitida* in Europe, one containing individuals from Poland and most of Sweden (East), and the other containing UK individuals (West). Both lineages were present in Germany, but no genetic mixture between the two was observed there. The two genetic lineages discovered here can be discerned by both shell morphology and genetic markers which further supports their status as separate evolutionary units and possibly separate species.

Understanding how these genetic patterns arose would need to be explored in greater detail with sampling in additional countries throughout Europe allowing greater resolution for STRUCTURE analysis and understanding the gene flow between different populations. Additionally, a molecular clock analysis would help identify when the two lineages diverged. The existence of two lineages of *S. nitida* may be of significant importance to potential reintroduction efforts in accordance with the UK Biodiversity Action Plan for *S. nitida*, especially in relation to choosing appropriate breeding stock. If individuals from a lineage not currently present in the UK are introduced to UK habitats, local populations could be outcompeted if this is a separate species, or - if mating does occur - incompatible offspring may be produced, potentially further imperilling UK populations. This illustrates the importance of having detailed knowledge of the genetic structure and providence of *S. nitida* populations that are the subject of management and conservation efforts. Additionally, if these are two separate species, this has great implications for the currently understood range of the West lineage of *S. nitida*, with this lineage being restricted to far fewer extant populations and a smaller geographical range than thought before, thus making it more imperilled than currently assumed. Full anatomical, genetic and shell morphology data are required to clarify the status of the two lineages, especially in relation to individuals from the type locality in Denmark.

Chapter 6- General Discussion

6.1. Introduction

Prior to this study, the main body of information about the Shining Ramshorn Snail, *Segmentina nitida,* related to its general biology and habitat requirements (Watson, 2002; Hill-Cottingham, 2004; Watson and Ormerod, 2004b, 2004a; Ormerod *et al.*, 2010; Clark, 2011). This supported the development of management advice in the UK to maintain drainage ditches in late stages of hydroseral succession that *S. nitida* inhabits there. However, additional research and research-informed actions are needed in order to more effectively protect this previously International Union for Conservation of Nature (IUCN) Red Listed species (Kerney, 1991b). *Segmentina nitida* is also included as a 'priority' species as part of the UK Biodiversity Action Plan (BAP) (JNCC, 2010b), due to the ongoing decline of the species. The specific BAP for *S. nitida* identified a set of four actions that would facilitate the conservation of *S. nitida*. These were: 1) the application of the management techniques identified by the previous studies with focus on working with land owners; 2) research into the environmental factors favouring current established populations of *S. nitida* and management techniques which allow the best population recovery post-habitat clearance; 3) increased surveying and monitoring of populations of *S. nitida* to be able to understand current range and success of management techniques; 4) research on the colonisation and translocation methodologies to facilitate an increased extent of occupied habitats (JNCC, 2010b). This thesis has attempted to contribute to methods needed to address point three (increased monitoring of *S. nitida* populations and understanding of current range) as well as address the needs of point four (translocation) through captive breeding experiments, and morphological and population genetic analyses, which are summarised in this chapter. Additionally, implications for the continued conservation of *S. nitida* are discussed.

6.2. Surveying and monitoring *Segmentina nitida* populations

Monitoring natural populations is important in assessing management decisions and their impact on a species, and assessing the conservation status of a species (Yoccoz *et al.*, 2001; Martin *et al.*, 2007). However, monitoring for species, especially those occurring in low abundance or in cryptic habitats,

can be difficult. Historically, multiple techniques have been used to survey for *S. nitida* specifically (e.g. Killeen and Willing 1997; Killeen 2000; Watson 2002; Hill-Cottingham 2004), each requiring different levels of labour. Some of these techniques include washing vegetation sampled to facilitate the snails on the surface of the vegetation to fall off into the water column where they can be easily identified and counted (Killeen, 2000; Hill-Cottingham, 2004). However, none of these sampling methods have been evaluated in comparison to a more traditional method of going through all the vegetation collected in a sample by hand. This latter method may be more thorough for larger species, but may also lead to small, cryptic species such as *S. nitida* that are attached to the surface of the vegetation being underreported in terms of presence and abundance. In Chapter 2 a sample assessment method was described and evaluated that involved washing vegetation and allowing snails to release from the vegetation. This sample assessment method was found to save time whilst being comparable in accuracy to the 'traditional' sample assessment method of sorting through vegetation by hand, including for the recording of *S. nitida* individuals, even at low densities. This effect was consistent for inexperienced and experienced surveyors, making the proposed sample assessment method suitable for use with volunteers and 'citizen scientists' (Dickinson and Bonney, 2012; Shirk *et al.*, 2012). The Non-Government Organisations (NGOs) Kent Wildlife Trust (KWT) and Natural England (NE) supported this project by helping with land access and providing historical knowledge of sites previously known to contain *S. nitida* across the Preston Marshes, and the Ash Level (Sadler, 2012). Both of these organisations rely heavily on volunteers to facilitate their work in conservation. Having a fast and effective sampling method that can be utilised by volunteers could free up resources that would allow a greater range of habitats to be sampled or for increased sampling at sites undergoing management in accordance with the BAP for *S. nitida*.

Segmentina nitida proved difficult to breed in a laboratory setting (Chapter 3). High mortality rates were observed in all experiments and were attributed predominantly to water chemistry fluctuations. Some success was achieved with outdoor macrocosms; however this was experiment was affected by low levels of dissolved oxygen and harsh winter weather, resulting in the death of most of the snails.

The difficulty of breeding *S. nitida* in captivity may pose a significant obstacle for reintroduction of the species to historical sites in the UK, one of the key actions detailed in the Biodiversity Action Plan for *S. nitida* (JNCC, 2010b).

Evidence of *S. nitida* being unable to self-fertilise even in the absence of potential mates has been found in the population genetic analyses described in Chapter 5. The UK populations of *S. nitida* investigated showed a very low, non-significant inbreeding coefficient (F_{IS}), indicating that there was no evidence of inbreeding that would be expected in a selfing hermaphroditic species. Lamy *et al.* (2012) investigated a tropical species of hermaphroditic freshwater snail, *Drepanotrema depressissimum* (Basommotophora: Planorbidae) and found that even in temporary habitats and in situations where selfing would be beneficial for survival in natural habitats, *D. depressissimum* were extremely reluctant to self (Lamy *et al.*, 2012). This species also showed very low levels of inbreeding in the wild, indicating that outcrossing is the dominant reproductive mode of *D. depressissimum* populations, at least in the wild. If this is also the case for *S. nitida* it may go some way to explain the lack of reproduction in these early breeding experiments. A way to increase the chance of mating in predominantly outcrossing species is to isolate sexually mature individuals for a few days, then introducing the snails together. In *D. depressissimum* and *Physa acuta* (both predominantly outcrossing species) swift mating after a few days of isolation was observed, with an increase in overall mating probability (Escobar *et al.*, 2009; Lamy *et al.*, 2012). Future breeding experiments for *S. nitida* could attempt to incorporate this to explore outcrossing preference and mating probability. Monitoring the mating behaviour of sexually mature individuals, which have been isolated for a long enough period of time, is a simple and informative way to characterise the dominant mating system, at least in freshwater snails (Doums *et al.*, 1996; Escobar *et al.*, 2011).

6.3. Translocation and reintroduction of *Segmentina nitida* populations

In conservation, the intentional movement of populations, individuals, or species to new locations to increase or maintain biodiversity, or increase the number or size of populations of a threatened

species, is referred to as translocation (Weeks *et al.*, 2011). There are three different types of translocations: 1) augmentation- movement of individuals into a population of conspecifics; 2) introduction- movement of an organism outside its historical range; 3) re-introduction- movement of an organism into a part of its native/historical range from which it has disappeared (IUCN 1987). The type of translocation undertaken for a species ultimately depends on the requirements and aims for conservation (Weeks *et al.*, 2011). Whilst initially one of the aims of translocation of *S. nitida* within the UK would be to expand its range again following the recent decline (Hill-Cottingham, 2004; JNCC, 2010b; Clark, 2011), the genetic analyses shown in Chapter 5 also indicate that there is already very low genetic diversity within the sampled UK populations, at least in the relatively small geographic area sampled. This infers that the UK populations of *S. nitida* may need augmentation translocation to facilitate genetic restoration. Reintroduction for genetic augmentation of a population aims to alleviate detrimental genetic effects that arise in small fragmented populations, such as genetic load, inbreeding depression, and reduced genetic variation (Hedrick, 1995; Westermeier *et al.*, 1998; Vila *et al.*, 2003; Pickup and Young, 2008; Hedrick and Fredrickson, 2010). Two of the potential criteria for successful translocations with the goal of species conservation are improving population resilience (genetic variation, resistance to perturbation) and persistence (Pavlik, 1996; Vallee *et al.*, 2004). To be able to improve the genetic variation of a population, one first needs to understand the breadth of genetic variation within that population, as well as other populations of that species that may represent a source of individuals for translocation. Chapter 5 focussed on the population genetics of European populations of *S. nitida* to obtain a baseline genetic diversity of UK populations, as well as identify potential continental European populations that could be used in a translocation programme. In general terms, augmentation of a population with individuals from a different lineage or sub-population could lead to outbreeding depression, a reduction in reproductive fitness because of the attempted crossing of distinct lineages, populations, sub-species or even species (Weeks *et al.*, 2011). Two separate lineages of *S. nitida* were identified through Maximum Likelihood trees for COI and ITS2 and the structure analysis of microsatellites in Chapter 5. There is currently no evidence of

interbreeding between the two lineages in the German sites where they co-exist. Whilst this means there is probably no risk of outbreeding depression when introducing European snails to the UK, it also suggests that they are unlikely to be unsuitable for reintroduction to the UK.

This genetic structure in *S. nitida* in Europe is also represented phenotypically, as explored in Chapter 4. Because shell shape differences seem to correlate with genetic differences in European *S. nitida* it appears that phenotypic variation is not purely caused by phenotypic plasticity (Stearns, 1989; DeWitt, 1998; Kistner and Dybdahl, 2013). Phenotypic plasticity refers to the expression of alternative phenotypes by the same genotype, usually in response to environmental conditions (Stearns 1989). The shape variation observed in *S. nitida* across Europe corresponds with the two genetic lineages found in Chapter 5, and it was possible to assign snails to their genetic lineage based on shell shape approximately 85% of the time, a distinction almost as clear as that found between the two recognised species *S. nitida* and *Hippeutis complanatus* (~96% of snails correctly assigned to each species). In Chapter 4 the shape of *S. nitida* shells from the Czech Republic were analysed, but no samples with preserved soft tissue could be obtained, so these populations could not be included in the genetic analysis in Chapter 5. Morphologically, these samples were grouped with individuals from Poland. This may indicate that the Czech samples are genetically part of the East lineage represented by Sweden and Poland. Sampling for fresh samples in the Czech Republic would allow exploration of this hypothesis.

Further sampling for *S. nitida* in the UK would allow exploration of the evolutionary pressures on *S. nitida* at different locations, and could reveal if the low genetic diversity in the Kent marsh populations is also observed in other locations such as Pevensey Levels, Norfolk, and Yorkshire (Mensch, 2009). Of particular interest is the population of *S. nitida* discovered in a freshwater lake in Hornsea Mere Yorkshire (Mensch 2009). Not only is this population interesting because it occupies a lake instead of the more typical drainage ditch habitat of the species in the UK, but in previous AFLP genetic analysis of *S. nitida*, this population was found to be genetically similar to individuals from Poland (Mensch

2009). Using the microsatellite markers developed for this study on snails from that location would allow corroboration of this result.

6.4. Implications for the future conservation and research of *Segmentina nitida*

Of key relevance for future research into the conservation of *S. nitida* is the evidence of two distinct genetic lineages of the species found in the structure analysis of the European populations. Whilst these lineages are represented both genetically and morphologically, additional data are needed for the full description of a new species. Dissections of individuals from both lineages would need to be undertaken to assess and compare reproductive anatomy and radula structure differences between them. This analysis would need to include individuals from populations in Denmark, detailed as the source locality of the type specimens (Müller 1774; Nekhaev *et al.,* 2015) to assign the name *Segmentina nitida* to the appropriate lineage, and describe and name the other lineage as a new species. Compelling evidence of separate species based on genetic and anatomical data would affect the sourcing of appropriate stock of the species for potential reintroduction and also inform the conservation status of *S. nitida* with regards to the IUCN Red List and BAP. Whilst *S. nitida* is not currently listed on the IUCN Red List following the 1994 guideline changes, it was previously classified as Endangered (Kerney, 1991b). In this classification, the distribution of *S. nitida* was listed as throughout lowland Europe and Scandinavia, and other studies have also described this range (Wells and Chatfield, 1992; Hill-Cottingham, 2004). The BAP for *S. nitida* also states that the populations are unlikely to be significant in relation to global distribution of the species (JNCC, 2010b). If the East and West lineages described in the population genetics analyses (Chapter 5) are in fact separate species, one endangered species may become two, with the range of the East lineage of *S. nitida* that is present in the UK considerably.

6.5. Conclusion

This thesis addresses some of the issues identified in the Biodiversity Action Plan for *Segmentina nitida*, but also raises new issues that could influence future decisions on the conservation of declining UK populations. New, or quicker sample assessment methods, such as that devised Chapter 2, would allow rapid surveying of ditches and speed up assessment of habitats and populations of rare planorbids, such as *S. nitida*. Rapid sampling of sites with management strategies in place for the conservation of *S. nitida* would allow monitoring if the effectiveness of said management and allow adaptation of methods if required. Captive breeding of *S. nitida* still remains problematic, and further work on laboratory-based breeding experiments would need to be conducted to establish stable and viable programmes.

Significant shell morphology differences exist between European populations of *S. nitida* and evidence of strong population genetic structure shows two distinct lineages of *S. nitida* Europe that is also expressed in morphological differences. Augmentation of current UK populations or reintroduction to historical locations of *S. nitida* in the UK with snails from other countries in Europe could result in either outbreeding depression with the mating of these two lineages, production of infertile offspring if the two lineages are reproductively isolated, or in a worst-case scenario, result in the inadvertent introduction a non-native species. The work presented here highlights the importance of detailed research into all aspects of a species of interest before major conservation strategies are devised.

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Appendices

Appendix A1. Combinations of substrates for each of the treatments of the breeding experiment substrate influence on survival and fecundity of *Segmentina nitida* in microcosms

Treatment	***Enteromorpha* sp.**	**Paper**	***Lemna trisulca***	**Sponge**
A	Yes	Yes	No	Yes
B	No	Yes	No	Yes
C	Yes	Yes	Yes	Yes
D	No	Yes	Yes	Yes
E	Yes	No	No	Yes
F	No	No	No	Yes
G	Yes	No	Yes	Yes
H	No	No	Yes	Yes
I	Yes	Yes	No	No
J	No	Yes	No	No
K	Yes	Yes	Yes	No
L	No	Yes	Yes	No
M	Yes	No	No	No
N	No	No	No	No
O	Yes	No	Yes	No
P	No	No	Yes	No

Appendix A2. Randomised locations of microcosms in substrate breeding experiment

E3	D3
O3	J3
F1	H1
P1	H3
P2	K1
B3	M3

L2	G2
M1	J2
L3	C1
D1	E1
O1	G1
A3	P3

O2	C2
B1	I2
N2	A2
A1	E2
D2	K3
K2	F3

L1	N1
F2	G3
C3	M2
J1	B2
I1	I3
N3	H2

Appendix B1. *Segmentina nitida* samples used for geometric morphometrics analysis, with X and Y coordinates used for mapping, population number, and country of origin**.**

Sample ID	Country	Population	X Coordinate	Y Coordinate
CZ1.1	Czech Rep	CZ1	49.7312	18.1145
CZ1.2	Czech Rep	CZ1	49.7312	18.1145
CZ1.3	Czech Rep	CZ1	49.7312	18.1145
CZ1.4	Czech Rep	CZ1	49.7312	18.1145
CZ1.5	Czech Rep	CZ1	49.7312	18.1145
CZ1.6	Czech Rep	CZ1	49.7312	18.1145
CZ1.7	Czech Rep	CZ1	49.7312	18.1145
CZ1.8	Czech Rep	CZ1	49.7312	18.1145
CZ1.9	Czech Rep	CZ1	49.7312	18.1145
CZ1.10	Czech Rep	CZ1	49.7312	18.1145
CZ1.11	Czech Rep	CZ1	49.7312	18.1145
CZ1.12	Czech Rep	CZ1	49.7312	18.1145
CZ1.13	Czech Rep	CZ1	49.7312	18.1145
CZ1.14	Czech Rep	CZ1	49.7312	18.1145
CZ2.1	Czech Rep	CZ2	49.8604	18.1938
CZ2.2	Czech Rep	CZ2	49.8604	18.1938
CZ2.4	Czech Rep	CZ2	49.8604	18.1938
CZ2.6	Czech Rep	CZ2	49.8604	18.1938
CZ2.7	Czech Rep	CZ2	49.8604	18.1938
CZ2.8	Czech Rep	CZ2	49.8604	18.1938
CZ2.9	Czech Rep	CZ2	49.8604	18.1938
CZ2.10	Czech Rep	CZ2	49.8604	18.1938
CZ2.11	Czech Rep	CZ2	49.8604	18.1938
CZ2.12	Czech Rep	CZ2	49.8604	18.1938
CZ2.13	Czech Rep	CZ2	49.8604	18.1938
CZ2.15	Czech Rep	CZ2	49.8604	18.1938
CZ2.16	Czech Rep	CZ2	49.8604	18.1938
CZ2.17	Czech Rep	CZ2	49.8604	18.1938

Sample ID	Country	Population	X Coordinate	Y Coordinate
CZ2.18	Czech Rep	CZ2	49.8604	18.1938
CZ2.19	Czech Rep	CZ2	49.8604	18.1938
CZ2.20	Czech Rep	CZ2	49.8604	18.1938
CZ3.1	Czech Rep	CZ3	48.7981	14.7314
CZ3.2	Czech Rep	CZ3	48.7981	14.7314
CZ3.3	Czech Rep	CZ3	48.7981	14.7314
CZ3.4	Czech Rep	CZ3	48.7981	14.7314
CZ3.5	Czech Rep	CZ3	48.7981	14.7314
CZ3.6	Czech Rep	CZ3	48.7981	14.7314
CZ3.7	Czech Rep	CZ3	48.7981	14.7314
CZ3.8	Czech Rep	CZ3	48.7981	14.7314
CZ3.9	Czech Rep	CZ3	48.7981	14.7314
CZ3.10	Czech Rep	CZ3	48.7981	14.7314
CZ3.11	Czech Rep	CZ3	48.7981	14.7314
CZ4.1	Czech Rep	CZ4	48.5903	16.9403
CZ4.2	Czech Rep	CZ4	48.5903	16.9403
CZ4.3	Czech Rep	CZ4	48.5903	16.9403
CZ4.4	Czech Rep	CZ4	48.5903	16.9403
CZ4.5	Czech Rep	CZ4	48.5903	16.9403
CZ4.6	Czech Rep	CZ4	48.5903	16.9403
CZ4.8	Czech Rep	CZ4	48.5903	16.9403
CZ4.9	Czech Rep	CZ4	48.5903	16.9403
CZ4.10	Czech Rep	CZ4	48.5903	16.9403
CZ4.11	Czech Rep	CZ4	48.5903	16.9403
CZ4.12	Czech Rep	CZ4	48.5903	16.9403
CZ5.1	Czech Rep	CZ5	48.8140	16.7973
CZ5.2	Czech Rep	CZ5	48.8140	16.7973
CZ5.3	Czech Rep	CZ5	48.8140	16.7973
CZ5.4	Czech Rep	CZ5	48.8140	16.7973
CZ5.5	Czech Rep	CZ5	48.8140	16.7973
CZ5.6	Czech Rep	CZ5	48.8140	16.7973
CZ5.7	Czech Rep	CZ5	48.8140	16.7973
CZ5.8	Czech Rep	CZ5	48.8140	16.7973
CZ6.1	Czech Rep	CZ6	50.2647	14.8647
CZ6.2	Czech Rep	CZ6	50.2647	14.8647
CZ6.3	Czech Rep	CZ6	50.2647	14.8647
CZ6.4	Czech Rep	CZ6	50.2647	14.8647
CZ6.5	Czech Rep	CZ6	50.2647	14.8647
CZ6.6	Czech Rep	CZ6	50.2647	14.8647
CZ6.7	Czech Rep	CZ6	50.2647	14.8647
CZ6.8	Czech Rep	CZ6	50.2647	14.8647
CZ7.1	Czech Rep	CZ7	49.3480	16.0980
CZ7.2	Czech Rep	CZ7	49.3480	16.0980
CZ7.3	Czech Rep	CZ7	49.3480	16.0980

Sample ID	Country	Population	X Coordinate	Y Coordinate
CZ7.4	Czech Rep	CZ7	49.3480	16.0980
CZ7.5	Czech Rep	CZ7	49.3480	16.0980
CZ7.6	Czech Rep	CZ7	49.3480	16.0980
CZ7.7	Czech Rep	CZ7	49.3480	16.0980
G1.1	Germany	G1	53.9420	13.0661
G1.2	Germany	G1	53.9420	13.0661
G1.3	Germany	G1	53.9420	13.0661
G1.4	Germany	G1	53.9420	13.0661
G1.5	Germany	G1	53.9420	13.0661
G1.7	Germany	G1	53.9420	13.0661
G1.8	Germany	G1	53.9420	13.0661
G1.9	Germany	G1	53.9420	13.0661
G1.10	Germany	G1	53.9420	13.0661
G1.11	Germany	G1	53.9420	13.0661
G1.12	Germany	G1	53.9420	13.0661
G1.13	Germany	G1	53.9420	13.0661
G1.14	Germany	G1	53.9420	13.0661
G1.15	Germany	G1	53.9420	13.0661
G2.1	Germany	G2	53.9942	12.8204
G2.2	Germany	G2	53.9942	12.8204
G2.3	Germany	G2	53.9942	12.8204
G2.4	Germany	G2	53.9942	12.8204
G2.5	Germany	G2	53.9942	12.8204
G2.6	Germany	G2	53.9942	12.8204
G2.7	Germany	G2	53.9942	12.8204
G2.8	Germany	G2	53.9942	12.8204
G2.9	Germany	G2	53.9942	12.8204
G2.10	Germany	G2	53.9942	12.8204
G2.11	Germany	G2	53.9942	12.8204
G2.12	Germany	G2	53.9942	12.8204
G2.13	Germany	G2	53.9942	12.8204
G2.14	Germany	G2	53.9942	12.8204
G2.15	Germany	G2	53.9942	12.8204
G2.16	Germany	G2	53.9942	12.8204
G2.17	Germany	G2	53.9942	12.8204
G2.18	Germany	G2	53.9942	12.8204
G2.19	Germany	G2	53.9942	12.8204
G2.20	Germany	G2	53.9942	12.8204
G2.21	Germany	G2	53.9942	12.8204
G2.22	Germany	G2	53.9942	12.8204
G2.23	Germany	G2	53.9942	12.8204
G2.24	Germany	G2	53.9942	12.8204
G2.25	Germany	G2	53.9942	12.8204
G2.26	Germany	G2	53.9942	12.8204

Sample ID	Country	Population	X Coordinate	Y Coordinate
G2.27	Germany	G2	53.9942	12.8204
G2.28	Germany	G2	53.9942	12.8204
G2.29	Germany	G2	53.9942	12.8204
G2.30	Germany	G2	53.9942	12.8204
G2.31	Germany	G2	53.9942	12.8204
G2.32	Germany	G2	53.9942	12.8204
G2.33	Germany	G2	53.9942	12.8204
G2.34	Germany	G2	53.9942	12.8204
G3.1	Germany	G3	53.9929	12.8224
G3.2	Germany	G3	53.9929	12.8224
G3.3	Germany	G3	53.9929	12.8224
G3.4	Germany	G3	53.9929	12.8224
G3.5	Germany	G3	53.9929	12.8224
G3.6	Germany	G3	53.9929	12.8224
G3.7	Germany	G3	53.9929	12.8224
G3.8	Germany	G3	53.9929	12.8224
G3.9	Germany	G3	53.9929	12.8224
G3.10	Germany	G3	53.9929	12.8224
G3.11	Germany	G3	53.9929	12.8224
G3.12	Germany	G3	53.9929	12.8224
G3.14	Germany	G3	53.9929	12.8224
G3.15	Germany	G3	53.9929	12.8224
G4.1	Germany	G4	53.8526	12.8977
G4.2	Germany	G4	53.8526	12.8977
G4.3	Germany	G4	53.8526	12.8977
G4.4	Germany	G4	53.8526	12.8977
G4.5	Germany	G4	53.8526	12.8977
G4.6	Germany	G4	53.8526	12.8977
G4.7	Germany	G4	53.8526	12.8977
G4.8	Germany	G4	53.8526	12.8977
G4.9	Germany	G4	53.8526	12.8977
G4.10	Germany	G4	53.8526	12.8977
G4.11	Germany	G4	53.8526	12.8977
G4.12	Germany	G4	53.8526	12.8977
G5.1	Germany	G5	53.8325	12.8325
G5.2	Germany	G5	53.8325	12.8325
G5.3	Germany	G5	53.8325	12.8325
GB1.1	UK	UK1	51.3130	1.3168
GB1.2	UK	UK1	51.3130	1.3168
GB1.3	UK	UK1	51.3130	1.3168
GB1.4	UK	UK1	51.3130	1.3168
GB1.5	UK	UK1	51.3130	1.3168
GB1.6	UK	UK1	51.3130	1.3168
GB1.7	UK	UK1	51.3130	1.3168

Sample ID	Country	Population	X Coordinate	Y Coordinate
GB1.8	UK	UK1	51.3130	1.3168
GB1.10	UK	UK1	51.3130	1.3168
GB1.11	UK	UK1	51.3130	1.3168
GB1.12	UK	UK1	51.3130	1.3168
GB1.14	UK	UK1	51.3130	1.3168
GB1.15	UK	UK1	51.3130	1.3168
GB2.1	UK	UK2	51.3148	1.3164
GB2.2	UK	UK2	51.3148	1.3164
GB2.3	UK	UK2	51.3148	1.3164
GB2.4	UK	UK2	51.3148	1.3164
GB2.5	UK	UK2	51.3148	1.3164
GB2.6	UK	UK2	51.3148	1.3164
GB2.7	UK	UK2	51.3148	1.3164
GB2.8	UK	UK2	51.3148	1.3164
GB2.9	UK	UK2	51.3148	1.3164
GB3.1	UK	UK3	51.3025	1.2132
GB3.2	UK	UK3	51.3025	1.2132
GB3.3	UK	UK3	51.3025	1.2132
GB3.4	UK	UK3	51.3025	1.2132
GB3.5	UK	UK3	51.3025	1.2132
GB3.6	UK	UK3	51.3025	1.2132
GB4.1	UK	UK4	53.9903	-1.0814
GB4.2	UK	UK4	53.9903	-1.0814
GB4.3	UK	UK4	53.9903	-1.0814
GB4.4	UK	UK4	53.9903	-1.0814
GB4.5	UK	UK4	53.9903	-1.0814
GB4.6	UK	UK4	53.9903	-1.0814
GB4.7	UK	UK4	53.9903	-1.0814
GB4.8	UK	UK4	53.9903	-1.0814
GB4.9	UK	UK4	53.9903	-1.0814
GB4.10	UK	UK4	53.9903	-1.0814
GB5.1	UK	UK5	51.4693	-0.2379
GB5.3	UK	UK5	51.4693	-0.2379
GB5.5	UK	UK5	51.4693	-0.2379
GB5.7	UK	UK5	51.4693	-0.2379
GB5.8	UK	UK5	51.4693	-0.2379
GB5.9	UK	UK5	51.4693	-0.2379
GB6.1	UK	UK6	53.4377	0.1134
GB6.2	UK	UK6	53.4377	0.1134
GB6.3	UK	UK6	53.4377	0.1134
GB6.4	UK	UK6	53.4377	0.1134
GB6.5	UK	UK6	53.4377	0.1134
GB6.6	UK	UK6	53.4377	0.1134
GB6.7	UK	UK6	53.4377	0.1134

Sample ID	Country	Population	X Coordinate	Y Coordinate
GB6.8	UK	UK6	53.4377	0.1134
GB6.9	UK	UK6	53.4377	0.1134
GB6.10	UK	UK6	53.4377	0.1134
GB7.2	UK	UK7	51.3533	1.1943
GB7.3	UK	UK7	51.3533	1.1943
GB7.4	UK	UK7	51.3533	1.1943
GB7.5	UK	UK7	51.3533	1.1943
GB7.6	UK	UK7	51.3533	1.1943
GB7.8	UK	UK7	51.3533	1.1943
GB7.9	UK	UK7	51.3533	1.1943
GB7.10	UK	UK7	51.3533	1.1943
GB8.1	UK	UK8	52.6120	1.4397
GB8.2	UK	UK8	52.6120	1.4397
GB8.3	UK	UK8	52.6120	1.4397
GB8.4	UK	UK8	52.6120	1.4397
GB8.5	UK	UK8	52.6120	1.4397
GB8.6	UK	UK8	52.6120	1.4397
GB8.7	UK	UK8	52.6120	1.4397
GB8.8	UK	UK8	52.6120	1.4397
GB8.9	UK	UK8	52.6120	1.4397
GB8.10	UK	UK8	52.6120	1.4397
GB9.1	UK	UK9	52.3915	0.2554
GB9.2	UK	UK9	52.3915	0.2554
GB9.3	UK	UK9	52.3915	0.2554
GB9.4	UK	UK9	52.3915	0.2554
GB9.6	UK	UK9	52.3915	0.2554
GB9.7	UK	UK9	52.3915	0.2554
GB9.8	UK	UK9	52.3915	0.2554
GB9.9	UK	UK9	52.3915	0.2554
GB9.10	UK	UK9	52.3915	0.2554
GB10.1	UK	UK10	51.4359	-2.6612
GB10.2	UK	UK10	51.4359	-2.6612
GB10.3	UK	UK10	51.4359	-2.6612
GB10.4	UK	UK10	51.4359	-2.6612
GB10.6	UK	UK10	51.4359	-2.6612
GB10.7	UK	UK10	51.4359	-2.6612
GB10.8	UK	UK10	51.4359	-2.6612
GB10.9	UK	UK10	51.4359	-2.6612
GB10.10	UK	UK10	51.4359	-2.6612
GB11.1	UK	UK11	51.7418	-0.0153
GB11.2	UK	UK11	51.7418	-0.0153
GB11.3	UK	UK11	51.7418	-0.0153
GB11.4	UK	UK11	51.7418	-0.0153
GB11.5	UK	UK11	51.7418	-0.0153

Sample ID	Country	Population	X Coordinate	Y Coordinate
GB11.6	UK	UK11	51.7418	-0.0153
GB11.7	UK	UK11	51.7418	-0.0153
GB11.8	UK	UK11	51.7418	-0.0153
GB11.9	UK	UK11	51.7418	-0.0153
GB11.10	UK	UK11	51.7418	-0.0153
P2.1	Poland	P2	52.4729	16.6303
P2.2	Poland	P2	52.4729	16.6303
P2.3	Poland	P2	52.4729	16.6303
P2.4	Poland	P2	52.4729	16.6303
P2.5	Poland	P2	52.4729	16.6303
P2.7	Poland	P2	52.4729	16.6303
P2.8	Poland	P2	52.4729	16.6303
P2.9	Poland	P2	52.4729	16.6303
P2.10	Poland	P2	52.4729	16.6303
P2.11	Poland	P2	52.4729	16.6303
P2.12	Poland	P2	52.4729	16.6303
P2.13	Poland	P2	52.4729	16.6303
P2.14	Poland	P2	52.4729	16.6303
P2.15	Poland	P2	52.4729	16.6303
P2.16	Poland	P2	52.4729	16.6303
P2.17	Poland	P2	52.4729	16.6303
P2.18	Poland	P2	52.4729	16.6303
P3.1	Poland	P3	52.4750	16.5427
P3.2	Poland	P3	52.4750	16.5427
P3.3	Poland	P3	52.4750	16.5427
P3.4	Poland	P3	52.4750	16.5427
P3.5	Poland	P3	52.4750	16.5427
P3.6	Poland	P3	52.4750	16.5427
P3.7	Poland	P3	52.4750	16.5427
P3.8	Poland	P3	52.4750	16.5427
P3.9	Poland	P3	52.4750	16.5427
P3.10	Poland	P3	52.4750	16.5427
P3.11	Poland	P3	52.4750	16.5427
P5.1	Poland	P5	52.4714	16.5302
P5.2	Poland	P5	52.4714	16.5302
P5.3	Poland	P5	52.4714	16.5302
P5.4	Poland	P5	52.4714	16.5302
P5.5	Poland	P5	52.4714	16.5302
P5.6	Poland	P5	52.4714	16.5302
P5.7	Poland	P5	52.4714	16.5302
P5.8	Poland	P5	52.4714	16.5302
P5.9	Poland	P5	52.4714	16.5302
P5.10	Poland	P5	52.4714	16.5302
P5.11	Poland	P5	52.4714	16.5302

Sample ID	Country	Population	X Coordinate	Y Coordinate
P5.12	Poland	P5	52.4714	16.5302
P5.13	Poland	P5	52.4714	16.5302
P5.14	Poland	P5	52.4714	16.5302
P5.15	Poland	P5	52.4714	16.5302
P5.16	Poland	P5	52.4714	16.5302
P5.17	Poland	P5	52.4714	16.5302
P5.18	Poland	P5	52.4714	16.5302
P5.19	Poland	P5	52.4714	16.5302
P5.20	Poland	P5	52.4714	16.5302
P5.21	Poland	P5	52.4714	16.5302
P5.22	Poland	P5	52.4714	16.5302
P6.1	Poland	P6	52.4892	16.8967
P6.2	Poland	P6	52.4892	16.8967
P6.3	Poland	P6	52.4892	16.8967
P6.4	Poland	P6	52.4892	16.8967
P6.5	Poland	P6	52.4892	16.8967
P6.6	Poland	P6	52.4892	16.8967
P6.7	Poland	P6	52.4892	16.8967
P6.8	Poland	P6	52.4892	16.8967
P6.9	Poland	P6	52.4892	16.8967
P6.10	Poland	P6	52.4892	16.8967
P6.11	Poland	P6	52.4892	16.8967
P6.12	Poland	P6	52.4892	16.8967
P6.13	Poland	P6	52.4892	16.8967
P6.14	Poland	P6	52.4892	16.8967
P6.15	Poland	P6	52.4892	16.8967
P6.16	Poland	P6	52.4892	16.8967
P6.17	Poland	P6	52.4892	16.8967
P7.1	Poland	P7	52.4897	16.8978
P7.2	Poland	P7	52.4897	16.8978
P7.3	Poland	P7	52.4897	16.8978
P7.4	Poland	P7	52.4897	16.8978
P7.5	Poland	P7	52.4897	16.8978
P7.6	Poland	P7	52.4897	16.8978
P7.7	Poland	P7	52.4897	16.8978
P7.8	Poland	P7	52.4897	16.8978
P7.9	Poland	P7	52.4897	16.8978
P7.10	Poland	P7	52.4897	16.8978
P7.11	Poland	P7	52.4897	16.8978
P7.12	Poland	P7	52.4897	16.8978
P7.13	Poland	P7	52.4897	16.8978
P7.14	Poland	P7	52.4897	16.8978
P7.15	Poland	P7	52.4897	16.8978
P7.16	Poland	P7	52.4897	16.8978

Sample ID	Country	Population	X Coordinate	Y Coordinate
P7.17	Poland	P7	52.4897	16.8978
P7.18	Poland	P7	52.4897	16.8978
P7.19	Poland	P7	52.4897	16.8978
P8.1	Poland	P8	52.4902	16.8967
P8.2	Poland	P8	52.4902	16.8967
P8.3	Poland	P8	52.4902	16.8967
P8.4	Poland	P8	52.4902	16.8967
P8.5	Poland	P8	52.4902	16.8967
P8.6	Poland	P8	52.4902	16.8967
P8.7	Poland	P8	52.4902	16.8967
P8.8	Poland	P8	52.4902	16.8967
P8.9	Poland	P8	52.4902	16.8967
P8.10	Poland	P8	52.4902	16.8967
P8.11	Poland	P8	52.4902	16.8967
P8.12	Poland	P8	52.4902	16.8967
P8.13	Poland	P8	52.4902	16.8967
P8.14	Poland	P8	52.4902	16.8967
P8.15	Poland	P8	52.4902	16.8967
P8.16	Poland	P8	52.4902	16.8967
P8.17	Poland	P8	52.4902	16.8967
P8.18	Poland	P8	52.4902	16.8967
P8.19	Poland	P8	52.4902	16.8967
P8.20	Poland	P8	52.4902	16.8967
P8.21	Poland	P8	52.4902	16.8967
P8.22	Poland	P8	52.4902	16.8967
P8.23	Poland	P8	52.4902	16.8967
P8.24	Poland	P8	52.4902	16.8967
P8.25	Poland	P8	52.4902	16.8967
P8.26	Poland	P8	52.4902	16.8967
P8.28	Poland	P8	52.4902	16.8967
S1.1	Sweden	S1	55.6385	14.2541
S1.2	Sweden	S1	55.6385	14.2541
S1.3	Sweden	S1	55.6385	14.2541
S1.4	Sweden	S1	55.6385	14.2541
S1.5	Sweden	S1	55.6385	14.2541
S1.6	Sweden	S1	55.6385	14.2541
S1.7	Sweden	S1	55.6385	14.2541
S1.8	Sweden	S1	55.6385	14.2541
S1.9	Sweden	S1	55.6385	14.2541
S1.11	Sweden	S1	55.6385	14.2541
S1.12	Sweden	S1	55.6385	14.2541
S1.13	Sweden	S1	55.6385	14.2541
S1.14	Sweden	S1	55.6385	14.2541
S1.15	Sweden	S1	55.6385	14.2541

Sample ID	Country	Population	X Coordinate	Y Coordinate
S1.16	Sweden	S1	55.6385	14.2541
S1.17	Sweden	S1	55.6385	14.2541
S1.18	Sweden	S1	55.6385	14.2541
S1.19	Sweden	S1	55.6385	14.2541
S1.20	Sweden	S1	55.6385	14.2541
S1.21	Sweden	S1	55.6385	14.2541
S1.22	Sweden	S1	55.6385	14.2541
S1.23	Sweden	S1	55.6385	14.2541
S1.24	Sweden	S1	55.6385	14.2541
S1.25	Sweden	S1	55.6385	14.2541
S1.26	Sweden	S1	55.6385	14.2541
S1.27	Sweden	S1	55.6385	14.2541
S1.28	Sweden	S1	55.6385	14.2541
S1.30	Sweden	S1	55.6385	14.2541
S1.31	Sweden	S1	55.6385	14.2541
S1.32	Sweden	S1	55.6385	14.2541
S1.33	Sweden	S1	55.6385	14.2541
S1.34	Sweden	S1	55.6385	14.2541
S1.35	Sweden	S1	55.6385	14.2541
S1.36	Sweden	S1	55.6385	14.2541
S1.37	Sweden	S1	55.6385	14.2541
S2.1	Sweden	S2	55.3866	13.4945
S2.2	Sweden	S2	55.3866	13.4945
S2.3	Sweden	S2	55.3866	13.4945
S2.4	Sweden	S2	55.3866	13.4945
S2.5	Sweden	S2	55.3866	13.4945
S2.6	Sweden	S2	55.3866	13.4945
S2.7	Sweden	S2	55.3866	13.4945
S2.8	Sweden	S2	55.3866	13.4945
S2.9	Sweden	S2	55.3866	13.4945
S2.10	Sweden	S2	55.3866	13.4945
S2.11	Sweden	S2	55.3866	13.4945
S2.12	Sweden	S2	55.3866	13.4945
S2.13	Sweden	S2	55.3866	13.4945
S2.14	Sweden	S2	55.3866	13.4945
S2.15	Sweden	S2	55.3866	13.4945
S2.16	Sweden	S2	55.3866	13.4945
S2.17	Sweden	S2	55.3866	13.4945
S2.18	Sweden	S2	55.3866	13.4945
S2.19	Sweden	S2	55.3866	13.4945
S2.20	Sweden	S2	55.3866	13.4945
S2.21	Sweden	S2	55.3866	13.4945
S2.22	Sweden	S2	55.3866	13.4945
S2.23	Sweden	S2	55.3866	13.4945

Sample ID	Country	Population	X Coordinate	Y Coordinate
S3.1	Sweden	S3	55.5142	13.4359
S3.2	Sweden	S3	55.5142	13.4359
S3.3	Sweden	S3	55.5142	13.4359
S3.4	Sweden	S3	55.5142	13.4359
S3.5	Sweden	S3	55.5142	13.4359
S3.7	Sweden	S3	55.5142	13.4359
S3.8	Sweden	S3	55.5142	13.4359
S3.11	Sweden	S3	55.5142	13.4359
S3.12	Sweden	S3	55.5142	13.4359
S3.13	Sweden	S3	55.5142	13.4359
S3.14	Sweden	S3	55.5142	13.4359
S3.15	Sweden	S3	55.5142	13.4359
S3.17	Sweden	S3	55.5142	13.4359
S3.19	Sweden	S3	55.5142	13.4359
S3.20	Sweden	S3	55.5142	13.4359
S3.21	Sweden	S3	55.5142	13.4359
S3.22	Sweden	S3	55.5142	13.4359
S3.23	Sweden	S3	55.5142	13.4359
S3.24	Sweden	S3	55.5142	13.4359
S3.26	Sweden	S3	55.5142	13.4359
S3.27	Sweden	S3	55.5142	13.4359
S3.28	Sweden	S3	55.5142	13.4359
S3.29	Sweden	S3	55.5142	13.4359
S3.30	Sweden	S3	55.5142	13.4359
S3.31	Sweden	S3	55.5142	13.4359
S3.33	Sweden	S3	55.5142	13.4359
S3.35	Sweden	S3	55.5142	13.4359
S3.36	Sweden	S3	55.5142	13.4359
S3.37	Sweden	S3	55.5142	13.4359
S3.39	Sweden	S3	55.5142	13.4359
S3.40	Sweden	S3	55.5142	13.4359
S3.41	Sweden	S3	55.5142	13.4359
S3.43	Sweden	S3	55.5142	13.4359
S3.44	Sweden	S3	55.5142	13.4359
S3.45	Sweden	S3	55.5142	13.4359
S4.1	Sweden	S4	55.5596	13.2494
S4.2	Sweden	S4	55.5596	13.2494
S4.3	Sweden	S4	55.5596	13.2494
S4.4	Sweden	S4	55.5596	13.2494
S4.5	Sweden	S4	55.5596	13.2494
S4.6	Sweden	S4	55.5596	13.2494
S4.7	Sweden	S4	55.5596	13.2494
S4.8	Sweden	S4	55.5596	13.2494
S4.9	Sweden	S4	55.5596	13.2494

Sample ID	Country	Population	X Coordinate	Y Coordinate
S4.10	Sweden	S4	55.5596	13.2494
S4.11	Sweden	S4	55.5596	13.2494
S4.12	Sweden	S4	55.5596	13.2494
S4.13	Sweden	S4	55.5596	13.2494
S4.14	Sweden	S4	55.5596	13.2494
S4.15	Sweden	S4	55.5596	13.2494
S4.16	Sweden	S4	55.5596	13.2494
S4.17	Sweden	S4	55.5596	13.2494
S4.18	Sweden	S4	55.5596	13.2494
S4.19	Sweden	S4	55.5596	13.2494
S4.20	Sweden	S4	55.5596	13.2494
S4.21	Sweden	S4	55.5596	13.2494
S4.22	Sweden	S4	55.5596	13.2494
S4.23	Sweden	S4	55.5596	13.2494
S4.24	Sweden	S4	55.5596	13.2494
S4.25	Sweden	S4	55.5596	13.2494
S4.26	Sweden	S4	55.5596	13.2494
S4.27	Sweden	S4	55.5596	13.2494
S4.28	Sweden	S4	55.5596	13.2494
S4.29	Sweden	S4	55.5596	13.2494
S4.30	Sweden	S4	55.5596	13.2494
S4.31	Sweden	S4	55.5596	13.2494
S4.32	Sweden	S4	55.5596	13.2494
S4.33	Sweden	S4	55.5596	13.2494
S4.34	Sweden	S4	55.5596	13.2494
S4.35	Sweden	S4	55.5596	13.2494
S4.36	Sweden	S4	55.5596	13.2494
S4.37	Sweden	S4	55.5596	13.2494
S4.38	Sweden	S4	55.5596	13.2494
S4.39	Sweden	S4	55.5596	13.2494
S4.40	Sweden	S4	55.5596	13.2494
S4.42	Sweden	S4	55.5596	13.2494
S4.43	Sweden	S4	55.5596	13.2494
S4.44	Sweden	S4	55.5596	13.2494
S4.45	Sweden	S4	55.5596	13.2494
S4.46	Sweden	S4	55.5596	13.2494
S5.1	Sweden	S5	55.5612	13.2173
S5.2	Sweden	S5	55.5612	13.2173
S5.3	Sweden	S5	55.5612	13.2173
S5.4	Sweden	S5	55.5612	13.2173
S5.5	Sweden	S5	55.5612	13.2173
S5.7	Sweden	S5	55.5612	13.2173
S5.8	Sweden	S5	55.5612	13.2173
S5.9	Sweden	S5	55.5612	13.2173

Sample ID	Country	Population	X Coordinate	Y Coordinate
S5.10	Sweden	S5	55.5612	13.2173
S5.11	Sweden	S5	55.5612	13.2173
S5.12	Sweden	S5	55.5612	13.2173
S5.13	Sweden	S5	55.5612	13.2173
S5.14	Sweden	S5	55.5612	13.2173
S5.15	Sweden	S5	55.5612	13.2173
S5.16	Sweden	S5	55.5612	13.2173
S5.17	Sweden	S5	55.5612	13.2173
S5.18	Sweden	S5	55.5612	13.2173
S5.19	Sweden	S5	55.5612	13.2173
S5.20	Sweden	S5	55.5612	13.2173
S5.21	Sweden	S5	55.5612	13.2173
S5.22	Sweden	S5	55.5612	13.2173
S5.24	Sweden	S5	55.5612	13.2173
S5.25	Sweden	S5	55.5612	13.2173
S5.26	Sweden	S5	55.5612	13.2173
S5.27	Sweden	S5	55.5612	13.2173
S5.28	Sweden	S5	55.5612	13.2173
S5.29	Sweden	S5	55.5612	13.2173
S5.30	Sweden	S5	55.5612	13.2173
S5.31	Sweden	S5	55.5612	13.2173
S5.32	Sweden	S5	55.5612	13.2173
S5.33	Sweden	S5	55.5612	13.2173
S6.1	Sweden	S6	55.5619	13.2160
S6.2	Sweden	S6	55.5619	13.2160
S6.3	Sweden	S6	55.5619	13.2160
S6.4	Sweden	S6	55.5619	13.2160
S6.5	Sweden	S6	55.5619	13.2160
S6.6	Sweden	S6	55.5619	13.2160
S6.7	Sweden	S6	55.5619	13.2160
S6.8	Sweden	S6	55.5619	13.2160
S6.9	Sweden	S6	55.5619	13.2160
S6.10	Sweden	S6	55.5619	13.2160
S6.11	Sweden	S6	55.5619	13.2160
S6.12	Sweden	S6	55.5619	13.2160
S6.13	Sweden	S6	55.5619	13.2160
S6.14	Sweden	S6	55.5619	13.2160
S6.15	Sweden	S6	55.5619	13.2160
S6.16	Sweden	S6	55.5619	13.2160
S6.17	Sweden	S6	55.5619	13.2160
S6.18	Sweden	S6	55.5619	13.2160
S6.19	Sweden	S6	55.5619	13.2160
S6.20	Sweden	S6	55.5619	13.2160
S6.21	Sweden	S6	55.5619	13.2160

Sample ID	Country	Population	X Coordinate	Y Coordinate
S6.22	Sweden	S6	55.5619	13.2160
S6.23	Sweden	S6	55.5619	13.2160
S6.24	Sweden	S6	55.5619	13.2160
S6.25	Sweden	S6	55.5619	13.2160
S6.26	Sweden	S6	55.5619	13.2160
S6.27	Sweden	S6	55.5619	13.2160
S7.1	Sweden	S7	55.7183	13.4382
S7.2	Sweden	S7	55.7183	13.4382
S7.3	Sweden	S7	55.7183	13.4382
S7.4	Sweden	S7	55.7183	13.4382
S7.5	Sweden	S7	55.7183	13.4382
S7.6	Sweden	S7	55.7183	13.4382
S7.7	Sweden	S7	55.7183	13.4382
S7.8	Sweden	S7	55.7183	13.4382
S7.9	Sweden	S7	55.7183	13.4382
S7.10	Sweden	S7	55.7183	13.4382
S7.11	Sweden	S7	55.7183	13.4382
S7.12	Sweden	S7	55.7183	13.4382
S7.13	Sweden	S7	55.7183	13.4382
S7.14	Sweden	S7	55.7183	13.4382
S7.15	Sweden	S7	55.7183	13.4382
S7.18	Sweden	S7	55.7183	13.4382
S7.19	Sweden	S7	55.7183	13.4382
S7.20	Sweden	S7	55.7183	13.4382
S7.21	Sweden	S7	55.7183	13.4382
S7.22	Sweden	S7	55.7183	13.4382
S7.23	Sweden	S7	55.7183	13.4382
S7.24	Sweden	S7	55.7183	13.4382
S7.25	Sweden	S7	55.7183	13.4382
S7.26	Sweden	S7	55.7183	13.4382
S7.28	Sweden	S7	55.7183	13.4382
S7.31	Sweden	S7	55.7183	13.4382
S7.33	Sweden	S7	55.7183	13.4382
S7.34	Sweden	S7	55.7183	13.4382
S7.35	Sweden	S7	55.7183	13.4382
S7.37	Sweden	S7	55.7183	13.4382
S7.38	Sweden	S7	55.7183	13.4382

Appendix C1. *Segmentina nitida* samples used for population genetics analysis, with X and Y coordinates used for mapping, population number, and country of origin.

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
G1.1	Germany	G1	53.9488	13.7720
G1.10	Germany	G1	53.9488	13.7720
G1.11	Germany	G1	53.9488	13.7720
G1.12	Germany	G1	53.9488	13.7720
G1.13	Germany	G1	53.9488	13.7720
G1.14	Germany	G1	53.9488	13.7720
G1.15	Germany	G1	53.9488	13.7720
G1.2	Germany	G1	53.9488	13.7720
G1.3	Germany	G1	53.9488	13.7720
G1.4	Germany	G1	53.9488	13.7720
G1.5	Germany	G1	53.9488	13.7720
G1.6	Germany	G1	53.9488	13.7720
G1.7	Germany	G1	53.9488	13.7720
G1.9	Germany	G1	53.9488	13.7720
G2.1	Germany	G2	53.9942	12.8204
G2.10	Germany	G2	53.9942	12.8204
G2.11	Germany	G2	53.9942	12.8204
G2.12	Germany	G2	53.9942	12.8204
G2.13	Germany	G2	53.9942	12.8204
G2.14	Germany	G2	53.9942	12.8204
G2.15	Germany	G2	53.9942	12.8204
G2.16	Germany	G2	53.9942	12.8204
G2.17	Germany	G2	53.9942	12.8204
G2.18	Germany	G2	53.9942	12.8204
G2.19	Germany	G2	53.9942	12.8204
G2.2	Germany	G2	53.9942	12.8204
G2.20	Germany	G2	53.9942	12.8204
G2.21	Germany	G2	53.9942	12.8204
G2.22	Germany	G2	53.9942	12.8204
G2.23	Germany	G2	53.9942	12.8204
G2.24	Germany	G2	53.9942	12.8204
G2.25	Germany	G2	53.9942	12.8204
G2.26	Germany	G2	53.9942	12.8204
G2.27	Germany	G2	53.9942	12.8204
G2.28	Germany	G2	53.9942	12.8204
G2.29	Germany	G2	53.9942	12.8204
G2.3	Germany	G2	53.9942	12.8204
G2.30	Germany	G2	53.9942	12.8204
G2.4	Germany	G2	53.9942	12.8204
G2.5	Germany	G2	53.9942	12.8204
G2.6	Germany	G2	53.9942	12.8204

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
G2.7	Germany	G2	53.9942	12.8204
G2.8	Germany	G2	53.9942	12.8204
G2.9	Germany	G2	53.9942	12.8204
G3.1	Germany	G3	53.9929	12.8224
G3.10	Germany	G3	53.9929	12.8224
G3.11	Germany	G3	53.9929	12.8224
G3.12	Germany	G3	53.9929	12.8224
G3.13	Germany	G3	53.9929	12.8224
G3.14	Germany	G3	53.9929	12.8224
G3.15	Germany	G3	53.9929	12.8224
G3.2	Germany	G3	53.9929	12.8224
G3.3	Germany	G3	53.9929	12.8224
G3.4	Germany	G3	53.9929	12.8224
G3.5	Germany	G3	53.9929	12.8224
G3.6	Germany	G3	53.9929	12.8224
G3.7	Germany	G3	53.9929	12.8224
G3.8	Germany	G3	53.9929	12.8224
G3.9	Germany	G3	53.9929	12.8224
G4.1	Germany	G4	53.8526	12.8977
G4.10	Germany	G4	53.8526	12.8977
G4.11	Germany	G4	53.8526	12.8977
G4.12	Germany	G4	53.8526	12.8977
G4.13	Germany	G4	53.8526	12.8977
G4.14	Germany	G4	53.8526	12.8977
G4.15	Germany	G4	53.8526	12.8977
G4.2	Germany	G4	53.8526	12.8977
G4.3	Germany	G4	53.8526	12.8977
G4.4	Germany	G4	53.8526	12.8977
G4.5	Germany	G4	53.8526	12.8977
G4.6	Germany	G4	53.8526	12.8977
G4.7	Germany	G4	53.8526	12.8977
G4.8	Germany	G4	53.8526	12.8977
G4.9	Germany	G4	53.8526	12.8977
G5.1	Germany	G5	53.8325	12.8325
G5.2	Germany	G5	53.8325	12.8325
G5.3	Germany	G5	53.8325	12.8325
GB1.1	UK	GB1	51.3130	1.3168
GB1.11	UK	GB1	51.3130	1.3168
GB1.12	UK	GB1	51.3130	1.3168
GB1.13	UK	GB1	51.3130	1.3168
GB1.14	UK	GB1	51.3130	1.3168
GB1.15	UK	GB1	51.3130	1.3168
GB1.5	UK	GB1	51.3130	1.3168

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
GB1.6	UK	GB1	51.3130	1.3168
GB1.7	UK	GB1	51.3130	1.3168
GB1.8	UK	GB1	51.3130	1.3168
GB1.9	UK	GB1	51.3130	1.3168
GB1B	UK	GB1	51.3130	1.3168
GB1C	UK	GB1	51.3130	1.3168
GB1D	UK	GB1	51.3130	1.3168
GB1E	UK	GB1	51.3130	1.3168
GB1F	UK	GB1	51.3130	1.3168
GB2.1	UK	GB2	51.3148	1.3164
GB2.2	UK	GB2	51.3148	1.3164
GB2.3	UK	GB2	51.3148	1.3164
GB2.4	UK	GB2	51.3148	1.3164
GB2.5	UK	GB2	51.3148	1.3164
GB2.6	UK	GB2	51.3148	1.3164
GB2.7	UK	GB2	51.3148	1.3164
GB2.8	UK	GB2	51.3148	1.3164
GB2.9	UK	GB2	51.3148	1.3164
GB3.1	UK	GB3	51.3025	1.2132
GB3.2	UK	GB3	51.3025	1.2132
GB3.3	UK	GB3	51.3025	1.2132
GB3.4	UK	GB3	51.3025	1.2132
GB3.5	UK	GB3	51.3025	1.2132
GB3.6	UK	GB3	51.3025	1.2132
P2.1	Poland	P2	52.4729	16.6303
P2.10	Poland	P2	52.4729	16.6303
P2.11	Poland	P2	52.4729	16.6303
P2.12	Poland	P2	52.4729	16.6303
P2.13	Poland	P2	52.4729	16.6303
P2.14	Poland	P2	52.4729	16.6303
P2.15	Poland	P2	52.4729	16.6303
P2.2	Poland	P2	52.4729	16.6303
P2.3	Poland	P2	52.4729	16.6303
P2.4	Poland	P2	52.4729	16.6303
P2.5	Poland	P2	52.4729	16.6303
P2.6	Poland	P2	52.4729	16.6303
P2.7	Poland	P2	52.4729	16.6303
P2.8	Poland	P2	52.4729	16.6303
P2.9	Poland	P2	52.4729	16.6303
P3.1	Poland	P3	52.4750	16.5427
P3.10	Poland	P3	52.4750	16.5427
P3.11	Poland	P3	52.4750	16.5427
P3.12	Poland	P3	52.4750	16.5427

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
P3.2	Poland	P3	52.4750	16.5427
P3.3	Poland	P3	52.4750	16.5427
P3.4	Poland	P3	52.4750	16.5427
P3.5	Poland	P3	52.4750	16.5427
P3.6	Poland	P3	52.4750	16.5427
P3.7	Poland	P3	52.4750	16.5427
P3.8	Poland	P3	52.4750	16.5427
P3.9	Poland	P3	52.4750	16.5427
P5.1	Poland	P5	52.4714	16.5302
P5.10	Poland	P5	52.4714	16.5302
P5.11	Poland	P5	52.4714	16.5302
P5.12	Poland	P5	52.4714	16.5302
P5.13	Poland	P5	52.4714	16.5302
P5.14	Poland	P5	52.4714	16.5302
P5.15	Poland	P5	52.4714	16.5302
P5.2	Poland	P5	52.4714	16.5302
P5.20	Poland	P5	52.4714	16.5302
P5.21	Poland	P5	52.4714	16.5302
P5.3	Poland	P5	52.4714	16.5302
P5.4	Poland	P5	52.4714	16.5302
P5.5	Poland	P5	52.4714	16.5302
P5.6	Poland	P5	52.4714	16.5302
P5.7	Poland	P5	52.4714	16.5302
P5.8	Poland	P5	52.4714	16.5302
P5.9	Poland	P5	52.4714	16.5302
P6.1	Poland	P6	52.4892	16.8967
P6.10	Poland	P6	52.4892	16.8967
P6.11	Poland	P6	52.4892	16.8967
P6.12	Poland	P6	52.4892	16.8967
P6.13	Poland	P6	52.4892	16.8967
P6.14	Poland	P6	52.4892	16.8967
P6.15	Poland	P6	52.4892	16.8967
P6.2	Poland	P6	52.4892	16.8967
P6.3	Poland	P6	52.4892	16.8967
P6.4	Poland	P6	52.4892	16.8967
P6.5	Poland	P6	52.4892	16.8967
P6.7	Poland	P6	52.4892	16.8967
P6.8	Poland	P6	52.4892	16.8967
P6.9	Poland	P6	52.4892	16.8967
P7.1	Poland	P7	52.4897	16.8978
P7.11	Poland	P7	52.4897	16.8978
P7.12	Poland	P7	52.4897	16.8978
P7.13	Poland	P7	52.4897	16.8978

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
P7.15	Poland	P7	52.4897	16.8978
P7.2	Poland	P7	52.4897	16.8978
P7.3	Poland	P7	52.4897	16.8978
P7.4	Poland	P7	52.4897	16.8978
P7.5	Poland	P7	52.4897	16.8978
P7.7	Poland	P7	52.4897	16.8978
P7.8	Poland	P7	52.4897	16.8978
P8.1	Poland	P8	52.4902	16.8967
P8.10	Poland	P8	52.4902	16.8967
P8.11	Poland	P8	52.4902	16.8967
P8.13	Poland	P8	52.4902	16.8967
P8.14	Poland	P8	52.4902	16.8967
P8.15	Poland	P8	52.4902	16.8967
P8.2	Poland	P8	52.4902	16.8967
P8.3	Poland	P8	52.4902	16.8967
P8.4	Poland	P8	52.4902	16.8967
P8.5	Poland	P8	52.4902	16.8967
P8.6	Poland	P8	52.4902	16.8967
P8.7	Poland	P8	52.4902	16.8967
P8.8	Poland	P8	52.4902	16.8967
P8.9	Poland	P8	52.4902	16.8967
S1.1	Sweden	S1	55.6385	14.2541
S1.10	Sweden	S1	55.6385	14.2541
S1.11	Sweden	S1	55.6385	14.2541
S1.12	Sweden	S1	55.6385	14.2541
S1.13	Sweden	S1	55.6385	14.2541
S1.14	Sweden	S1	55.6385	14.2541
S1.15	Sweden	S1	55.6385	14.2541
S1.16	Sweden	S1	55.6385	14.2541
S1.17	Sweden	S1	55.6385	14.2541
S1.19	Sweden	S1	55.6385	14.2541
S1.2	Sweden	S1	55.6385	14.2541
S1.20	Sweden	S1	55.6385	14.2541
S1.21	Sweden	S1	55.6385	14.2541
S1.22	Sweden	S1	55.6385	14.2541
S1.25	Sweden	S1	55.6385	14.2541
S1.26	Sweden	S1	55.6385	14.2541
S1.27	Sweden	S1	55.6385	14.2541
S1.28	Sweden	S1	55.6385	14.2541
S1.29	Sweden	S1	55.6385	14.2541
S1.3	Sweden	S1	55.6385	14.2541
S1.30	Sweden	S1	55.6385	14.2541
S1.31	Sweden	S1	55.6385	14.2541

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
S1.4	Sweden	S1	55.6385	14.2541
S1.5	Sweden	S1	55.6385	14.2541
S1.6	Sweden	S1	55.6385	14.2541
S1.7	Sweden	S1	55.6385	14.2541
S1.8	Sweden	S1	55.6385	14.2541
S1.9	Sweden	S1	55.6385	14.2541
S2.10	Sweden	S2	55.3866	13.4945
S2.11	Sweden	S2	55.3866	13.4945
S2.12	Sweden	S2	55.3866	13.4945
S2.13	Sweden	S2	55.3866	13.4945
S2.15	Sweden	S2	55.3866	13.4945
S2.16	Sweden	S2	55.3866	13.4945
S2.18	Sweden	S2	55.3866	13.4945
S2.19	Sweden	S2	55.3866	13.4945
S2.2	Sweden	S2	55.3866	13.4945
S2.21	Sweden	S2	55.3866	13.4945
S2.23	Sweden	S2	55.3866	13.4945
S2.3	Sweden	S2	55.3866	13.4945
S2.4	Sweden	S2	55.3866	13.4945
S2.5	Sweden	S2	55.3866	13.4945
S2.6	Sweden	S2	55.3866	13.4945
S2.8	Sweden	S2	55.3866	13.4945
S3.1	Sweden	S3	55.5142	13.4359
S3.10	Sweden	S3	55.5142	13.4359
S3.11	Sweden	S3	55.5142	13.4359
S3.12	Sweden	S3	55.5142	13.4359
S3.13	Sweden	S3	55.5142	13.4359
S3.14	Sweden	S3	55.5142	13.4359
S3.15	Sweden	S3	55.5142	13.4359
S3.16	Sweden	S3	55.5142	13.4359
S3.17	Sweden	S3	55.5142	13.4359
S3.18	Sweden	S3	55.5142	13.4359
S3.2	Sweden	S3	55.5142	13.4359
S3.22	Sweden	S3	55.5142	13.4359
S3.24	Sweden	S3	55.5142	13.4359
S3.26	Sweden	S3	55.5142	13.4359
S3.27	Sweden	S3	55.5142	13.4359
S3.28	Sweden	S3	55.5142	13.4359
S3.3	Sweden	S3	55.5142	13.4359
S3.30	Sweden	S3	55.5142	13.4359
S3.32	Sweden	S3	55.5142	13.4359
S3.33	Sweden	S3	55.5142	13.4359
S3.35	Sweden	S3	55.5142	13.4359

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
S3.38	Sweden	S3	55.5142	13.4359
S3.39	Sweden	S3	55.5142	13.4359
S3.4	Sweden	S3	55.5142	13.4359
S3.5	Sweden	S3	55.5142	13.4359
S3.6	Sweden	S3	55.5142	13.4359
S3.7	Sweden	S3	55.5142	13.4359
S3.8	Sweden	S3	55.5142	13.4359
S3.9	Sweden	S3	55.5142	13.4359
S4.1	Sweden	S4	55.5596	13.2494
S4.10	Sweden	S4	55.5596	13.2494
S4.11	Sweden	S4	55.5596	13.2494
S4.12	Sweden	S4	55.5596	13.2494
S4.14	Sweden	S4	55.5596	13.2494
S4.15	Sweden	S4	55.5596	13.2494
S4.16	Sweden	S4	55.5596	13.2494
S4.17	Sweden	S4	55.5596	13.2494
S4.19	Sweden	S4	55.5596	13.2494
S4.2	Sweden	S4	55.5596	13.2494
S4.21	Sweden	S4	55.5596	13.2494
S4.22	Sweden	S4	55.5596	13.2494
S4.23	Sweden	S4	55.5596	13.2494
S4.24	Sweden	S4	55.5596	13.2494
S4.25	Sweden	S4	55.5596	13.2494
S4.26	Sweden	S4	55.5596	13.2494
S4.27	Sweden	S4	55.5596	13.2494
S4.28	Sweden	S4	55.5596	13.2494
S4.29	Sweden	S4	55.5596	13.2494
S4.3	Sweden	S4	55.5596	13.2494
S4.4	Sweden	S4	55.5596	13.2494
S4.5	Sweden	S4	55.5596	13.2494
S4.6	Sweden	S4	55.5596	13.2494
S4.7	Sweden	S4	55.5596	13.2494
S4.8	Sweden	S4	55.5596	13.2494
S4.9	Sweden	S4	55.5596	13.2494
S5.1	Sweden	S5	55.5612	13.2173
S5.10	Sweden	S5	55.5612	13.2173
S5.11	Sweden	S5	55.5612	13.2173
S5.13	Sweden	S5	55.5612	13.2173
S5.14	Sweden	S5	55.5612	13.2173
S5.16	Sweden	S5	55.5612	13.2173
S5.17	Sweden	S5	55.5612	13.2173
S5.18	Sweden	S5	55.5612	13.2173
S5.19	Sweden	S5	55.5612	13.2173

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
S5.2	Sweden	S5	55.5612	13.2173
S5.20	Sweden	S5	55.5612	13.2173
S5.21	Sweden	S5	55.5612	13.2173
S5.22	Sweden	S5	55.5612	13.2173
S5.23	Sweden	S5	55.5612	13.2173
S5.24	Sweden	S5	55.5612	13.2173
S5.25	Sweden	S5	55.5612	13.2173
S5.27	Sweden	S5	55.5612	13.2173
S5.28	Sweden	S5	55.5612	13.2173
S5.30	Sweden	S5	55.5612	13.2173
S5.4	Sweden	S5	55.5612	13.2173
S5.5	Sweden	S5	55.5612	13.2173
S5.6	Sweden	S5	55.5612	13.2173
S5.7	Sweden	S5	55.5612	13.2173
S5.8	Sweden	S5	55.5612	13.2173
S5.9	Sweden	S5	55.5612	13.2173
S6.1	Sweden	S6	55.5619	13.2160
S6.10	Sweden	S6	55.5619	13.2160
S6.11	Sweden	S6	55.5619	13.2160
S6.12	Sweden	S6	55.5619	13.2160
S6.15	Sweden	S6	55.5619	13.2160
S6.16	Sweden	S6	55.5619	13.2160
S6.17	Sweden	S6	55.5619	13.2160
S6.18	Sweden	S6	55.5619	13.2160
S6.19	Sweden	S6	55.5619	13.2160
S6.2	Sweden	S6	55.5619	13.2160
S6.20	Sweden	S6	55.5619	13.2160
S6.21	Sweden	S6	55.5619	13.2160
S6.22	Sweden	S6	55.5619	13.2160
S6.24	Sweden	S6	55.5619	13.2160
S6.25	Sweden	S6	55.5619	13.2160
S6.26	Sweden	S6	55.5619	13.2160
S6.27	Sweden	S6	55.5619	13.2160
S6.3	Sweden	S6	55.5619	13.2160
S6.4	Sweden	S6	55.5619	13.2160
S6.5	Sweden	S6	55.5619	13.2160
S6.6	Sweden	S6	55.5619	13.2160
S6.7	Sweden	S6	55.5619	13.2160
S6.8	Sweden	S6	55.5619	13.2160
S6.9	Sweden	S6	55.5619	13.2160
S7.1	Sweden	S7	55.7183	13.4382
S7.10	Sweden	S7	55.7183	13.4382
S7.11	Sweden	S7	55.7183	13.4382

Sample ID	Country of Origin	Population	X Coordinate	Y Coordinate
S7.12	Sweden	S7	55.7183	13.4382
S7.13	Sweden	S7	55.7183	13.4382
S7.14	Sweden	S7	55.7183	13.4382
S7.15	Sweden	S7	55.7183	13.4382
S7.16	Sweden	S7	55.7183	13.4382
S7.17	Sweden	S7	55.7183	13.4382
S7.18	Sweden	S7	55.7183	13.4382
S7.19	Sweden	S7	55.7183	13.4382
S7.2	Sweden	S7	55.7183	13.4382
S7.20	Sweden	S7	55.7183	13.4382
S7.21	Sweden	S7	55.7183	13.4382
S7.22	Sweden	S7	55.7183	13.4382
S7.23	Sweden	S7	55.7183	13.4382
S7.24	Sweden	S7	55.7183	13.4382
S7.29	Sweden	S7	55.7183	13.4382
S7.3	Sweden	S7	55.7183	13.4382
S7.30	Sweden	S7	55.7183	13.4382
S7.32	Sweden	S7	55.7183	13.4382
S7.33	Sweden	S7	55.7183	13.4382
S7.34	Sweden	S7	55.7183	13.4382
S7.35	Sweden	S7	55.7183	13.4382
S7.5	Sweden	S7	55.7183	13.4382
S7.6	Sweden	S7	55.7183	13.4382
S7.7	Sweden	S7	55.7183	13.4382
S7.8	Sweden	S7	55.7183	13.4382
S7.9	Sweden	S7	55.7183	13.4382

Appendix C2. *Segmentina nitida* individuals used for COI sequencing

Sample ID	Country	Population	X Coordinate	Y Coordinate
G1.2	Germany	G1	53.9488	13.772
G1.12	Germany	G1	53.9488	13.772
G1.14	Germany	G1	53.9488	13.772
G1.15	Germany	G1	53.9488	13.772
G2.3	Germany	G2	53.9942	12.8204
G2.7	Germany	G2	53.9942	12.8204
G2.8	Germany	G2	53.9942	12.8204
G2.14	Germany	G2	53.9942	12.8204
G2.15	Germany	G2	53.9942	12.8204
G3.1	Germany	G3	53.9929	12.8224
G3.2	Germany	G3	53.9929	12.8224
G3.3	Germany	G3	53.9929	12.8224
G3.14	Germany	G3	53.9929	12.8224

Sample ID	Country	Population	X Coordinate	Y Coordinate
G4.12	Germany	G4	53.8526	12.8977
G4.13	Germany	G4	53.8526	12.8977
G4.14	Germany	G4	53.8526	12.8977
G4.15	Germany	G4	53.8526	12.8977
G5.2	Germany	G5	53.8325	12.8325
G5.3	Germany	G5	53.8325	12.8325
GB1.11	UK	GB1	51.313	1.3168
GB1.13	UK	GB1	51.313	1.3168
GB1.14	UK	GB1	51.313	1.3168
GB1.15	UK	GB1	51.313	1.3168
GB1C	UK	GB1	51.313	1.3168
GB2.1	UK	GB2	51.3148	1.3164
GB2.2	UK	GB2	51.3148	1.3164
GB2.7	UK	GB2	51.3148	1.3164
GB2.8	UK	GB2	51.3148	1.3164
GB2.9	UK	GB2	51.3148	1.3164
GB3.1	UK	GB3	51.3025	1.2132
GB3.3	UK	GB3	51.3025	1.2132
GB3.4	UK	GB3	51.3025	1.2132
GB3.5	UK	GB3	51.3025	1.2132
GB3.6	UK	GB3	51.3025	1.2132
P2.2	Poland	P2	52.4729	16.6303
P2.6	Poland	P2	52.4729	16.6303
P2.14	Poland	P2	52.4729	16.6303
P2.15	Poland	P2	52.4729	16.6303
P3.2	Poland	P3	52.475	16.5427
P3.3	Poland	P3	52.475	16.5427
P3.4	Poland	P3	52.475	16.5427
P3.7	Poland	P3	52.475	16.5427
P3.9	Poland	P3	52.475	16.5427
P5.8	Poland	P5	52.4714	16.5302
P5.12	Poland	P5	52.4714	16.5302
P5.14	Poland	P5	52.4714	16.5302
P5.15	Poland	P5	52.4714	16.5302
P6.1	Poland	P6	52.4892	16.8967
P6.4	Poland	P6	52.4892	16.8967
P6.9	Poland	P6	52.4892	16.8967
P6.10	Poland	P6	52.4892	16.8967
P6.15	Poland	P6	52.4892	16.8967
P7.2	Poland	P7	52.4897	16.8978
P7.3	Poland	P7	52.4897	16.8978
P7.6	Poland	P7	52.4897	16.8978
P7.8	Poland	P7	52.4897	16.8978
P7.15	Poland	P7	52.4897	16.8978

Sample ID	Country	Population	X Coordinate	Y Coordinate
P8.1	Poland	P8	52.4902	16.8967
P8.4	Poland	P8	52.4902	16.8967
P8.5	Poland	P8	52.4902	16.8967
P8.14	Poland	P8	52.4902	16.8967
P8.15	Poland	P8	52.4902	16.8967
S1.1	Sweden	S1	55.6385	14.2541
S1.3	Sweden	S1	55.6385	14.2541
S1.4	Sweden	S1	55.6385	14.2541
S1.5	Sweden	S1	55.6385	14.2541
S1.6	Sweden	S1	55.6385	14.2541
S2.1	Sweden	S2	55.3866	13.4945
S2.2	Sweden	S2	55.3866	13.4945
S2.3	Sweden	S2	55.3866	13.4945
S2.14	Sweden	S2	55.3866	13.4945
S2.15	Sweden	S2	55.3866	13.4945
S3.6	Sweden	S3	55.5142	13.4359
S3.10	Sweden	S3	55.5142	13.4359
S3.11	Sweden	S3	55.5142	13.4359
S3.13	Sweden	S3	55.5142	13.4359
S3.14	Sweden	S3	55.5142	13.4359
S3.15	Sweden	S3	55.5142	13.4359
S4.10	Sweden	S4	55.5596	13.2494
S4.11	Sweden	S4	55.5596	13.2494
S4.14	Sweden	S4	55.5596	13.2494
S4.15	Sweden	S4	55.5596	13.2494
S5.4	Sweden	S5	55.5612	13.2173
S5.5	Sweden	S5	55.5612	13.2173
S5.6	Sweden	S5	55.5612	13.2173
S5.14	Sweden	S5	55.5612	13.2173
S5.25	Sweden	S5	55.5612	13.2173
S6.2	Sweden	S6	55.5619	13.216
S6.11	Sweden	S6	55.5619	13.216
S6.12	Sweden	S6	55.5619	13.216
S6.15	Sweden	S6	55.5619	13.216
S6.17	Sweden	S6	55.5619	13.216
S7.8	Sweden	S7	55.7183	13.4382
S7.9	Sweden	S7	55.7183	13.4382
S7.10	Sweden	S7	55.7183	13.4382
S7.11	Sweden	S7	55.7183	13.4382
S7.12	Sweden	S7	55.7183	13.4382

Appendix C3. *Segmentina nitida* individuals used for ITS2 sequencing

Sample ID	Country	Population	X Coordinate	Y Coordinate
G1.2	Germany	G1	53.9488	13.772
G1.12	Germany	G1	53.9488	13.772
G1.13	Germany	G1	53.9488	13.772
G1.14	Germany	G1	53.9488	13.772
G1.15	Germany	G1	53.9488	13.772
G2.14	Germany	G2	53.9942	12.8204
G2.3	Germany	G2	53.9942	12.8204
G2.5	Germany	G2	53.9942	12.8204
G2.7	Germany	G2	53.9942	12.8204
G2.8	Germany	G2	53.9942	12.8204
G3.1	Germany	G3	53.9929	12.8224
G3.14	Germany	G3	53.9929	12.8224
G3.15	Germany	G3	53.9929	12.8224
G3.2	Germany	G3	53.9929	12.8224
G3.3	Germany	G3	53.9929	12.8224
G4.11	Germany	G4	53.8526	12.8977
G4.12	Germany	G4	53.8526	12.8977
G4.13	Germany	G4	53.8526	12.8977
G4.14	Germany	G4	53.8526	12.8977
G4.15	Germany	G4	53.8526	12.8977
G5.1	Germany	G5	53.8325	12.8325
G5.2	Germany	G5	53.8325	12.8325
G5.3	Germany	G5	53.8325	12.8325
GB1.11	UK	GB1	51.313	1.3168
GB1.12	UK	GB1	51.313	1.3168
GB1.13	UK	GB1	51.313	1.3168
GB1.15	UK	GB1	51.313	1.3168
GB1C	UK	GB1	51.313	1.3168
GB2.1	UK	GB2	51.3148	1.3164
GB2.2	UK	GB2	51.3148	1.3164
GB2.7	UK	GB2	51.3148	1.3164
GB2.8	UK	GB2	51.3148	1.3164
GB2.9	UK	GB2	51.3148	1.3164
GB3.3	UK	GB3	51.3025	1.2132
GB3.4	UK	GB3	51.3025	1.2132
GB3.5	UK	GB3	51.3025	1.2132
GB3.6	UK	GB3	51.3025	1.2132
P2.14	Poland	P2	52.4729	16.6303
P2.15	Poland	P2	52.4729	16.6303
P2.2	Poland	P2	52.4729	16.6303
P2.4	Poland	P2	52.4729	16.6303
P2.6	Poland	P2	52.4729	16.6303

Sample ID	Country	Population	X Coordinate	Y Coordinate
P3.2	Poland	P3	52.475	16.5427
P3.4	Poland	P3	52.475	16.5427
P3.6	Poland	P3	52.475	16.5427
P3.7	Poland	P3	52.475	16.5427
P3.9	Poland	P3	52.475	16.5427
P5.12	Poland	P5	52.4714	16.5302
P5.14	Poland	P5	52.4714	16.5302
P5.15	Poland	P5	52.4714	16.5302
P5.7	Poland	P5	52.4714	16.5302
P5.8	Poland	P5	52.4714	16.5302
P6.1	Poland	P6	52.4892	16.8967
P6.10	Poland	P6	52.4892	16.8967
P6.15	Poland	P6	52.4892	16.8967
P6.4	Poland	P6	52.4892	16.8967
P6.9	Poland	P6	52.4892	16.8967
P7.15	Poland	P7	52.4897	16.8978
P7.2	Poland	P7	52.4897	16.8978
P7.3	Poland	P7	52.4897	16.8978
P7.6	Poland	P7	52.4897	16.8978
P7.8	Poland	P7	52.4897	16.8978
P8.1	Sweden	P8	52.4902	16.8967
P8.14	Sweden	P8	52.4902	16.8967
P8.15	Sweden	P8	52.4902	16.8967
P8.4	Sweden	P8	52.4902	16.8967
P8.5	Sweden	P8	52.4902	16.8967
S1.1	Sweden	S1	55.6385	14.2541
S1.3	Sweden	S1	55.6385	14.2541
S1.4	Sweden	S1	55.6385	14.2541
S1.5	Sweden	S1	55.6385	14.2541
S1.6	Sweden	S1	55.6385	14.2541
S2.1	Sweden	S2	55.3866	13.4945
S2.14	Sweden	S2	55.3866	13.4945
S2.15	Sweden	S2	55.3866	13.4945
S2.2	Sweden	S2	55.3866	13.4945
S2.3	Sweden	S2	55.3866	13.4945
S3.13	Sweden	S3	55.5142	13.4359
S3.14	Sweden	S3	55.5142	13.4359
S3.15	Sweden	S3	55.5142	13.4359
S4.10	Sweden	S4	55.5596	13.2494
S4.11	Sweden	S4	55.5596	13.2494
S4.12	Sweden	S4	55.5596	13.2494
S4.14	Sweden	S4	55.5596	13.2494
S4.15	Sweden	S4	55.5596	13.2494
S5.14	Sweden	S5	55.5612	13.2173

Sample ID	Country	Population	X Coordinate	Y Coordinate
S5.4	Sweden	S5	55.5612	13.2173
S5.5	Sweden	S5	55.5612	13.2173
S5.6	Sweden	S5	55.5612	13.2173
S6.11	Sweden	S6	55.5619	13.216
S6.12	Sweden	S6	55.5619	13.216
S6.15	Sweden	S6	55.5619	13.216
S6.17	Sweden	S6	55.5619	13.216
S6.2	Sweden	S6	55.5619	13.216
S7.10	Sweden	S7	55.7183	13.4382
S7.11	Sweden	S7	55.7183	13.4382
S7.12	Sweden	S7	55.7183	13.4382
S7.8	Sweden	S7	55.7183	13.4382
S7.9	Sweden	S7	55.7183	13.4382

Appendix C4. Delta K values for Evanno analysis for number of clusters in STRUCTURE analysis of microsatellite markers

K	Delta K
1	—
2	1249.217224
3	9.917547
4	73.871360
5	1.325439
6	1.148886
7	0.416368
8	0.989778
9	0.579903
10	3.287288
11	3.975548
12	4.178777
13	0.688533
14	3.653501
15	0.740975
16	0.145296
17	1.562281
18	1.729717
19	1.123199
20	0.976700
21	0.471898
22	1.025240
23	0.399811
24	0.338761
25	1.221811
26	—