THE UNIVERSITY OF HULL

Genetic structure and colonisation history of European and UK populations of *Gammarus pulex*

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by

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Abstract

The structure of populations has been studied for many years and there have been three main factors that have been suggested as the cause for present-day distributions of species, those being environment, biology and history. With the use of molecular data and advanced phylogeographic approaches it is now possible to distinguish between the main causes of population structuring. The present study considers the extent of population structure in *G. pulex* on regional (UK) and large geographic (Europe) scales using studies of molecular genetic (allozymes, mtDNA sequencing and microsatellites) and morphological variation.

Molecular analysis of *Gammarus pulex* in Europe revealed more diversity than previously thought. This was thought to be a consequence of two separate waves of colonisation after the formation of the major drainages in the Miocene. The UK appears to have been colonised once from either the Elbe, Mosel and Rhine drainages separately or cumulatively across the drainage basins late in the Pleistocene before a land bridge connection to mainland Europe was submerged. Limited molecular variation in the UK is thought to be a result of reduced genetic variation in the colonising individuals. This in turn was caused by repeated founder events during population expansion and contraction from European refugia.

A detailed analysis of a transplantation experiment in 1950 in the Isle of Man revealed little genetic impoverishment of the introduced population when compared to the source. In contrast, morphological variation increased in the introduced population. Unlike in mainland Europe there was no historical explanation for the diversity recorded (as the introduced population was so young) and, in the absence of fragmentation, speciation and colonisation the contemporary forces of gene flow, selection and limited genetic drift are thought to be the determining factors in population structure.

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CHAPTER 1

General Introduction

1.1. Context and rationale of thesis

Populations of most species display, to some degree, a level of population structuring across their geographic range (McGlashan, 2000). The degree and nature of the structuring has implications for the survival of populations and their evolutionary potential (Hartl & Clark, 1997). Traditionally, population structure has been studied using an ecological approach, assessing key demographic factors such as age structure, population size, population dynamics and the distribution of individuals. More recently, molecular approaches have been used to determine population genetic structure. These two sets of methods have been used largely independently in most studies of population structure, though both should be considered ideally as interdependent. Using molecular techniques alone cannot necessarily provide answers to the patterns and causes of population structure without some consideration of the possible ecological interactions and historical factors involved in shaping populations. Not only does a molecular approach provide an insight into the nature and extent of genetic structuring within and among populations, but it can also aid in the ecological explanations underlying the patterns observed. In this thesis I present an investigation into the patterns of genetic and morphological diversity in the freshwater shrimp, *Gammarus pulex pulex*, and examine the role of biological, environmental and historical factors that impact on the levels and distribution described.

1.1.1 Population structure in freshwater systems

Within freshwater systems there are several key factors that are thought to be responsible for the nature and extent of population structuring of species. The relative

importance of key processes, such as gene flow, patterns of colonisation and environmental factors in the formation of extant populations does, however, remain unclear (Schultheis *et al.*, 2002). One dominant factor is the patchiness of freshwater habitats and the associated restrictions to movement between populations and across drainages. Although many organisms have significant population sub-structuring imposed by environmental patchiness (Hartl & Clark, 1997) this situation is enhanced in most freshwater species. Within freshwaters, the network of lotic systems (running water) within drainages, and lenthic systems (standing waters such as lakes and ponds), can be thought of as 'habitat islands' surrounded by a 'sea' of un-crossable land (Kelly *et al.*, 2001). Freshwater riverine habitats have been described as one-dimensional in structure, with restricted migration routes for gene flow (Gooch & Hetrick, 1979; Siegismund & Müller, 1991).

Classically, the riverine systems display a hierarchical structure with several levels: interbreeding animals within a stream form the lowest level; streams flowing into a common river form the next level; rivers that flow in to the same drainage or watershed usually form the next highest level; and sometimes drainages within a continent can be considered as the highest level. As the level of the hierarchy increases, the genetic distance between individuals often increases in parallel, as movement across a level becomes more restricted. The natural hierarchy of freshwater habitats has been suggested as one of the important extrinsic factors in defining the nature of subdivided populations and genetic structuring (Bilton *et al.*, 2001; Siegismund & Müller, 1991; Scheepmaker, 1990). However a hierarchical population structure in relation to habitat is not always observed, especially where there is patchy habitat availability within streams (Kelly *et al.*, 2001).

Compounding the effects of drainage structure can be the small size of established populations (compared to marine species) that promote differentiation and

structuring (Ward *et al.*, 1994). Smaller populations are generally found in more isolated habitats, such as headwaters, and which through genetic drift and bottlenecks are thought to promote differentiation within a species (Siegismund *et al.*, 1985) and bring about allelic fixation (Gooch & Hetrick, 1979; Shaw *et al.*, 1994). In some of the most geographically fragmented freshwater systems genetic differentiation between populations can be related directly to environmental structure (Gooch & Hetrick, 1979), though this is not necessarily the case where species have a high dispersal ability (Kelly *et al.*, 2001). Geographic barriers to migration can be seen to restrict gene flow directly between populations and promote differentiation (Gooch & Hetrick, 1979; Müller, 1998).

Not only does the genetic structure of populations reflect the level of isolation within the hierarchical riverine system, but it also may reflect the dispersal ability and life history of species within them. The range of dispersal mechanisms and behaviours to aid dispersal within freshwater invertebrates is both extensive and diverse, including active flight, passive wind dispersal, the use of animal vectors and passive water transport (see review by Bilton *et al.*, 2001). Figure 1.1 summarises the key behaviours associated with invertebrate dispersal within freshwaters.

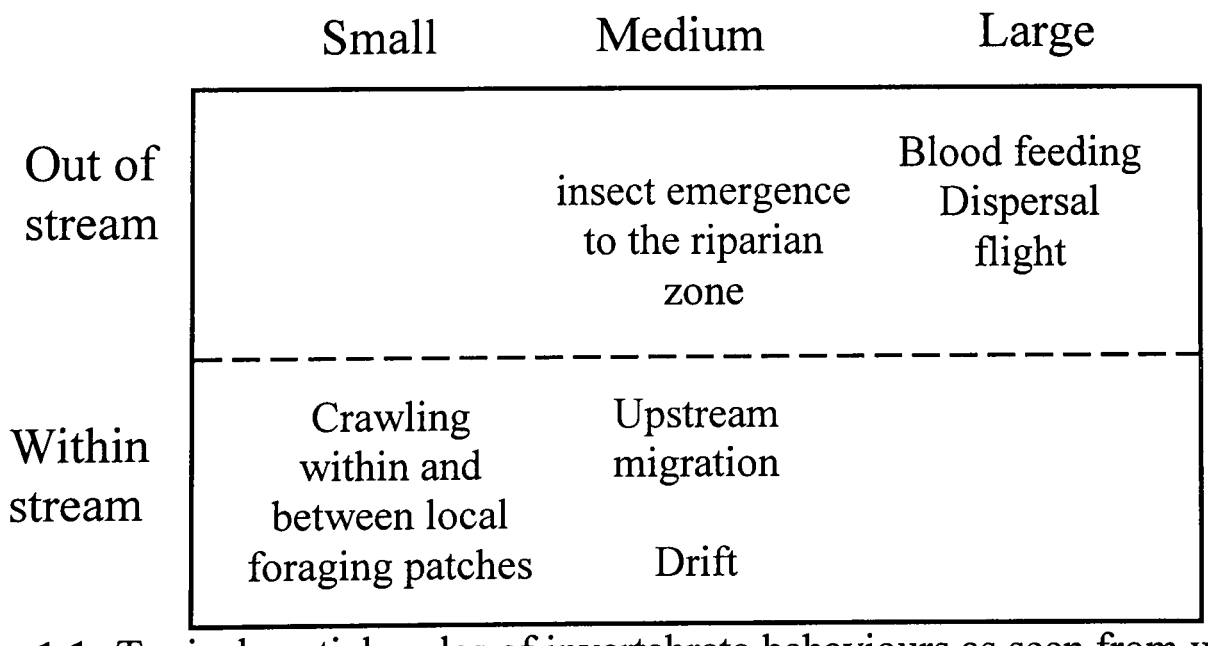


Figure 1.1: Typical spatial scales of invertebrate behaviours as seen from within-stream and out-of-stream perspectives in the movement of carbon along a scale (small, medium & large). Redrawn from Malmqvist (2002)

Not all invertebrates inhabiting freshwater systems have specific life history stages to aid their movement between and within drainages. Most freshwater insects possess an active dispersal stage, with the primary mechanism for dispersal being an adult flight stage (Schultheis *et al.*, 2002; Wilcock *et al.*, 2001). However, within freshwater crustaceans, dispersal is predominantly passive and reliant on vectors and desiccation resistant life history stages (normally eggs) for dispersal between drainages (see table 1 in review by Bilton *et al.*, 2001). The fairy shrimp (*Brachinecta colorandensis*) relies on salamanders ingesting its eggs for transportation (Bohonak, 1998), cladocerans appear to utilise waterfowl for passive transportation of resting eggs (Bilton *et al.*, 2001), and anostracans rely on wind for egg dispersal (Brendonck & Riddoch, 1999). Even when compared to other Crustacea which have draught-tolerant life history stages, the gammarids are unique in their restricted dispersal ability and absence of any specific life history stage for dispersal.

If a species has restricted dispersal ability the population genetic structure would be expected to reflect the hierarchy of the drainage system through genetic drift or natural selection (Wilcock *et al.*, 2001). Conversely, if a species has a high dispersal ability then genetic structuring would be predicted to be weak and bear little relation to the spatial pattern of the drainage system. Data comparing genetic distance and geographic distance between populations can be used to examine such predictions. Figure 1.2 shows the difference in relationship between geographic and genetic distance for two freshwater amphipods. *Gammarus fossarum* inhabits headwater habitats that are isolated and hence show a positive correlation between both distance measures. Conversely, *G. pulex* that inhabits lower reaches of streams, has less isolated populations and shows little correlation between distance measures. These data suggest that not all freshwater gammarids have restricted dispersal ability, and that genetic differentiation is not always determined solely by the hierarchical riverine structure.

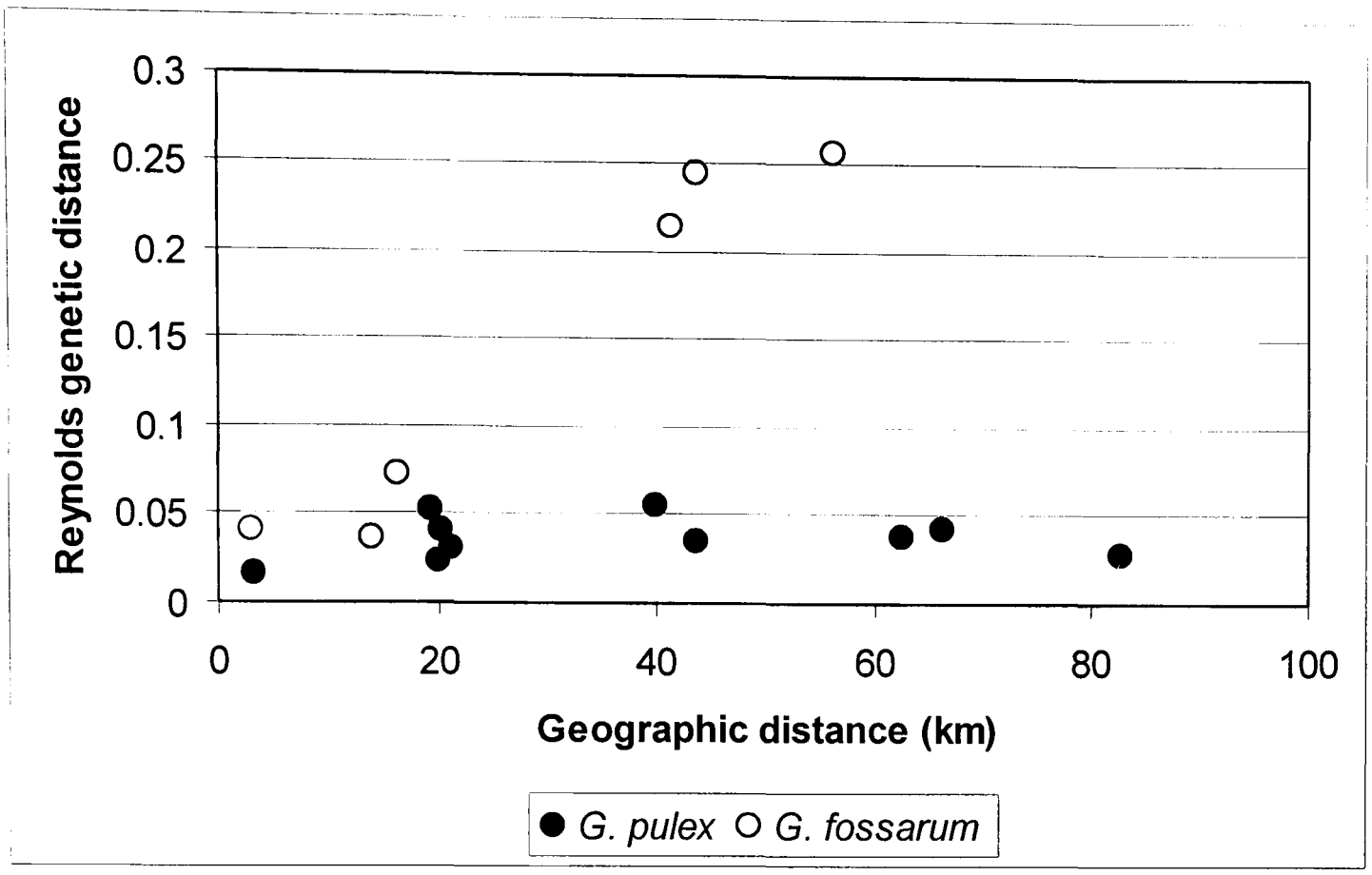


Figure 1.2: Genetic and geographic distance between populations of amphipods sampled in Belgium (De Meester, *unpublished data*).

The extent of present-day freshwater invertebrate population structure will reflect both the contemporary genetic processes (gene flow, drift and selection) and the past history of a population (colonisation, range expansion and bottlenecks). The ability to separate the contemporary forces (gene flow and drift) from historical processes (colonisation and range expansion) can now be achieved using phylogeographic approaches, such as nested clade analysis (Templeton, 1998). Phylogeographic methods are particularly important in freshwater systems, where geography and gene flow (through dispersal) are such dominant forces in shaping populations. Many river systems and catchments in Europe have changed since they were formed in the Miocene (Gibbard, 1988), and it is these historical changes that have been proposed as the underlying basis for population structure in many freshwater species (Durand *et al.*, 1999; Englbrecht *et al.*, 2000; Müller, 1998; Nesbo *et al.*, 2000). Where populations have been established for a relatively short period of time, contemporary forces are unlikely to have sufficient time to act, and populations would be expected to reflect the

past history (Bohonak, 1999; Wilcock *et al.*, 2001).

G. pulex is thought to be genetically homogeneous on a large scale across its massive geographic distribution (Afghanistan to Sweden) (Scheepmaker & Van Dalfsen, 1989). Caution should, however, be used when interpreting this statement as Adams *et al.* (1987) suggest that even cosmopolitan species like *G. pulex* can have a patchy distribution and hence greater population structuring than previously thought. The homogeneity of *G. pulex* appears to be due to its ability to migrate along 'inhabitable' rivers and watersheds, possibly by waterfowl or through human activity (Müller, 1998). The migration and dispersal ability of the gammarids depends largely on their habitat, with marine gammarids showing less structuring and differentiation between populations than their freshwater counterparts (Seigismund *et al.*, 1985). The dispersal ability of freshwater gammarids would appear to be limited without a specific life history stage, but observations by Elliott (1971) and Litterick (1973) suggest that *G. pulex* is capable of upstream movements of 14m per day and the ability to traverse small waterfalls. Overall, the general consensus from the literature suggests that *G. pulex* is truly aquatic, and dispersal is by passive drift and active upstream movements (see review in Litterick, 1973). The lack of a specific dispersal life history stage does not appear to restrict gammarids from long distance movements within drainages, and the possession of an adult dispersal stage (such as flight) is therefore not a requirement to prevent extinction of upstream reaches (Humphries & Ruxton, 2002). However, it remains unclear how common such long-distance dispersal actually is; the frequency of such events will determine the extent of genetic structure (Slatkin, 1985). The ability to disperse will determine the geographic distribution of *G. pulex*, with historical processes defining the scale of population genetic differentiation (Slatkin, 1985).

The geographic range over which *G. pulex* can disperse is potentially high, but the actual dispersal recorded indirectly in the field has been lower than the suggested

potential (Scheepmaker, 1990). Thus, *G. pulex* has a high gene flow within drainages and a low gene flow between drainages, despite its apparent limited dispersal ability. The dispersal can be constrained further by patchiness, all of which will determine the overall population genetic structure. However, particular attention should be given to the historical forces that shape populations, which often has a significant influence on the present-day distribution and structure of freshwater species (Durand *et al.*, 1999; Englbrecht *et al.*, 2000; Müller, 1998; Nesbo *et al.*, 2000).

1.2 Molecular markers

Over the last forty years the use of molecular markers in biology has increased rapidly. Both the decreased costs and increased availability of protocols have led to molecular markers being utilised in virtually all fields of biology. Since the first allozyme electrophoresis methods were described (Smithies, 1955), there has been a rapid development of new markers and techniques, along with a parallel increase in statistical power. Most recent techniques (10 years) have been designed to assess DNA variation directly, aided by the polymerase chain reaction (PCR). The aim of this section is to cover, albeit briefly, the techniques used (and available) in molecular ecology, with particular reference to the objectives of this thesis.

1.2.1 Allozyme electrophoresis

Since its introduction (Smithies, 1955), allozyme electrophoresis has had a major impact on the fields of evolutionary biology, population ecology and systematics (Avise, 1994). Most major taxa in the animal kingdom have been investigated with allozyme electrophoresis. Allozymes are enzymes differing in electrophoretic mobility as a result of allelic differences at a single gene (Park & Moran, 1995). Isozymes are functionally similar but electrophoretically separable forms of enzymes, encoded for by

one or more loci (Markert & Moller, 1959), and unlike allozymes, may include non-genetic variability.

Electrophoresis (DNA or protein) is the separation of molecules based on charge, size or shape through a gel matrix (starch, agarose, cellulose acetate or polyacrylamide). In DNA electrophoresis, separation is by size alone, as all DNA is negatively charged (Park and Moran, 1995). Protein electrophoresis occurs by both charge and size separation (in a starch matrix only), as the amino acid sequence of a protein will determine the net charge and quaternary structure of the molecule. When an electric potential is applied to a gel matrix, proteins migrate at differential rates (their specific electrophoretic mobility), with small highly charged proteins moving fastest. Once the proteins have migrated a sufficient distance to resolve the different alleles shown by the protein types, they need to be stained and recorded.

Allelic products are not visible to the naked eye, and visualisation relies on the ability of the enzymes to catalyse specific reactions resulting in a visible product. The most common method is to use a stain that will produce a dark purple precipitate of formazan (Hillis & Moritz, 1990) at the site of the enzyme on the gel, which can be viewed under normal light. Alternatively, a negative stain can be used, with the enzyme acting to inhibit a stain reaction, producing a clear band. Harris and Hopkinson (1976) include an extensive section on recipes and the reactions of stains for allozyme electrophoresis.

Allelic variation at allozyme loci can be used to compare species, populations and stocks. Allozyme electrophoresis is an ideal tool for distinguishing stocks of fish, where a given locus (or loci) may show fixed differences between the given stocks, though allele frequency differences may be less discrete (Carvalho & Hauser, 1995). The functional study of polymorphisms at specific loci has no parallel in DNA techniques (Skibinski, 1994). However due to their often low variability, allozyme

electrophoresis has usually lower statistical power compared to direct DNA techniques, limiting the detection of differentiation at the population and individual level (Grant & Utter, 1980).

Allozyme electrophoresis can underestimate the total genetic variation of an organism, as only DNA sequences that result in either a change in protein structure or net charge will be detected (Hartl, 1981). The limitation in the power of detection of DNA sequence alteration by allozyme electrophoresis cannot be improved upon. However increased resolution can only be achieved by increasing the number of enzyme loci scored, thereby increasing the number of genes (and proportion of whole genome) screened.

Another limitation of allozyme electrophoresis is the need for a minimum amount of either fresh or frozen tissue. This limits the marker to organisms larger than a fish larva or a mussel spat (Skibinski, 1994). Though allozyme electrophoresis has limited resolving power it still provides a basic tool in the field of molecular and evolutionary biology.

1.2.3 Mitochondrial DNA

Vertebrate mitochondrial DNA (mtDNA) is a closed circular molecule, 16-18 kilobases long with 37 genes that code for 13 polypeptides, rRNAs and 22 tRNAs (Ferris & Berg, 1986). As mtDNA is haploid and maternally (cytoplasmically) inherited, the effective population (N_e) size is one quarter of that of nuclear DNA (nDNA) for the same organism (Nei & Tajima, 1981). This is a very useful attribute as the smaller N_e will be more susceptible to the effect of genetic drift compared to nDNA (Park and Moran, 1995). Although mtDNA contains over thirty genes, it is effectively a single locus in population genetic analyses because of the absence of recombination (Park and Moran, 1995).

The evolutionary rate of mtDNA varies throughout the molecule and depends in part on the function of the region (coding/non-coding). In mtDNA there are fewer repair mechanisms than in nDNA, and the mutation rate is correspondingly higher for similar genes. Comparative sequencing and restriction enzyme mapping indicate that the greatest rate of mutation occurs in the D-loop, and the least in the RNA genes (Ferris and Berg, 1986). Meyran *et al* (1997) used the cytochrome c oxidase I subunit (COI) as a taxonomic and phylogenetic marker to separate six species of *Gammarus*. Although parts of cytochrome oxidase I are highly conserved, there are large sequence differences between species of penaeid shrimps (Palumbi & Benzi, 1991).

1.2.4 Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs are a molecular tool that can be applied to either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). Restriction enzymes (endonucleases) cut and digest DNA at specific sites (to the given enzyme), known as restriction sites (Park and Moran, 1995). The restrictions sites vary in length, from four to eight nucleotides (bases) long. Most enzymes cut DNA in an asymmetric fashion, leaving short single-stranded overhangs, "sticky ends". The products of a restriction digest can be separated according to size on a gel matrix (either agarose or polyacrylamide) and visualised with staining (ethidium bromide or silver staining,). If there are no restriction sites within the sample, there will be only a single product on the gel (same size as an unrestricted sample). However if there are restriction sites, there will be several fragments visible that in total will be equal in length to the unrestricted DNA.

RFLPs can be utilised in population and species differentiation, using restriction enzymes that produce variable fragments. To target such enzyme(s), there are two approaches, sequencing and enzyme screening. Where possible, the DNA sample can be sequenced and searched for known restriction sites, and the optimal enzyme selected.

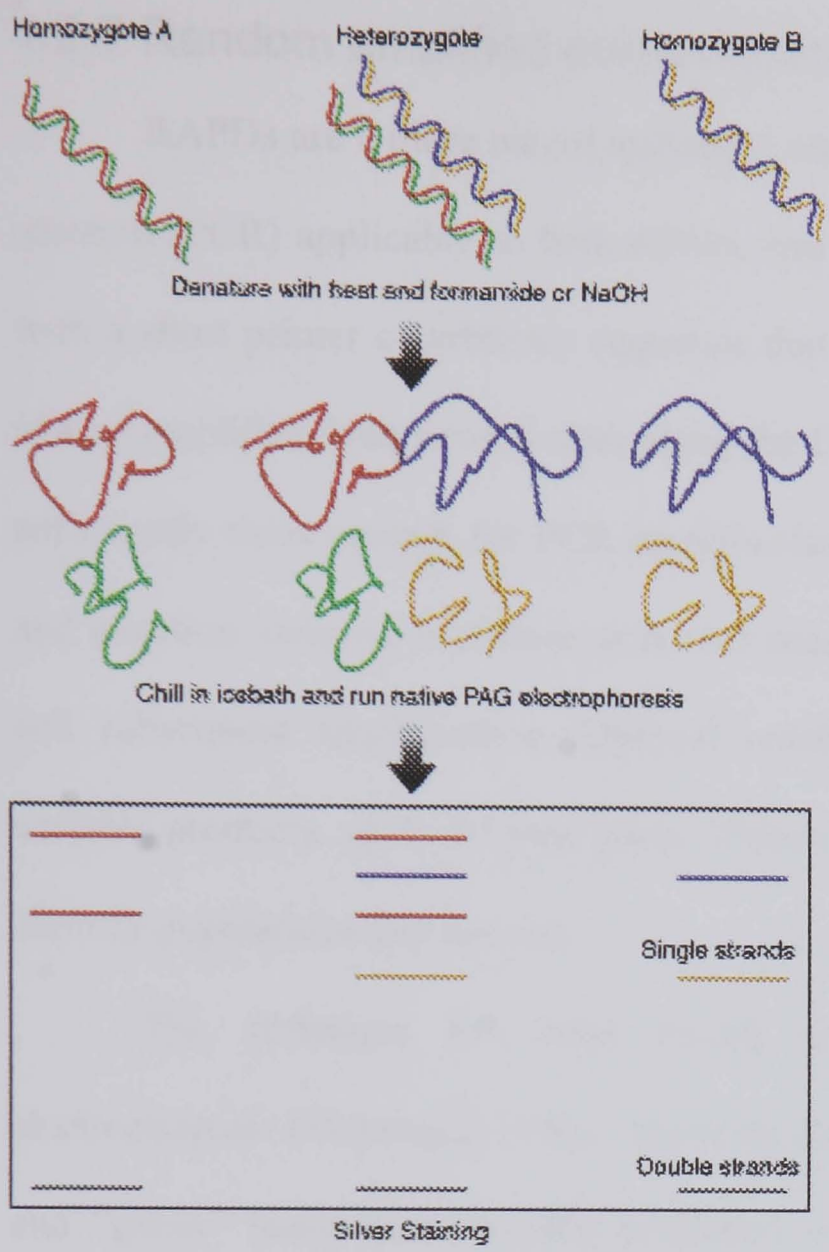
Enzyme screening utilises many enzymes, and from a few individuals screened the optimal enzymes can be picked. Both approaches are valid and offer advantages where sequencing or large numbers of enzymes are unavailable.

Fragment polymorphism can be the result of base substitution or from insertion/deletion mutations, causing a loss (or gain) of a restriction sites (Park and Moran, 1995). Base substitutions and insertions/deletions that occur at restriction sites alter the sequence, preventing the enzyme recognition and cleavage of the DNA. The resolution of RFLPs relies on variability of restriction sites and the type of DNA sequence selected.

1.2.5 Single Strand Confirmation Polymorphisms

SSCPs are a technique based on single strands of DNA in a non-denatured state forming folded secondary structures. These structures will be determined by intramolecular interactions, which will cause the DNA to fold and adhere to itself, where sequences align. DNA molecules (nuclear and organelle) with mutations (even a single base) show different secondary structures than the wild type, resulting in a different electrophoretic mobility (Figure 1.3). Electrophoresis can be carried out in various media, agarose and polyacrylamide, and DNA is visualised by ethidium bromide under UV or by silver staining.

The sensitivity of the method is optimal for DNA of 200 bases and decreases with increasing strand length. The method is also affected by gel running conditions, temperature, gel additives, ionic strength and sequence composition (Innis *et al.*, 1995). Sensitivity measurements (the ability to detect mutation) in the literature ranges from 35% (Sarkar *et al.*, 1992) to nearly 100% (Orita *et al.*, 1989).



To test for a mutant gene or polymorphism, PCR products from control and test samples are prepared by heat denaturation in the presence of formamide, causing the separation of the two DNA strands. During cooling, folded single-stranded secondary structures emerge as well as the original double stranded DNA. The three dimensional structures are unique to the primary sequence, and move at different rates through a nondenaturing MDE gel. The sensitivity of mutation detection is high because single-base changes may radically alter the migration of the nucleic acid.

Figure 1.3 From (Innis *et al.*, 1995)

1.2.6 Nuclear DNA

There are several different types of DNA that can be found in plants and animals, which can be partitioned into nuclear and organelle DNA (mitochondrial, chloroplast and plasmid). The function of nuclear DNA regions has led to the classification into exon regions (coding) and intron regions (non-coding) within coding genes. Intron regions have been referred to as "junk DNA", which is an over-simplistic view as some introns act as precursors to synthesis of gene sequences. Both the rates of mutation and of evolutionary change (through selection)are greatest in intron regions, where there are reduced selective pressures and DNA repair mechanisms. In nuclear DNA (nDNA), repair mechanisms (exonucleases) repair mutations that occur mainly in intron regions.

1.2.7 Random amplified polymorphic DNAs (RAPDs)

RAPDs are a more recent technique and a consequence of the polymerase chain reaction (PCR) applicable to both nDNA and mtDNA. A DNA template is amplified with a short primer of arbitrary sequence that binds many times to the DNA. As the primer amplifies from several sites along the DNA template the resultant fragments are sufficiently short enough for PCR amplification. Similar to RFLPs, base substitutions and insertion /deletion mutations at primer sites will prevent the annealing of the primer and subsequent amplification. Optimal results are with ten-base primers that give variable products, up to 20 base pairs. Primer product polymorphisms can be used to identify populations and species.

The technique has been hailed as the DNA equivalent of allozyme electrophoresis (Skibinski, 1994). However RAPDs are dependant on PCR conditions and "ghost" bands (non specific products) can be produced as a result of the low annealing temperatures and the primers used

1.2.8 Repetitive DNA

Non-coding DNA has regions of highly repetitive DNA motifs that are termed satellite DNA. Satellite DNA accounts for less than 30% of the mammalian genome, with the remaining 70% dominated by non-repeat coding or non-coding regions (single copy DNA). The number of repeat regions in satellite DNA is anywhere between tens and thousands of copies, of various sequences. The numbers of repeat units (motifs) are diverse, depending on the variable number of tandem repeats in the sequence (VNTRs). Minisatellites have repeat motifs of ten to one hundred nucleotides long (Jefferys *et al.*, 1985). Microsatellites contain smaller repeat motifs, normally one to four nucleotides long (Tautz, 1989). The structure and mutation of microsatellite loci will be covered in more detail in Chapter 6.

1.2.9 DNA Sequencing

DNA sequencing provides the highest level of resolution for examining genetic differences among populations. The most common method is the dideoxy-termination method, or Sanger method. Labelled primers are used to initiate the synthesis of new strands of DNA, but unlike PCR, only single strands are synthesised. Four PCR reactions are set up, each with all four deoxynucleotides (dA, dC, dG and dT) plus a single dideoxynucleotide (Avise, 1994). When the dideoxynucleotide (lacks the 3' -OH group) is incorporated no further synthesis occurs due to the inhibition of phosphate bonding. The primer will then anneal to the template again and the polymerase begins to synthesis a new DNA strand. Again, random termination of the synthesis will occur, when a dideoxynucleotide is incorporated instead of a normal nucleotide. As the incorporation of the modified nucleotides is random there will be a large number of DNA fragments terminated at different lengths in each of the four reactions. The four reactions are then run on a high-resolution gel (normally polyacrylamide) and visualised with radioactivity or fluorescence.

Automated sequencing can run the four reactions simultaneously in the same lane and do not require the use of expensive labelled primers. With the development of advanced sequencing machines it is possible to sequence longer sections of DNA on a regular basis (Livack *et al.*, 1995).

1.3 The ecology of Gammarus

At this point a consideration of some basic ecological features of the target genus, *Gammarus*, in relation to the questions under study will be undertaken. *Gammarus*, the genus (Amphipoda; Crustacea) was first described in 1758 (Karaman & Pinkster, 1977a). The classification of *Gammarus* has until recently been based on reputedly stable morphological characters and hybridisation experiments. These

species-diagnostic stable characters have, however, been shown to be variable across geographic areas (Goedmakers, 1972; Pinkster, 1983). More recent work has concentrated on classification based on molecular markers and morphology (Scheepmaker, 1990; Kane, 1992; Meyran *et al.*, 1997; Müller, 1998). From these studies there is general support for the morphological classification, with some exceptions in isolated populations.

The present distribution of the genus *Gammarus* covers all continents of the globe. Most species within the genus are found in marine habitats (80%), with the remaining species found in freshwater, except 10 terrestrial species. By far the richest freshwater *Gammarus* fauna in the world is to be found in Lake Baikal with over 200 endemic species (Yampolsky *et al.*, 1994). Within Europe, the diversity is much less, with almost 100 freshwater species described. These have been classified into three species groups based on morphological similarities (Karaman & Pinkster, 1977a; Karaman & Pinkster, 1977b; Karaman & Pinkster, 1987). The *Gammarus pulex* group contains 31 species, including two sub species of *G. pulex* (*Gammarus pulex pulex* and *Gammarus pulex gallicus*); the *Gammarus balcanicus* group contains 45 species, and the *Gammarus roeseli* group contains at least 12 species (Meyran *et al.*, 1997).

The UK has a lower diversity of *Gammarus* species than much of the rest of Europe. Isolation from Europe during the last glacial period has been thought to have prevented the spread of *Gammarus* species into the UK. Migration was possible only towards the end of the glacial period, when a land bridge existed between the UK and Europe, (Hynes, 1954).

1.3.1 Native *Gammarus* species

Within Britain, there are at least 17 species of *Gammarus*, living in marine, freshwater and brackish water environments. There are only three freshwater species

(not including introduced species), one of which can also be associated with brackish water (*Gammarus duebeni*). The preferred habitat of the three species differs (Fitter & Manuel, 1986), with the range of the three species depending on the environment and anthropogenic effects (see section 1.3.4).

G. dubeni and *Gammarus lacustris* are pre-glacial inhabitants of Britain and Ireland and show a wide distribution in estuaries and lakes respectively (Hynes, 1954). Survival of these species during ice coverage is thought to occur in limited brackish coastal regions (Bilton, *pers comm*). *G. pulex* is a post-glacial inhabitant of the British Isles, which was thought to have invaded via the land bridge across the English Channel, 8000 years ago (Hynes, 1954). The English Channel land bridge was submerged after the separation of Ireland and the Western Isles from the mainland (Hynes, 1954), so preventing *G. pulex* from colonising Ireland naturally. In England, relic populations of *G. dubeni* are to be found in the absence of *G. pulex* in brackish habitats.

Within Europe, *G. pulex* has a distribution dating back to before the last glacial event (Thienemann, 1950). During times of ice coverage, populations and species retreated into refugia, from where subsequent re-colonisation occurred (Taberlet *et al.*, 1998). In southern France there has been a subspecies of *G. pulex* described, *G. pulex gallicus*, which retreated into the Mediterranean basin during glaciation, unlike most other *G. pulex* populations. Expansion from these isolated refugia resulted in a geographically limited distribution of *G. pulex gallicus* due to the fragmented habitat.

1.3.2 Known Introductions of *Gammarus* species

Introductions have had a major effect on *Gammarus* species composition and distribution throughout England and Ireland. *Gammarus tigrinus* Sexton (native to North America) was found on the western coast of parts of England in 1931, colonising

freshwater and brackish water sites where the ion content was high due to pollution (Sexton & Cooper, 1939). In 1957, *G. tigrinus* extended its range into mainland Europe via two introductions, the first occurred in the river Werra in Germany, (Schmitz, 1960) where industrial pollution of potassium salts had destroyed the native *Gammarus* populations. The second introduction occurred in the Yssellake River, Holland, where little is known about the actual mechanisms involved. Colonisation in Holland by *G. tigrinus* was very rapid, and by 1979 it had reached the German border, replacing the native *Gammarus* fauna (*G. fossarum*). Rapid colonisation was linked to the reproductive capacity of *G. tigrinus* and its ability to withstand greater levels of pollution than the native species (Schmitz, 1960).

Introductions in Ireland have been well documented, and there have been at least two species of *Gammarus* introduced (*G. pulex* and *G. tigrinus*). In the 1950's, there was an introduction of *G. pulex* and associated elimination of the native species *Gammarus dubeni celticus* occurred in several river systems (Dick *et al.*, 1993). The speed of replacement was caused by predation of *G. pulex* on *G. d. celticus*. However in some rivers, *G. d. celticus* withstood an introduction of *G. pulex*, and even displaced *G. pulex* under certain circumstances (Dick *et al.*, 1993).

G. tigrinus was introduced to Ireland during the First World War (Costello, 1993). The native habitat of *G. tigrinus* is freshwater and brackish water environments in North America (Dick & Platvoet, 1996). Around the same time a second species was introduced, *Crangonyx pseudogracilis* from an unknown source and mechanism. Recent work (Dick, 1996) on the four Irish species (*G. d. celticus*, *G. pulex*, *G. tigrinus* and *C. pseudogracilis*) showed that the recently introduced species were in decline in relation to the more established species (*G. d. celticus* and *G. pulex*). Predation studies revealed a species hierarchy with *G. pulex* at the top and *C. pseudogracilis* at the bottom. The effect of *G. pulex* on other introduced species is marked; both *C.*

pseudogracilis and *G. tigrinus* have been displaced from several river systems. Such ecological displacement has occurred, even though both *G. tigrinus* and *C. pseudogracilis* have higher reproductive outputs.

The most detailed records of a set of introductions occurred in the streams in the south of Isle of Man between 1949 and 1955. Hynes' (Hynes, 1954; 1955; Dick *et al.*, 1997) introduced *G. pulex* from three source populations (n= 50-3000) to eleven selected streams in the south of the island. The native gammarid fauna, *G. d. celticus* was in most cases succeeded by *G. pulex*. However, in some cases, *G. d. celticus* was able to withstand the *G. pulex* introduction (and associated predation) and even co-exist without significant predation occurring between the two species. Such co-existence was also shown in introductions in Brittany, where again under some circumstances the native species survived the predation of introduced species (Stock, 1993).

1.3.3 Morphological variation and identification

Until recently the classification of Gammarid species has been based on morphological characteristics. In some cases, 17 characters have been used to separate species (Karaman and Pinkster, 1977a; Karaman and Pinkster, 1977b; Karaman and Pinkster, 1987). Meyran *et al* (1997) only used stable characters, such as shape and setation of the peduncle and flagellum of antenna 2, to reduce incorrect identification. The identification by these means is very time consuming and subjective, though several good keys have been produced (Karaman and Pinkster, 1977a; Karaman and Pinkster, 1977b; Karaman and Pinkster, 1987) along with several basic ones (Barnes, 1994, Hayward & Ryland, 1995 and Fitter & Manuel, 1986), which still provide sufficient morphological data to identify an individual down to species level.

Where species show morphological variation within a population, identification to the species level increases in complexity. (See *G. minus* example in section 1.3.3.1).

In some cases, the use of morphometrics alone are insufficient to identify a species from individual specimens. Hybridisation experiments allow the separation of species and populations, but are limited by numerous technical problems (Karaman and Pinkster, 1977a; Karaman and Pinkster, 1977b). One possible solution is the use of molecular techniques in conjunction with morphometric data, as employed in some more recent studies (Scheepmaker, 1990; Kane *et al.*, 1992; Meyran *et al.*, 1997; Müller, 1998)

1.3.3.1 Environment and morphology

Within a species there may be considerable morphological variability resulting from population separation and exposure to different environments (Gooch & Hetrick, 1979). *G. pulex* has limited morphological variation when compared to other European species, especially *G. fossarum* and *G. roeseli* (Scheepmaker, 1990; Müller, 1998). The most extensive study on morphological variation has been on *G. minus* between cave and spring populations (Gooch and Hetrick, 1979; Culver, 1987; Fong, 1989). *G. minus* has invaded a cave network and adapted to the dark environment, displaying greatly reduced eye size and a transparent body form. At the other extreme (spring populations), selection favours large eyes and extensive body pigmentation to avoid visual predators (Gooch and Hetrick, 1979). The two extremes show no more genetic differentiation than that found among populations of the same habitat.

1.3.4 Habitat preference

This section will concentrate on the three native freshwater species of *Gammarus* in England, with some reference to other species. *G.pulex* is normally found in lotic systems with a regular flow, especially in clear running streams, but also in subterranean systems. *G. lacustris* is able to tolerate the lower oxygen levels associated with lakes and is found rarely with other *Gammarus* species (Hynes, 1955). *G. dubeni* is a brackish water species that can also adapt to pure freshwater systems, where *G.*

pulex is absent (Fitter and Manuel, 1986). The ecological niches of the species have large overlaps, especially in the absence of competition.

As with all organisms, the distribution of *Gammarus* species depends on physio-chemical factors and biological interactions in the environment. In the case of *Gammarus*, anthropogenic interactions have had a marked effect on species distribution. *G. pulex* itself has been able to monopolise most freshwater systems by its formidable predatory nature excluding other gammarids. Similarly, a higher reproductive output (than the native *G.fossarum*) of *G. tigrinus* in Europe allows colonisation of both oligohaline systems and freshwater systems (Dick and Platvoet, 1996).

1.3.4.1 Habitat availability

The range of niches available to a *Gammarus* species will be constrained by environmental factors and biological factors, especially predation. Habitat preference is related to animal size, with smaller *G. pulex* (and juvenile life stages) inhabiting gravel regions, and larger (older) individuals more commonly associated with rocks and stones (Ward, 1988). Selection of habitat has also been linked to the availability of food matter (Kaushik & Hynes, 1971). Predator size and substrate size shows a negative relationship (Dahl & Greenberg, 1996). *G. pulex* is preyed on by leeches and trout, and shows preference for cobbles in areas of high trout density, compared to a preference for rocks and stones when leeches are dense. The habitat affects the efficiency of the predators; cobbles affording *G. pulex* protection from brown trout, but not leeches and visa versa. Parasitic loading of *Gammarus* species has a marked effect on habitat selection (see section 1.3.7).

1.3.5 Feeding and growth

The diets of *Gammarus* species are similar, especially within the native freshwater species; *G. dubeni*, *G. pulex* and *G. lacustris*. Food preference of *G. pulex*

was of animal and vegetable matter (Hynes, 1954). However dependency on decaying leaf matter has been shown in *G. pulex* to cause seasonal limitations in production, until the next leaf fall (Gee, 1988). *G. pulex* is able to reduce its respiration rate to compensate for reduced food quality and quantity, but this appears the only major difference between native species (Graca *et al*, 1993).

Both *G. fossarum* and *G. roeseli* show maximum growth rates when fed naturally decaying leaf matter and fine organic detritus (Pockl, 1995). This was significantly lower on green living and dead yellow macrophytes and lowest in algae (*Cladophora*), indicating that the food matter needs a degree of decomposition before effective consumption is possible. These results agree with the distribution patterns of these two species in central European running water systems (Foeckler & Schrimpff, 1985). The general trend in preference for decaying vegetable matter over animal material seems common to all *Gammarus* species. The position of *Gammarus* species in freshwater lotic food webs is similar, and a specialisation to production based on seasonal leaf matter is common to all, explaining the dietary preferences shown.

An extensively studied example of diet selection is in *Gammarus fasciatus* (Ohio river USA), which again shows a preference for algae and dead animals over coarse and fine particulate matter. This preference was related in the field to the seasonal abundance of different food sources utilised by *G. fasciatus* (Delong *et al.*, 1993).

There is evidence in *Gammarus* species of predation between species and cannibalism (Sexton, 1928; Hynes, 1955). Animal matter analysed from the stomachs of *Gammarus duebeni* was identified as other *Gammarus* (Hynes, 1954). This supports predation studies between two *Gammarus* species in N. Ireland, where one species is forced from the system (Dick *et al.*, 1993; Dick and Platvoet, 1996). Another explanation for the presence of *Gammarus* in the stomachs of *G. duebeni* is cannibalism

within a localised population. Cannibalism even between males and their newly moulted female mates has been observed in *G. dubeni*, a phenomenon termed "reversed sexual cannibalism" (Dick, 1995). The frequency of these cannibalistic actions increases when males are deprived of food. *G. dubeni* females may even eat their own developing broods (Sheader, 1983).

The relationship between predation and cannibalism has been well studied in two gammarids *G. pulex* and *G. dubeni celticus* (Dick *et al.*, 1993). Where the two species co-exist predation dominates over any cannibalistic effects.

1.3.6 Reproduction

1.3.6.1 Reproductive structures

Gammarids are marsupial brooders, and as such have no specific dispersal stage in their life cycle (Gooch and Hetrick, 1979). The gonads are paired and positioned above the midgut and below the heart and are brightly coloured by carotenoid pigments (Sutcliffe, 1992). The oviducts in the female can stretch only immediately after a moult when the oviduct walls are flexible to allow the passage of eggs into the marsupium. A mature female will develop secondary sexual characteristics, including long guarding setae on the oostegite fringe, to prevent loss of the brood from the marsupium.

The males have a standard gonad structure with seminal vesicles leading to the vas deferens, which connects to genital papillae, through which sperm are ejected during copulation. A more detailed review of the reproductive structures in *Gammarus* is given in Sutcliffe (1992) and LeRoux (1933).

1.3.6.2 Precopulatory pairing

Several days or hours before the act of copulation, males select a female and grab hold of her and guard her in this pair forming action. The male holds the female so

that his ventral surface is against her dorsal surface; and he remains attached by placing his dactlyi between her thoracic segments. The male is then able to swim with the female attached, and she often aids the swimming by beating her pleopods (Birkhead & Pringle, 1986). When the female is larger than the male there is a chance that she can separate from him by rapid flexing. Apparently the female is exercising a choice over her potential mate (Sutcliffe, 1992). Pairing (or guarding) is size assortative, though the males are always the larger of the sexes. Large males are selected for sexually as they are able to retain large females, that produce more eggs than small females (Naylor *et al.*, 1988).

Duration of precopula guarding depends on three main factors: body size, water temperature and the sex ratio of the population. In the laboratory, guarding times for *G. pulex* at 10^0C were 11-12 days for large males and was approximately double in small males (Ward, 1984). Laboratory experiments show that the duration of precopula guarding is 8.8 days for *G. pulex* and 8.8-10.7 days for *G. dubeni* at 10°C. At 18^0C the duration of precopula is 4.1 days *G. pulex* and 4.8-5.0 days *G. dubeni* (Sutcliffe, 1992). Populations that have a low ratio of males to females have longer precopula guarding phases as male competition is higher (Hynes, 1954).

1.3.6.3 Fertilisation

At the end of the precopula guarding phase the female moults and the oviducts become flexible, allowing the passage of eggs into the marsupium. The production of ecdysone is the stimulus from the female for the male to ready himself for sperm production. Once the female moult has been shed, the males reconnects with the female and moves her so they are facing. Fertilisation is external and spermatozoa are ejected out of the genital papillae. The male than uses his pleopods to thrust bundles of sperm close to the oviduct openings with rapid bursts of pumping actions (Sutcliffe, 1992). There can be several of these copulatary acts, and as many as seven have been recorded

during a single mating (Heinze, 1932). Postcopulation guarding is minimal; in *G. pulex* precopula guarding was recorded at 9 days and postcopula was only 9 minutes (Birkhead & Pringle, 1986).

Once the female is released she remains receptive to other males, which readily copulate in laboratory conditions (Birkhead and Pringle, 1986). First males were shown to fertilise 90% of eggs, whereas second males only fertilised 7% of eggs. However the short postcopula guarding phase and observations suggest that multiple mating is rare in wild populations. Nevertheless, for a small investment (15-30 minutes), second males may produce a few extra offspring. Sperm storage in *Gammarus* does not occur between broods as fertilisation is external and much of the marsupial structure (setae on oostegites) is lost during moults (Sexton, 1935).

1.3.6.4 Development of young

The outline of the stages of embryonic development and the time at which they occur in *G. pulex* is given in Table 1.1. Developmental time of young, in this case the embryonic developmental time in the marsupium is chiefly temperature dependent. At low temperatures developmental times are very long compared to high temperatures (see Table 1.2). These data are based on constant temperatures and natural diel variations act to reduce the developmental time (Sarviro, 1983). At low temperatures (near 0^0C), embryonic development in freshwater species may virtually cease (Sutcliffe, 1992). However, saltwater species show better adaptation to low temperature, with development occurring at lower temperatures due to an increased egg size.

Temperature may be the dominant factor determining developmental time, but other environmental factors such as salinity and dissolved oxygen (Kolding, 1985), as well as and egg size will also have an effect. Smaller eggs show faster development and more broods per female can be produced as a result (Sutcliffe, 1992).

Table 1.1: Stages of egg development in *G. pulex* at 11^{0}C (Sutcliffe, 1992).

Stage	Mean Age (days)	Mean width (mm)	Length (mm)	Major characteristics
1	1-3	0.47	0.62	Newly fertilised, oval, black, undifferentiated egg with two membranes- the outer chorion and an inner embryonic layer enclosing the yolk
2	2-7	0.50	1.63	Cleavage cells divide to form large non- nucleated pigment yolk cells; blastoderm forms
3	6-13	0.52	0.66	Thorax and abdomen separated by a groove; dorsal organ present
4	9-15	0.56	0.68	Comma- like shape, orange-red thorax; midgut and appendages developing
5	16-19	0.55	0.70	Dorsal organ has regressed, eyespot pigmented; fully developed appendages but immobile
6	17-22	0.62	0.75	Fully formed animal with compound eye; movements of limbs, heart and midgut (peristalsis)
7 (hatch)	20-23	-	-	Embryo hatches, emerging in ca. 1 min, telson first, by vigorous body movements; length 1.6-1.8mm, with 5 antennal segments

Table 1.2: Mean embryonic developmental times of some saltwater and freshwater species of *Gammarus* (Sutcliffe, 1992).

		Embryonic development time (days)			
Species	**n**	**5^{0}C**	**10^{0}C**	**15^{0}C**	**20^{0}C**
G. aequicauda					
G. insensibilis	8	47-68	25-30	17-19	11-15
G. pseudolimnaeus	11	59-70	28-30	17-19	12-13
G. dubeni	5	65-83	31-35	18-23	13-17
G. pulex	12	54-80	32-38	22-26	16-20
G. fossarum	9	56-95	30-41	1-27	13-21
G. roeseli	6	116-176	40-51	20-26	12-17

The time to reach maturity in *Gammarus* has been well documented, especially in *G. pulex*; Table 1.3 shows some of the data. Instar numbers have been recorded for *G. pulex* and *G. dubeni* and are 10 and 13 respectively (Hynes, 1954; Hynes, 1955). The time to maturity is again temperature-dependent and will be affected directly by the nature of the species breeding cycle.

Table 1.3 Maturity time of *G. pulex*.

Time to maturity	Temperature	Author
120 days	15-20^{0}C	(Hynes, 1955)
100 days	unknown	(Mottram, 1934)
70 days	summer	(Sexton, 1924)
180 days	winter	(Sexton, 1924)

1.3.6.5 Breeding cycle

Gammarus have a life span of approximately 1 year depending on the time of year at which the individual was born. *G. pulex* young are born from March to September. Those born in March will reach maturity in July, and start to breed in August and September (and are able to overwinter as adults). The breeding will cease in November and for the rest of the winter and continue again in spring, with old adults dying in April or May. Later born individuals do not attain sexual maturity before winter and over winter as juveniles. They become mature in March and breed from April until their death in late June (Hynes, 1955).

G. lacustris has a strictly annual cycle with the first young hatching in April and becoming mature in February or March. Breeding occurs through May until their death at the end of June. Adult females do not overwinter, but die after breeding, unlike *G. pulex* and *G. dubeni*.

The breeding cycle of *G. dubeni* is similar to that of *G. pulex* with young hatching from March to May of the year. There is a cessation of breeding over winter and this is more marked in freshwater populations than in brackish ones (Hynes, 1954). The sex ratio of young produced during the breeding season alters with more males

being produced early rather than the late (environmental sex determination (see section 1.3.6.7)).

1.3.6.6 Diapause

Towards the end of the life of a female she will fail to join a male in precopula, ready for copulation at the next moult. Young being carried by the female are released during the copulatory moult, as normal. However no eggs are produced, and secondary sexual characters (setae on the fringe of the oostegites) are lost (Sutcliffe, 1992). The female has reached diapause, similar to the over wintering state, but in this case there is no reverse back to a reproductive state by environmental conditions. Males can also enter diapause, again there is loss of secondary sexual characters and no gamete production, though this appears limited to *G. pulex* and *G. tigrinus* (Hynes, 1955).

The onset of diapause was initially thought to be temperature induced (Hynes, 1955), but more recent work suggests that photoperiod is the control mechanism (Sutcliffe, 1992). Such environmental control is in line with the production of moulting hormones that are secreted from neurosecretory centres that are sensitive to photoperiod. Artificially induced diapause in *Gammarus lawrecianus* was possible by reduced photoperiod; conversely diapause was terminated by a longer photoperiod (Steele & Steele, 1986).

1.3.6.7 Environmental sex determination

In *G. dubeni*, *G. pulex* and *G. zaddachi*, sex is determined by a balanced polyfactorial system of allelic sex genes on several pairs of chromosomes (Bulnheim, 1972). *G. dubeni* and to a lesser extent *G. zaddachi* show varying sex ratios in offspring, dependent on seasonal photoperiod (Sutcliffe, 1992). Young produced early in the breeding season have the potential to grow larger (more time available, less competition between young). Sexual selection favours increased size in both sexes,

females to produce more eggs, and males to attain larger and more mates. The forces of sexual selection appear stronger in males, as they are more numerous early in the breeding season. The late breeding season is female dominated (McCabe & Dunn, 1997). Why the female selection is weaker is unknown, but females stop growing once they reach maturation unlike the males.

In a few cases, *G. dubeni* females will produce only daughters, who in turn only produce daughters. This form of inheritance is cytoplasmic and occurs by the transmission of microsporidia parasite via egg cytoplasm (McCabe & Dunn, 1997). The microsporidia cause a sex change in the genetic males, increasing the sex bias towards females in the brood. In stressful conditions (low oxygen and high salinity) the actions of the microsporidia are much reduced (Bulnheim, 1975). Cytoplasmic inheritance is specific to *G. dubeni* and has not been found in other gammarids.

1.3.7 Dispersal

The main dispersal mechanisms of all of the freshwater gammarids are passive downstream movement due to water currents and active upstream swimming or walking (Litterick. 1973). Terrestrial habitats provide a barrier to virtually all movements between drainages, though *G. fossarum* individuals have been observed to walk short distances out of water (Müller *pers comm*). Unlike the freshwater insects, gammarids have no distinct dispersal life history stage and rely on swimming and walking for upstream migrations. The ability of the gammarids to disperse through the water has been observed in *Dikerogammarus spp.* where an invasion from the Main-Danube canal (opened in 1992) resulted in the colonisation of water systems across Germany and into Belgium and France. Within populations of *G. pulex* upstream movements can still be extensive, with migration distances of up to 14m per day recorded in the UK (Litterick, 1973).

1.3.8 Parasitism

Gammarids are the final hosts for some parasites, but more commonly act as an intermediate host of parasites. There are at least five parasites that utilise gammarids as intermediate hosts (*Microphallus papillorobustus*, *Pomphorynchus laevis*, *Polymorphus paradoxus*, *and P. marilis* and *Corynosoma constrictum*) to aid their passage to a definitive host (normally a predator of the gammarids) (Hindsbo, 1972; Bethel & Holmes, 1973; Thomas *et al.*, 1996). The mode of infection is normally by consumption of faecal material from the definitive host and when the predator consumes the gammarid the cycle is complete.

1.3.9 Thesis objectives

Although much ecological work has been conducted on *G. pulex*, as yet there is a paucity of molecular genetic studies on this species, especially in the UK. The main objectives of this thesis are based on a central theme to examine the population structuring of *G. pulex* within the UK and on a wider geographic scale across Europe. Populations were chosen and sampled based on their known population history and ecological data, with the specific aim of separating contemporary and historical factors.

The first objective of the thesis was to investigate what the effect of a transplantation experiment, to the Isle of Man, had on the genetic diversity of the introduced population. Data from the source and introduced populations would provide an insight into the contemporary forces that determine population genetic structure within populations, without the compounding historical effects.

The second objective was to determine the pattern of population structuring in UK *G. pulex* populations and investigate the potential historical/ contemporary basis for the observed patterns. The diversity within the UK has been suggested as a consequence of a post glacial colonisation (Hynes, 1954) when a land-bridge connected

the UK to mainland Europe. Using molecular markers, the source of any such colonisation from European rivers could be identified and used to interpret the levels of diversity recorded in the UK.

The third objective was to investigate the effect of the historical factors on population structure using a phylogenetic approach. Here a wider geographic range was proposed (north west Europe) to cover important river systems that have changed direction of flow or connections with other rivers in the past.

Chapter 2

Sampling site locations and methods

2.1 Introduction and rationale

G. pulex has been used to answer a wide range of ecological questions, from the effects of pollution (Blockwell *et al*, 1998; Borlakoglu & Kickuth, 1990) to seasonal variation in density and production (Adams *et al*, 1987; Iversen *et al*, 1977; Welton, 1979). These studies have identified specific populations and provided fundamental ecological information that have in part formed the basis for the choice of populations studied in this thesis. In this chapter a summary of pertinent factors of the 43 *G. pulex* populations studied in this thesis are given.

The need for an ecological basis from which populations are selected for genetic and morphological analysis was clearly identified by a phylogeographic investigation by Avise *et al.* (1979). Genetic differentiation between populations was significant, but required knowledge of the population's ecology and history for a plausible conclusion to be drawn.

The populations selected for molecular and morphological analysis were based on known population history and/or previous molecular (allozyme) variation. Sampling was conducted at three scales: from three selected populations in relation to an introduction to the Isle of Man; 24 populations within the UK; and 19 populations within North-West Europe. Sampling was undertaken at these three geographical scales to address the three key objectives of this research. The first objective was to test for the loss of genetic and morphological variation in an introduced population and to determine if population differentiation had developed between the source and

introduced populations. To test this objective three populations were selected: the source of the introduction; the introduced population; and an unrelated population for comparison. At this relatively small scale, morphological and molecular markers (allozymes, mtDNA sequencing & microsatellites) were employed.

The second objective was to determine the level of molecular variation of populations within the UK and the extent of population differentiation. A series of populations within the UK, across as many drainages as possible, were sampled to examine population structure using molecular markers (allozymes, microsatellites and mtDNA).

Finally, samples from North West Europe were collected, in addition to those from the UK, to investigate the phylogeographic structure and colonization routes of *G. pulex*, using just mtDNA sequence variation.

2.2 Sampling methods

The method of animal collection depended on the habitat sampled. Lotic environments were sampled using a standard "kick-net" sampling method; sediment and animals upstream were disturbed, and any displaced animals were caught downstream in nets. Lenthic environments were intensively sampled by turning over rocks and stones and sweeping the substrate for animals with fine mesh hand nets.

Estimates of abundance were collected at most sites (Table 2.1) by calculating the number of animals collected in a given area of substrate. These estimates were then standardised to the number of animals in a square metre of substrate, for direct comparisons between sites and to published data. Where possible, sampling was repeated to detect any seasonal changes in abundance.

Seasonal cessation of copulation has been recorded in many populations of *G. pulex* (Hynes, 1955; Iversen & Jessen, 1977), though larger, more stable populations

appear to breed throughout the year (Welton, 1979). The first three populations were sampled at regular intervals, and the presence or absence of pre-copula pairs was recorded each time. The absence of pre-copula pairs was used as an indicator of a non-breeding resting period.

Water samples were collected from the Lowthorpe, River Terrig and Fleshwick Bay sites and analysed for conductivity, pH, copper, nitrite, nitrate and free chlorine. Conductivity and pH were measured using standard conductivity and pH metres. Copper, nitrite, nitrate and free chlorine were measured using portable Palintest kits (Palintest Ltd. England).

All samples, once collected, were transported either live (for breeding) or frozen in liquid nitrogen. Samples from each population for molecular analysis were kept frozen at -80°C for allozyme electrophoresis, and all other samples were kept in an ethanol-based preservative.

Samples required for both morphological and PCR-based molecular analysis were preserved in an ethanol-based substance that allowed DNA extraction as well as the maintenance of the morphological integrity of the animals. However, pure ethanol preserved the DNA but caused the animals to shrink and become brittle. Conversely, formalin (formaldehyde based) maintained the morphological integrity of the animals but caused extensive DNA degradation. After several trials, the optimum preservative for the dual-purpose samples was 70% ethanol solution with 10% sucrose (by weight).

2.3 The sample sites

Overall, the 43 populations sampled came from six different countries in north Western Europe (Belgium, France, Germany, Switzerland, Holland & UK). Figures 2.1 and 2.2 show the location of the populations sampled, and indicate the geographic range covered by the sampling scheme. The following section details some of the biological,

historical and physical conditions at the 43 sites, though where colleagues obtained samples site descriptions were not always provided. Table 2.1 summarises all the sample site locations and general information recorded while sampling.

2.4 Sampling within mainland Europe

There were eight main clusters of sampling in Europe covering 19 populations. The samples were collected mainly from the river systems that drain into the North Sea and the English Channel, with a view to identify a potential colonization route of the UK. The numbers in parenthesis below given after each geographic area indicate the sample site identifier presented in Figures 2.1 and 2.2.

2.4.1 Holland (24)

There was only one population sampled in Holland (Hierden), located on a tributary of the Rhine system. The location had been used previously by Scheepmaker (1990) and Scheepmaker and van Dalfsen (1989) for allozyme electrophoresis. Scheepmaker (1990) identified and reported that the population sampled contained only *G. pulex*.

2.4.2 Belgium and northern France (28-32)

In this cluster, five populations were sampled during June 2000 on a trip to sample the remaining river basins that drained in to the English Channel. Two sites were sampled from Belgium, (sites 28 & 29), both of which were on tributaries of the river Schelde. In France three sites were sampled, one on a tributary of the river Schelde (site 32) and the remaining two from a tributary of the river Seine (30 &31).

2.4.3 Central France (37)

A single population was sampled from a tributary of the Seine during 1995. The animals provided were collected and identified as *G. pulex* by Scheepmaker.

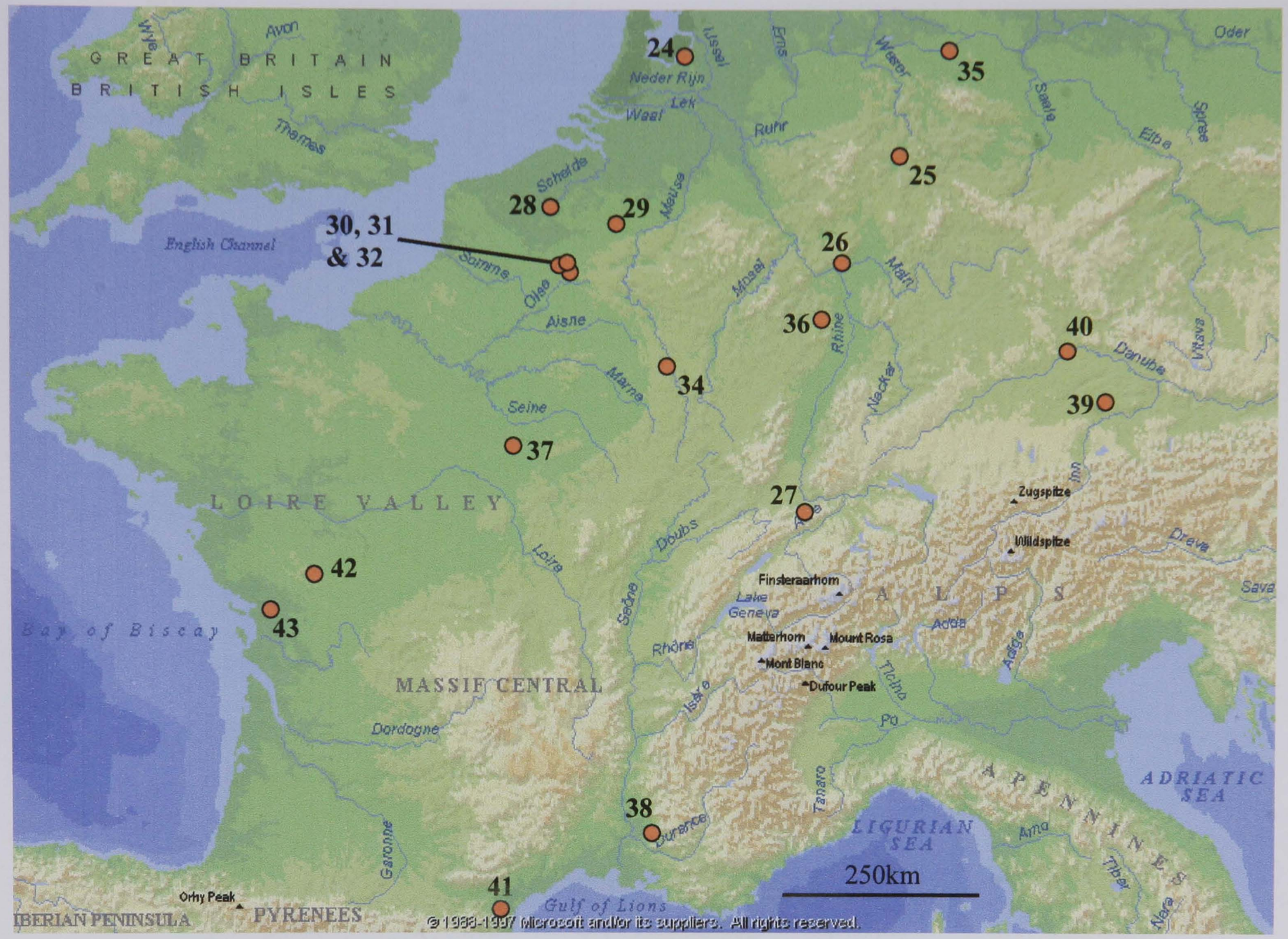


Figure 2.1: Sample site locations in mainland Europe, samples 1-23 are detailed in figure2.2

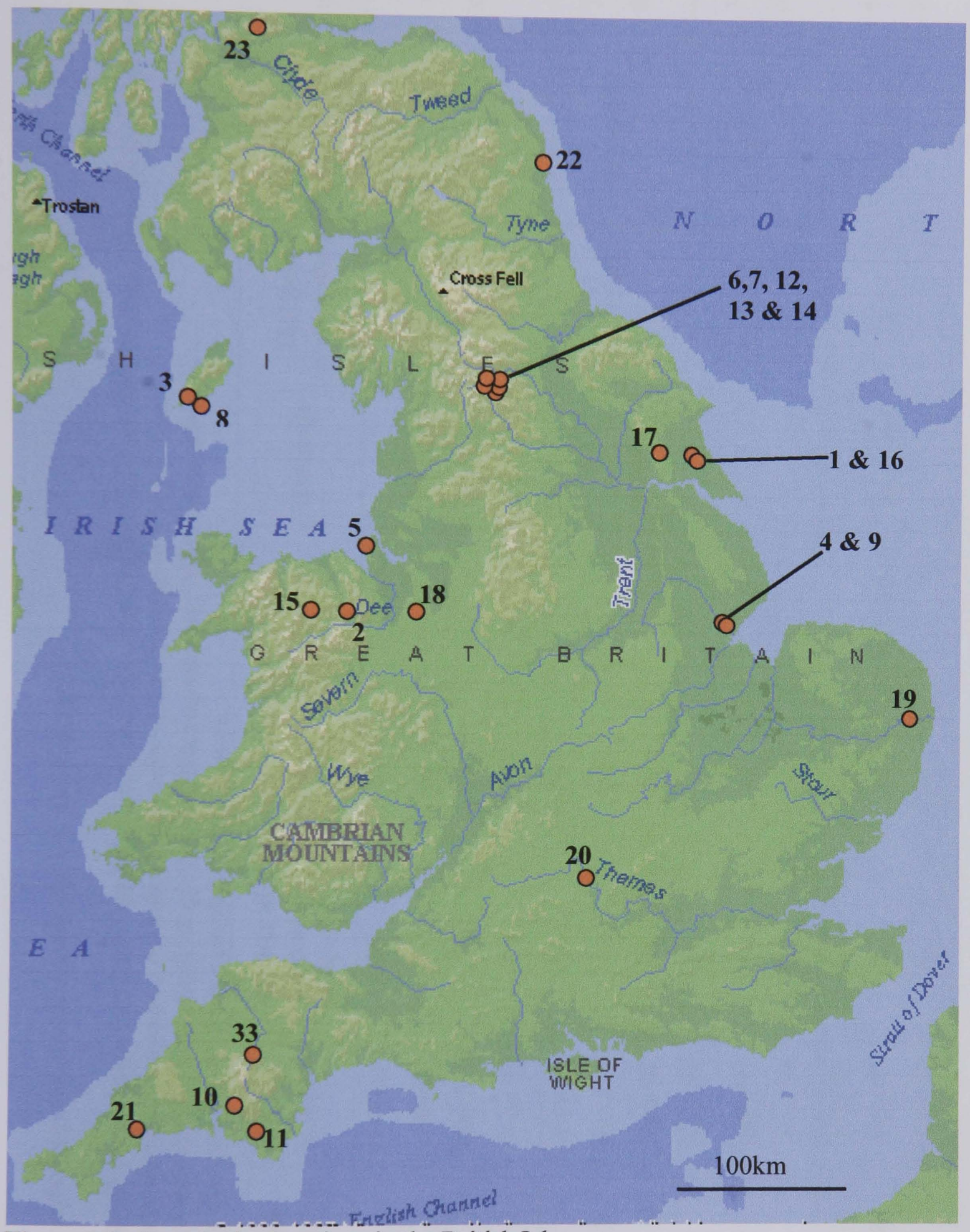


Figure 2.2: Sample site locations in the British Isles

Table 2.1: *Gammarus pulex* populations sampled in the UK and Europe.

River/drainage: IS= direct drainage into Irish Sea; EC= direct drainage into English Channel; MED= direct drainage into Mediterranean Sea; AO= direct drainage into Atlantic Ocean; NS= direct drainage into North Sea.
Marker system: A= Allozymes; M= morphology; S= mtDNA sequencing; MS= microsatellites
Sampling: π= denotes populations sampled by colleagues.

Population	Site number	Date sampled	Number of animals collected	Estimate of abundance (no.m^{-2})	River	Drainage	Markers system used	OS Landranger grid reference (UK only)	Position (degree minutes and seconds)
Lowthorpe	1	March 98 Jan 99 March 99 June 99	200 400 370 500	800 750 1000 3000	Hull	Humber estuary	A/M/S/MS	TA:084605	54 01 35 N 00 20 12 W
River Terrig	2	May 98 March 99 Dec 99	250 240 40	500 200 30	Dee	Dee estuary	A/M/S/MS	SJ:238515	53 05 12 N 03 08 30 W
Fleshwick Bay	3	April 98 May 99 Nov 99	500 300 200	500 900 100	Fleshwick beck	IS	A/M/S/MS	SC:207713	54 06 38 N 04 44 38 W
Stainfield Hall	4	May 98 July 99 π Nov 00	60 300 95	30 400 NA	Witham	The Wash	A/S	TF:135725	53 15 04 N 00 20 20 W

Greasby Brook	5	March 98	150	50	The Birket	Mersey	A/S	SJ:247874	53 21 50 N 03 07 43 W
Malham Tarn	6	April 98 Nov 99	32 30	0.5 1	Dibb	Derwent	A/S	SD:892674	54 06 00 N 02 10 19 W
Malham Village	7	April 98 Nov 99	55 45	20 15	Dibb	Derwent	A/S	SD:892627	54 03 22 N 02 10 11 W
River Crogga	8	April 98 May 99	100 150	80 150	Crogga	IS	A/S	SC:245679	54 05 18 N 04 40 47 W
Stainfield Village	9	May 98 July 99	400 100	600 250	Witham	The Wash	A	TF:116737	53 15 04 N 00 20 20 W
Horrabridge	10	Sept 98	80	50	Tavy	EC	A/S	SX:513694	50 30 21 N 04 05 54 W
Ivybridge	11	Sept 98	35	75	Erme	EC	A/S	SX:649562	50 23 27 N 03 54 04 W
Cowside Beck	12	Nov 99	35	4	Skirfare	Derwent	S	SD:889693	54 06 23 N 02 10 03 W
Malham Spring	13	Nov 99	16	2	Dibb	Derwent	S	SD:885671	54 06 00 N 02 10 33 W

Malham Outflow	14	Nov 99	34	11	Dibb	Derwent	S	SD:889664	54 05 39 N 02 10 14 W
River Alwen	15	Jan 00	50	8	Alwen	Dee Estuary	S	SH:966516	53 03 08 N 03 32 38 W
Driffield	16	Feb 98	100	150	Hull	Humber Estuary	S	TA:014575	54 00 12 N 00 26 07 W
Pocklington	17	March 99	42	10	Pockington canal	Derwent	S	SE:804473	53 54 58 N 00 46 35 W
Pickmere	18	Jan 00	35	7	Cheshire ring canal	Dee Estuary	S	SJ:685768	53 17 19 N 02 27 36 W
Norwich	19	π March 00	40	NA	Yare	NS	S	TG:237062	50 03 46 N 08 20 49 E
Oxford	20	π March 00	60	NA	Cherwell	Thames	S	SU:404904	51 36 41 N 01 25 04 W
Truro	21	π May 00	30	NA	EC	EC	S	SW:822441	50 15 26 N 05 03 24 W
Dunstanbrugh	22	π April 00	65	NA	Embleton Burn	NS	S	NU:238222	55 29 35 N 01 37 28 W

Lennoxtown	23	April 00	22	11	Endrick water	Forth & Clyde	S	NS:612791	55 59 08 N 04 13 34 W
Hierden, Holland	24	June 00	70	25	Hierdense beek	NS	S	Not available	52 21 51 N 05 43 36 E
Hopfgarten, Germany	25	π 1995	20	NA	Saale	Elbe	S	Not available	50 59 27 N 11 13 12 E
Mainz, Germany	26	π 1995	25	NA	Main	Main	S	Not available	50 03 46 N 08 20 49 E
Kleinlützel, Switzerland	27	π 1995	8	NA	Rhine	Rhine	S	Not available	47 25 47 N 07 24 51 E
Schendelbeke, Belgium	28	June 00	33	33	Schelde	Schelde	S	Not available	50 47 55 N 03 54 04 E
Corroy-le-Château, France	29	June 00	1	NA	Escaut	Schelde	S	Not available	50 32 09 N 04 39 56 E
Berlaimont, France	30	June 00	60	30	Sambre	Seine	S	Not available	50 12 07 N 03 48 49 E

Foret de Mormal, France	31	June 00	80	80	Sambre	Seine	S	Not available	50 12 22 N 03 42 03 E
St Georges, France	32	June 00	55	10	Sambre	Seine	S	Not available	50 12 39 N 03 33 44 E
Dartmoor	33	July 01	50	NA	Walden	Bristol Channel	S	SX: 585955	50 46 09 N 04 00 41 W
Sommedieue, France	34	π 1995	2	NA	Meuse	Meuse	S	Not available	49 05 06 N 05 27 48 E
Braunschweig, Germany	35	π August 00	20	NA	Oker	Wesser	S	Not available	52 16 30 N 10 30 29 E
Ramstein, Germany	36	π 2000	5	NA	Nahe	Rhine	S	Not available	49 27 25 N 07 33 16 E
St. Hilaire-sur-Puiseaux, France	37	π 1990	3	NA	Yonne	Seine	S	Not available	47 54 33 N 02 42 12 E
Fontain de Vauclus, France	38	π 1990	1	NA	Durance	Rhone	S	Not available	43 55 09 N 05 08 50 E

Isar, Germany	39	π April 01	6	NA	Isar	Danube	S	Not available	48 32 38N 12 08 55 E
Marching, Germany	40	π April 01	10	NA	Danube	Danube	S	Not available	48 49 00 N 11 43 02 E
Nefiach, France	41	π 1990	3	NA	Tet	MED	S	Not available	42 42 05 N 02 40 18 E
Ouzilly, France	42	π 2000	5	NA	Loire	Loire	S	Not available	46 47 25 N 00 22 56 E
Mortagne-sur-serve, France	43	π 2000	8	NA	Maine	AO	S	Not available	46 59 03 N 00 57 44 E

2.4.4 Western France (42, 43)

Two populations were sampled during the summer of 1999from the Loire and Vendee river captures by Catherine Soutty-Grosset (Unversite Poitiers). Animals were identified in the laboratory in Hull based on the morphological key by Karaman and Pinkster (1977a).

2.4.5 Southern France (38,41)

Two populations from the south of France were collected in 1990 and identified by Scheepmaker (1990). Samples from the Nefiach (41) population were identified as *G. p. gallicus* and samples from Fontain de Vauclus were identified as *G. p. pulex*.

2.4.6 Switzerland (27)

A single population was sampled on the upper Rhine in 1996 by Jakob Müller. Animals from this population had previously been identified by Müller (1998) to the species level as *G. pulex*.

2.4.7 Eastern Germany (39,40)

Two samples from the lower river Danube system were provided by Jakob Müller in 2000. The samples were identified as *G. pulex*, in the laboratory at Hull based on the morphological key by Karaman and Pinkster (1977a).

2.4.8 Western Germany (25,26,34-36)

Five populations were sampled from the Elbe, Rhine, Meuse and Weser river systems, all samples were provided by Jakob Müller (university of Mainz) and identified in the laboratory in Hull. Samples collected from the Elbe (25) and from a tributary of the Rhine (site 26) were both previously identified as *G. pulex* and used in an allozyme study (Siegismund & Müller, 1991; Müller, 1998).

2.5 Sampling within the UK

There were eight main clusters of populations sampled in the UK covering 24 populations. The aim of this sampling strategy was to cover as much of the present distribution of *G. pulex* in the British Isles as was reasonably possible. In addition to the three sample sites repeatedly sampled, a further 21 were sampled at least once (Table 2.1). The sample sites covered a geographic range of 800 kilometres and included both lotic and lenthic habitats.

2.5.1 Northern England and Scotland (22,23)

Two populations were sampled towards the northern and eastern range of *G. pulex*. samples were both collected from lotic systems.

2.5.2 Malham, North Yorkshire (6,7,12-14)

Five populations were sampled from in, or around, Malham Tarn. The Malham area is situated at the end of a complex of glacial valleys formed at the end of the last glacial period ~ 10,000 years ago (Fryer, 2000). Figure 2.5 illustrates the scale of the area investigated and the four sites sampled. The tarn is the highest part of the area situated on top of a limestone outcrop formed from glacial moraine deposits, and represents one of the few places in which both *G. pulex* and *G. lacustris* co-exist (Fryer, 1991a), the latter being associated with lenthic rather than lotic systems (Karaman & Pinkster, 1977). Water enters the tarn from a series of springs around its edge and water leaves via a weir at the south. After this, the water sinks into the porous limestone and re-emerges 2 km downstream at Aire Head. Malham cove forms the entrance to a cave network below the tarn, but the brook emerging from the caves is unconnected to the tarn as shown with dye tracing (Yorkshire Dales website). The brook actually originates from another water sink on Malham moor.

The five sites were chosen to cover a range of habitats in and around the tarn and identify any migration (gene flow) between them. The four sites sampled differ markedly in the populations of *G. pulex*. At one extreme the populations in the spring and tarn have very low densities, ~0.5 and ~1.0 per square metre, respectively (March sampling). This contrasts to densities of ~20-50 at the village and outflow sites.

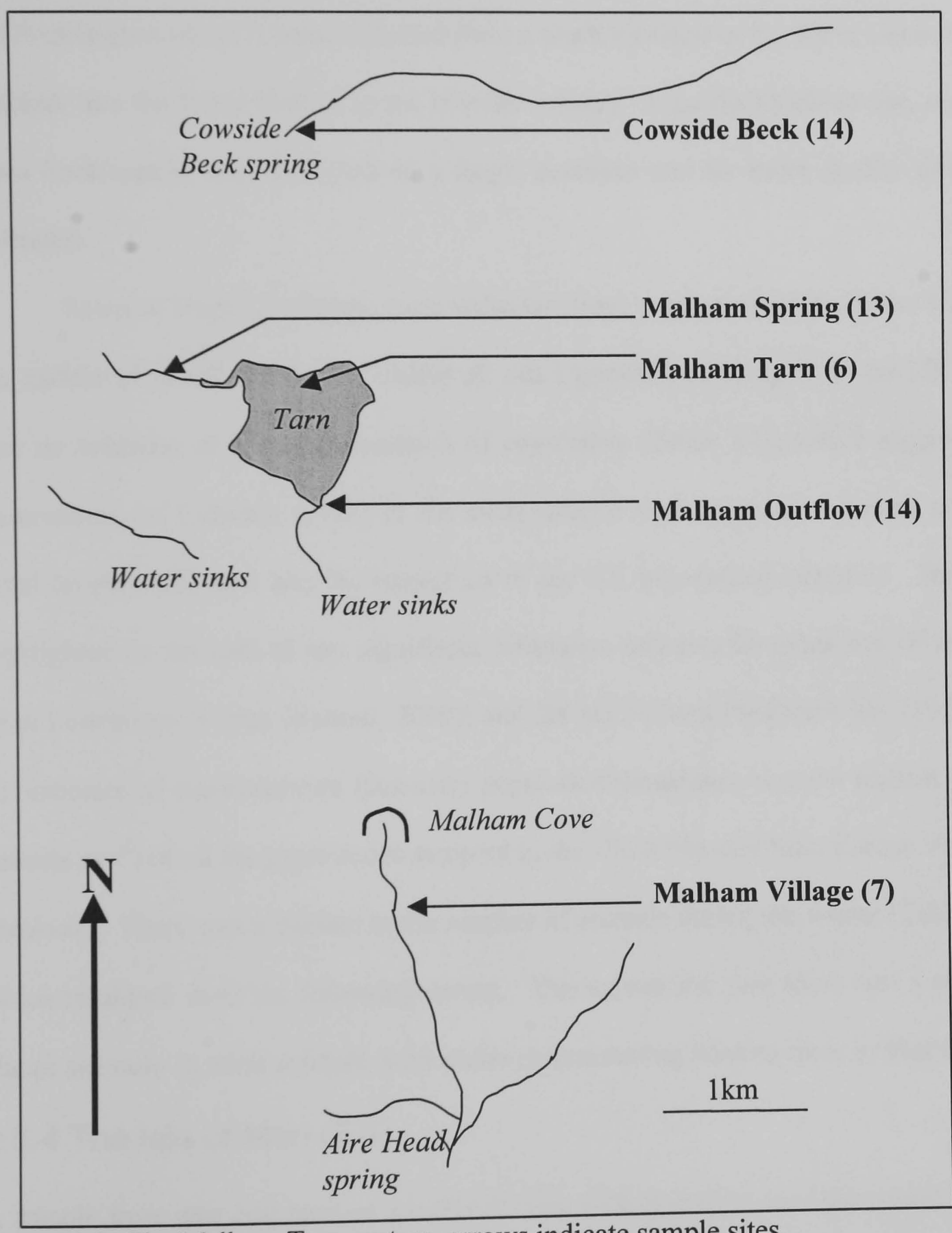


Figure 2.3: The Malham Tarn system, arrows indicate sample sites.

2.5.3 Humberside (1,16,17)

Three populations were sampled near to Hull including Lowthorpe which was sampled several times and for which water quality data were collected. Sample site at Lowthorpe and Driffield were located on the same tributary of the River Hull, and consequently only a single sampling trip was paid to the Driffield site. Samples from the Pocklington site (17) were collected from a small tributary of the River Derwent that drained, like the River Hull in to the Humber estuary. Like the Driffield site, samples from Pocklington were collected on a single occasion and no water quality data was collected.

Samples from Lowthorpe were collected from a tributary of the River Hull, in the middle of arable farmland. Unlike all other populations sampled in the UK there was no evidence of a winter cessation of copulation (Table 2.1), which supports the observations of Litterick (1973) at the same sample site. The mean size of animals (total length) collected was the largest all of the UK populations sampled. This was highlighted by the lack of any significant difference between the mean size of females from Lowthorpe (11mm (Farmer, 2000)) and the males from Fleshwick bay (10.5mm). An estimate of the maximum (summer) population abundance was the highest (3,000 animals m^{-2}) of all the populations sampled in the UK (estimates from Europe were not obtained). There was a decline in the number of animals during the winter (Table 2.1), which remained until the following spring. Throughout the year there was a strongly biased sex ratio in adult animals, with males outnumbering females three or four to one.

2.5.4 The Isle of Man (3,8)

A sample from east and west of the island were collected from two unconnected river systems that drained in to the Irish Sea. The first site, Fleshwick Bay, was located on the west of the island. Samples were taken a small isolated brook that drains surrounding grassland and moor land directly into the Irish Sea. In 1950 Noel Hynes

introduced 75 *G. pulex* to this site from the River Terrig (Dick *et al*, 1997). Prior to this time there were no records of any *Gammarus* at the site. During the two years following the introduction no *G. pulex* were found, but individuals were recorded by 1974. Estimates of abundance showed seasonal variation, between 900 m^{-2} during the summer "peak" and 100 m^{-2} in the winter (Table 2.1). In the winter the population showed evidence of a cessation in breeding (November 1999) coinciding with the low abundance. Unusually there was no wooded area near the sample site and instead of feeding on decaying leaf matter, animals were found grazing on dense mats of water moss (*Fontinalis sp*).

Like Fleshwick Bay, the presence of *G. pulex* in the river Crogga is thought to be artificial, in this case because of trout fish stocking in 1908 (Hynes *pers comm.*). The current population has been described as recent as there has been no evidence to show animals moving upstream of a twelfth century waterfall (Hynes, 1954). Additionally to being a source of introductions, animals were introduced to headwater regions of the river (Dick *et al*, 1997). The site on the Crogga is in grazing farmland.

2.5.5 North Wales and Liverpool (2,5,15,18)

Four populations were sampled in this area, including the River Terrig and Greasby brook, populations that were also used by Hynes (1954) for transplantation experiments. In addition to the populations used by Hynes (1954), samples were also collected from the River Alwen that drains into the Dee estuary, and the more isolated lenthic Pickmere in Cheshire.

Greasby Brook is situated on the Wirral across the river Mersey from Liverpool. The site was a shallow drainage ditch between grazing farmland, with visible signs of pollution (waste plastics etc.). The most striking feature was the spatial segregation of adult and juvenile animals, with the former inhabiting the sides of the ditch and the latter in the centre of the ditch under pebbles. Such a pattern has previously been

observed (Dahl & Greenberg, 1996) and is though to relate to prey size and predator avoidance.

The River Terrig population was sampled across a small area ($10m^2$) surrounded by grazing farmland near the village of Rhytalog. This population was the choice of Noel Hynes as a source for a series of introduction experiments to the Isle of Man during the 1950's, including the introduction to Fleshwick Bay (Dick *et al*, 1997; Hynes *pers comm*). Strong seasonal variation in both animal density and reproduction was evident (Table 2.1), with a reduced estimated abundance and cessation of copulation during the winter (December 1999). These fluctuations in seasonal density were concordant with the data from other rivers in the UK (Gee, 1988; Welton, 1979). However the seasonal variation was greater in this (smaller) population than either Lowthorpe or Fleshwick Bay.

2.5.6 Dartmoor and Cornwall (10,11,21,33)

Four populations were collected from this area; including three samples from around the Dartmoor National Park. The aim of collecting a high density of samples from this region was to look for genetic evidence of putative glacial refugia in southern England (Stewart & Lister, 2001), and the associated high genetic diversity.

The Dartmoor region in Devon and Cornwall is a large expanse of moorland the effectively creates a barrier to the movement of many species. It was also one of the few locations with populations of *G. pulex* and *G. duebeni*, the Isle of Man being another. Three sites were sampled from the area; first, Horrabridge (site 10) was a small flood plain adjacent to the main river that seasonally dries up; the second site Ivybridge (site 11) was 20km away in the middle of grazing farmland; the final site (33) was located on the northern fringe of the moor and was isolated from the other moor samples. The three sites contrasted in the substrate type; Horrabridge and Dartmoor

were granite, draining directly off the moor, and Ivybridge was surface limestone, draining lowland areas.

2.5.7 Lincolnshire and East Anglia (4,9,19)

Three samples were collected from the east of the UK, including two in Lincolnshire on the river Witham system separated by only 400m. The two sample sites were selected around this village to detect any possible fine scale effects over a small geographic distance. Stainfield Hall is located 400 metres upstream of Stainfield village on a tributary of the River Witham. The location was also chosen to include samples from the Wash estuary, a possible colonisation route for gammarids at the end of the last glacial cycle (Hynes, 1954). A sample from the River Yare in Norwich was collected by a colleague.

2.5.8 Oxford (20)

A single population was sampled in Oxford on the river Thames system no water quality data or abundance estimates were collected as samples were collected by colleagues.

2.6.Water quality at Lowthorpe (1), River Terrig (2) and Fleshwick Bay (3).

In the three most intensively studied populations, additional data on water quality was collected to highlight any differences between the sites. *G. pulex* has a restricted distribution in acidic waters and only occurs at pH 5.7 or higher (Sutcliffe & Carrick, 1973). In acidic waters the high levels of H^+ ions induce failure of osmoregulation and mortality (Hargeby & Patersen, 1988). Peeters and Gardiniers (1998) modeled the optimum habitat for *G. pulex* and found an ideal pH of 7.07- 9.16. The predicted optimum corresponds well with the present data set and no value lies outside the optimum (Table 2.2).

The substrate of a river system will affect the conductivity of the river itself. Soft substrate, such as limestone, will erode quickly releasing ions into the surrounding water and increasing the conductivity of the water. In areas of hard substrate (granite) erosion will be slower, with a corresponding reduced conductivity. Calcium is one of the major ions released from eroding substrates and plays a key role in the moult cycle of all gammarids (Meyran, 1994). Unlike many gammarids, *Gammarus* cannot store calcium in their bodies prior to moulting (Wright, 1980) and rely on the calcium in the water. Elevated levels of calcium can reduce the duration of the moult cycle and increase the size of animals (Meyran, 1994).

The conductivity of the water at Lowthorpe was almost twice that recorded at the other two sites (Table 2.2). The elevated conductivity and presence of large individuals at Lowthorpe suggest high levels of calcium in the water. The substrate type at the site (limestone) supports the finding, unlike River Terrig (granite) and Fleshwick Bay (sandstone). Data from Lowthorpe agree with the expectations of Meyran (1994) of increased body size with elevated calcium levels.

Table 2.2: Water conditions recorded at sample sites 1-3

Sample site	Date sampled	Temperature °C	pH	Conductivity µS
Lowthorpe (1)	13/01/99	8.0	7.8	565
Lowthorpe (1)	19/03/99	9.0	7.5	580
River Terrig (2)	17/03/99	10.2	7.6	330
Fleshwick (3)	05/05/99	10.5	7.1	370

At all sites, the lowest detectable value of organic pollution and metals was recorded (Table 2.3). The nitrate level detected in Lowthorpe was higher than the other two samples by an order of magnitude.

The substantial nitrate in the water in Lowthorpe was probably due to the surrounding land use in the area. Crops grown in the area are treated with nitrate based

fertilisers, which would run off in to the rivers. This contrasts with the grazing land around the River Terrig and grassland around Fleshwick Bay, neither of which have been treated with fertilisers. The increased nitrate levels would support a highly productive eutrophic system. This elevated productivity would increase the amount of flora in the system, which would be one of the key food resources for *G. pulex*.

Table 2.3: Water chemistry data recorded at sample sites 1-3

Sample site	Copper mgl^{-1}	Nitrite mgl^{-1}	Nitrate mgl^{-1}	Chlorine mgl^{-1}
Lowthorpe (1)	-	-	-	-
Lowthorpe (1)	<0.5	0.03	40.0	<0.1
River Terrig (2)	<0.5	<0.03	6.5	<0.1
Fleshwick (3)	<0.5	<0.03	4.5	<0.1

2.7 Summary

Samples of *G. pulex* were collected from 43 populations around Europe. The sampling strategy was intended to include: previously identified populations (Scheepmaker, 1990; Müller, 1998); as many drainage systems as possible in the UK; and to sample the most likely drainages that could have been utilised as potential colonisation routes of the UK from mainland Europe. On a smaller scale three populations were studied in significantly more detail, with water quality data collected, and repeated sampling trips during the year.

Table 2.4: Summary data for the detailed studies at three sample sites. Sample site location with identifiers in parenthesis with reference to Figures 2.1 and 2.2.

Sample site and location	**Area sampled at each location (m^2)**	**Surrounding land use**	**Substrate type**	**Estimated maximum population abundance (No./m^2)**	**Over winter copulation observed?**	**Conductivity (μS)**	**Nitrate levels (mg ml^{-1})**
Lowthorpe (1), East Yorkshire	10	Arable Farmland	Limestone	3000	YES	580	HIGH (40.0)
River Terrig (2), Wales	10	Grazing Farmland	Granite	500	NO	330	LOW (6.5)
Fleshwick Bay (3), Isle of Man	8	Grassland/ scrub	Sandstone	900	NO	370	LOW (4.5)

Chapter 3

Population differentiation of *Gammarus pulex* in the UK using allozyme electrophoresis

3.1 Introduction

With regard to freshwater invertebrates, movement between two populations is by two possible routes: directly over any terrestrial boundaries; or indirectly through interconnecting water courses. Clearly both of the alternatives are not open to all species, and in general only some of the insects with adult flighted stages and small invertebrates that can be carried by the wind can make use of the former route.

The structure and genetic differentiation of populations of the freshwater *Gammarus spp* have received increased research attention since the advent of molecular markers. However, this increased attention has been biased towards relatively few species. in Europe and North America (Bulnheim & Scholl, 1981; Gooch & Henrick, 1979; Scheepmaker, 1990; Siegismund *et al,* 1985; Siegismund, 1988). This chapter will consider variability and differentiation in populations of *G. pulex* in the UK, using allozyme electrophoresis.

Prior to 1997, the majority of molecular data available on differentiation in *Gammarus spp* (marine, brackish or freshwater) had been generated using allozymes. As such, there is much literature on both data (heterozygosities and allelic distribution to name but two) and associated methodologies. The technique is generally quick, cheap and relatively simple allowing many animals to be screened rapidly.

Previous allozyme studies of *Gammarus spp* have shown population differentiation to be associated with habitat. First, marine *Gammarus spp* generally

show weak differentiation and little variability. In one case, Siegismund *et al* (1985) found that it was impossible to detect any population genetic structure of *G. locusta* with 19 allozyme loci as a consequence of very low levels of variability across all loci. *G. oceanicus* also showed weak population structuring and differentiation, which Siegismund *et al* (1985) stated, was due to a lack of geographical barriers to migration.

Secondly, brackish *Gammarus spp* have a contrasting pattern to their marine relatives, showing moderate population differentiation and fluctuating levels of genetic variability. Habitat isolation and low population sizes in *G. duebeni* have been thought to cause, through genetic drift, genetic differentiation (Siegismund *et al,* 1985). Geographic isolation was also proposed as the cause of marked differentiation between *G. zaddachi* populations (Bulnheim & Scholl, 1981).

Thirdly, within the freshwater *Gammarus spp*, patterns of population differentiation are more complex than either marine or brackish species. One example that shows strong isolation and differentiation was observed in the North American *G. minus* (Gooch & Henrick, 1979) among populations with restricted gene flow. In contrast, *G. p. pulex* populations exhibit little genetic differentiation, and a high level of inferred gene flow in Europe (Scheepmaker, 1990). However, most freshwater gammarids fall between these two examples, and again habitat fragmentation and the associated low gene flow are the main causes of population differentiation. For example, *G. fossarum* inhabits isolated headwaters and shows greater differentiation (due to restricted movement between populations) than *G. roeseli,* which inhabits the lower reaches of rivers (Siegismund, 1988).

The few investigations in to population differentiation of freshwater animals in the UK that have been conducted suggest that historical events affect genetic variation (Hitchings & Beebee, 1996). For instance, the natterjack toad has a consistent lack of genetic variety in several isolated populations in the UK and consequently little or no

differentiation. The pattern of genetic differentiation in the natterjack toad is thought to be a consequence of limited genetic diversity in ancestral colonisers, rather than genetic drift since colonisation (Hitchings & Beebee, 1996). This pattern was also observed in the caddisfly (*Limnephilius elegans*) where insufficient time has passed for significant population differentiation to occur (Wilcock *et al.*, 2001).

Current literature on the population differentiation of *Gammarus* species outside the UK is extensive, especially for a few of the more widely distributed species. Information on population differentiation of freshwater taxa in the UK has received far less attention, by comparison. There has been no electrophoresis survey of UK *G. pulex* to date.

The predicted pattern of population differentiation of *G. pulex* in the UK depends upon the diversity of the ancestral colonisers and the level of habitat isolation. Like the natterjack toad, the presence of *G. pulex* in Western Europe is thought to be a result of post-glacial expansion from central European refugia (Scheepmaker, 1990; Thienemann, 1950). Unlike in mainland Europe, *G. pulex* in the UK can be found in isolated headwaters, lakes and lowland rivers. The utilisation of a more diverse set of habitats in the UK coincides with a reduced diversity in gammarid fauna. Hence, competition between gammarid species is reduced but competition with other freshwater invertebrates (*Asellus aquaticus*) still occurs (Graca *et al*, 1994).

Therefore population structuring and differentiation of *G. pulex* in the UK is expected to be weak and allelic variability low by virtue of postglacial colonisation, as in the natterjack toad, and high gene flow between populations (shown in European *G. pulex*, Scheepmaker, 1990).

The principal aim of this study was to estimate allozyme variation and patterns of differentiation in populations of *G. pulex* in the British Isles. Within the study there are three secondary aims:

1. To determine the effect of population isolation (by physical barriers to gene flow) on molecular diversity, using a case study of Malham Tarn.
2. To determine any loss of genetic variation between an introduced population on the Isle of Man (Fleshwick Bay) and its source population in Wales (River Terrig).
3. To assess the effect of small geographic distances (400m) on population differentiation.

3.2 Materials and method

3.2.1 Sample collection

Samples of between 10 and 50 animals were collected from 11 populations (Figure 3.1) encompassing several major drainage basins in the UK (Humber, Dee and the Wash). Population descriptions and sampling techniques are given in chapter 2. The choice of populations was based on known history and recorded ecological conditions.

3.2.2 Laboratory procedures

3.2.2.1 Starch gel electrophoresis

Standard starch gel (12% Connaught starch) electrophoresis (Harris & Hopkinson, 1976) was employed. Whole gammarids, fresh or frozen (in liquid nitrogen), were homogenised in 25µl distilled water using a Teflon plunger in a 1.5 ml ependorf tube. The homogenates were spun for 2 minutes at 13,000 G to separate cell debris. Filter paper wicks (3 x 6 mm) were used to soak up the supernatant and placed into the origin. Only a single origin was cut in each gel, allowing 30 samples and one control to be loaded. A single wick was stained with ferritin (Sigma chemicals) and acted as a marker to visualise the speed of the run.

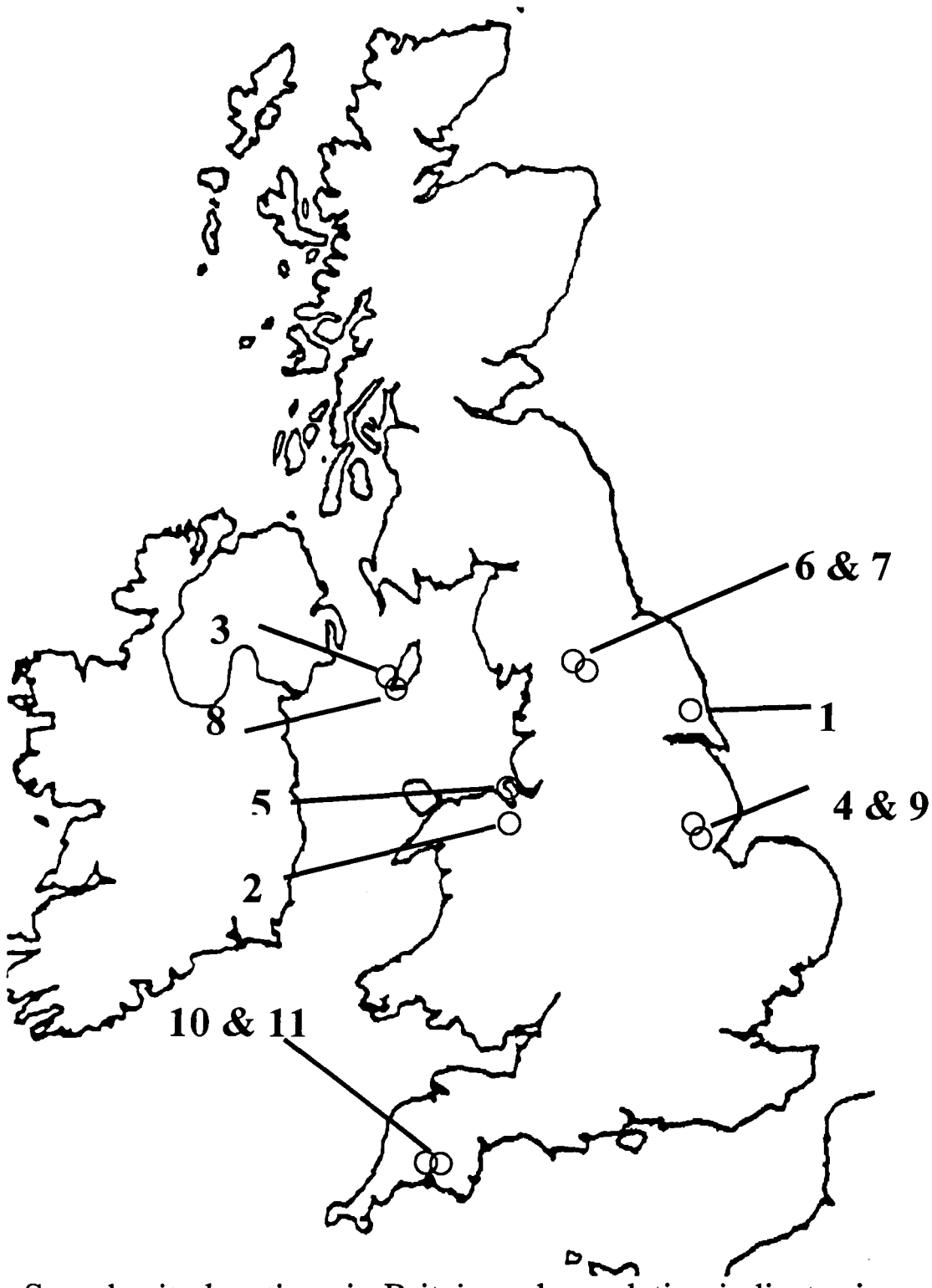


Figure 3.1: Sample site locations in Britain and population indicator in parenthesis (see Table 2.1). [1- Lowthorpe; 2- River Terrig; 3- Fleshwick Bay; 4- Stainfield Hall; 5- Greasby Brook; 6- Malham Tarn; 7- Malham Village; 8- River Crogga; 9- Stainfield Village; 10- Horrabridge; 11- Ivybridge.]

Electrophoresis (at 4°C) took between 6 and 15 hours depending on the buffer system (Appendix 3.1). Stain recipes (Appendix 3.2) were modified from Harris & Hopkinson, (1976), Murphy *et al* (1990), Piertney (1994) and Hauser (1996). All stains, except Esterase, were applied with an agar overlay and incubated at 37°C in the dark until bands appeared (normally 1 hour max). Gels were stained for Esterase-D and then incubated on the bench for 10 minutes, and examined using an UV trans-illuminator.

3.2.2.2 Cellulose acetate electrophoresis

The method for cellulose acetate gel electrophoresis follows that of Hebert & Beaton (1989) and was used to resolve a single locus, PGM. Cellulose acetate gels were chilled overnight prior to running in a Tris-glycine buffer (appendix 3.2) and placed in the electrophoresis chamber just before use. Supernatant from the starch gel preparation (whole animals homogenized in 25 µl of distilled water) was diluted 1:2 with distilled water, for a total loading volume of 6 µl.

Gels were run at 90 mA for 14 minutes on the bench and stained with an agar overlay, according to Hebert & Beaton (1989) (Appendix 3.2). The stained gels were then incubated at 37°C in the dark until band developed (30 minutes max).

3.2.3 Data analysis

Allele frequencies and heterozygosities (of all loci and all populations) were estimated with BIOSYS-1 (Swofford, 1981). Deviations from Hardy Weinberg expectations and tests for linkage between allozyme loci were calculated using GENEPOP V3.2b (Raymond & Rousset, 1995). Population differentiation based on allele frequency (genic) and heterozygosity (genotypic) were estimated by pair-wise comparisons in GENEPOP V3.2b. Differentiation between populations was also estimated by calculating F_{st} in FSTAT V2.8 (Goudet, 1995). Genotype frequencies were used to discriminate the 11 populations using a principal component analysis (PCA) program, PCA-GEN, (Goudet, 2001) and shown graphically as ordinate plots.

Genetic distances between populations were calculated based on Nei's (1972) and Reynolds' *et al* (1983) models, using PHYLIP (Felsenstein, 1993). Nei's distance assumes that the distances are generated as a result of both genetic drift and mutation (which is assumed to be constant across all loci), and that all populations have a constant effective population size. In contrast, Reynolds' distance assumes that all

genetic distances are a result of genetic drift and there is no mutation. The effective population size is assumed to change at a rate reciprocal to the genetic drift (drift = $^1/N_e$). Genetic distances were used to construct neighbour joining trees, using PHYLIP.

3.3 Results

Of 31 enzymes screened (Appendix 3.3), 11 had both sufficient activity and resolution for routine scoring. Activity and resolution were scored relative to the strongest and clearest locus, which was PGI (Figure 3.2b). The 11 enzymes scored encoded for 13 allozyme loci (PEP-1 and PEP-2; ADA-1 and ADA-2). One enzyme locus was scored on cellulose acetate (PGM) as the resolution and activity was superior to that of starch. At the LDH locus amplification was very weak, but in parasitised animals there was an additional band (Figure 3.2a).

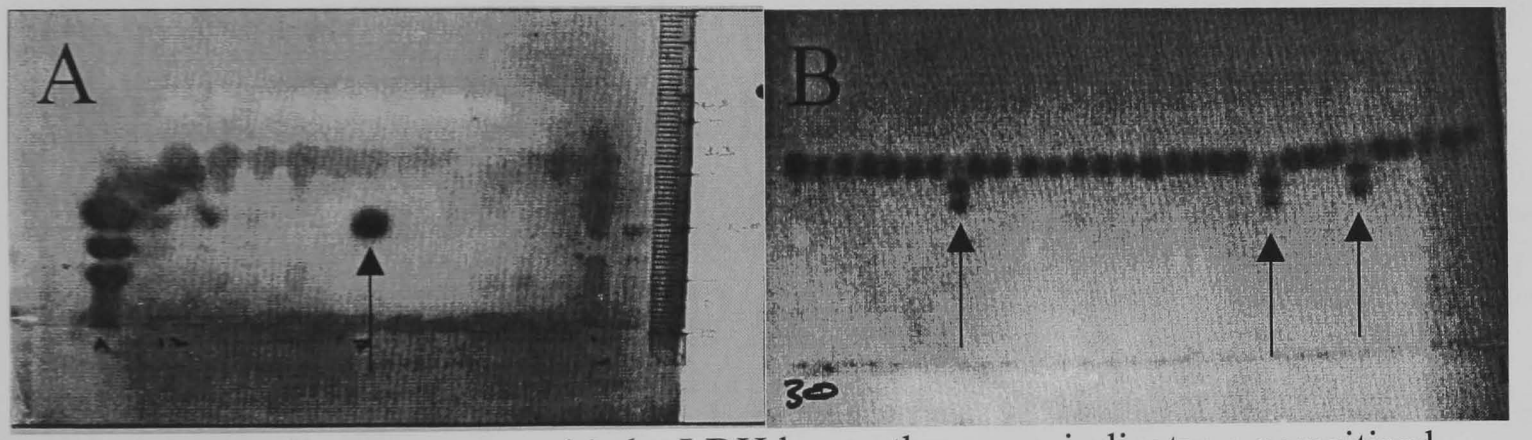


Figure 3.2: Banding patterns at (a) the LDH locus, the arrow indicates a parasitised animal and (b) the PGI locus, arrows indicate heterozygotes

3.3.1. Allozyme polymorphism

Of the 13 allozyme loci scored, 7 were polymorphic in at least one population at the 99% level (Table 3.1), though no single locus was polymorphic in all populations. Polymorphism at all loci was generally low, and the frequency of variant alleles never exceeded 14%, as shown by the number of rare alleles, with 10 of the 13 never exceeding frequencies of 5%. There were also six private alleles, which were only recorded in a single sample (Hartl & Clark, 1997), including all variant alleles at the GAPDH locus.

Table 3.1: Allele frequencies of the 7 polymorphic gene loci in *Gammarus pulex*. N= sample size, h= observed heterozygosity per locus (direct count method) and H= heterozygosity across all loci. Population locations refer to those in Figure 3.1. ADA-1, ADA-2, ARK, EST, PGDH and PEP-2 loci were monomorphic across all populations sampled

	POPULATION										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
LOCUS											
AAT											
(N)	30	30	30	40	30	40	40	20	40	50	10
91	0.050	0.000	0.017	0.013	0.017	0.038	0.013	0.000	0.013	0.000	0.000
100	0.950	1.000	0.983	0.988	0.983	0.962	0.988	1.000	0.988	1.000	1.000
h	**0.100**	**0.000**	**0.033**	**0.025**	**0.033**	**0.075**	**0.025**	**0.000**	**0.025**	**0.000**	**0.000**
GAPDH											
(N)	30	30	30	40	30	40	40	20	40	50	10
82	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
95	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000
100	1.000	0.983	0.967	1.000	1.000	0.975	1.000	1.000	1.000	1.000	1.000
108	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
h	**0.000**	**0.033**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
IDH											
(N)	30	20	30	40	30	40	40	20	40	50	10
90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000
100	1.000	1.000	1.000	1.000	0.983	1.000	1.000	1.000	0.962	1.000	1.000
122	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.013	0.000	0.000
h	**0.000**	**0.000**	**0.000**	**0.000**	**0.033**	**0.000**	**0.000**	**0.000**	**0.075**	**0.000**	**0.000**
MDH											
(N)	30	30	30	40	30	40	40	20	40	50	10
85	0.133	0.083	0.033	0.138	0.017	0.000	0.038	0.100	0.075	0.000	0.000
100	0.867	0.917	0.967	0.863	0.883	1.000	0.938	0.900	0.925	1.000	1.000
109	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000
h	**0.267**	**0.167**	**0.067**	**0.225**	**0.233**	**0.000**	**0.075**	**0.100**	**0.100**	**0.000**	**0.000**
PEP-1											
(N)	30	30	30	40	30	40	40	20	40	50	10
95	0.000	0.017	0.000	0.013	0.017	0.012	0.013	0.000	0.000	0.000	0.000
100	1.000	0.983	1.000	0.988	0.983	0.988	0.988	1.000	1.000	1.000	1.000
h	**0.000**	**0.033**	**0.000**	**0.025**	**0.033**	**0.025**	**0.025**	**0.000**	**0.000**	**0.000**	**0.000**
PGI											
(N)	30	30	20	30	20	40	40	20	40	50	10
80	0.000	0.033	0.017	0.025	0.033	0.025	0.000	0.050	0.038	0.000	0.000
85	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000
100	1.000	0.967	0.983	0.975	0.967	0.975	0.988	0.950	0.962	1.000	1.000
h	**0.000**	**0.067**	**0.033**	**0.050**	**0.067**	**0.050**	**0.025**	**0.100**	**0.075**	**0.000**	**0.000**
PGM											
(N)	30	30	30	40	30	40	39	20	40	50	10
89	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.075	0.000	0.000	0.000
95	0.117	0.000	0.067	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000
100	0.883	1.000	0.933	1.000	1.000	1.000	0.962	0.925	1.000	1.000	1.000
h	**0.167**	**0.000**	**0.133**	**0.000**	**0.000**	**0.000**	**0.075**	**0.150**	**0.000**	**0.000**	**0.000**
H	**0.041**	**0.023**	**0.021**	**0.025**	**0.031**	**0.012**	**0.017**	**0.027**	**0.019**	**0.000**	**0.000**

3.3.2 Linkage disequilibria and Hardy-Weinberg equilibrium

Of 54 tests for linkage disequlibria performed (across all populations and all pairs of loci) only one result was significant, between the PEP-1 locus and the PGM locus in the Malham Village samples (P=0.027). The association arose exclusively by the co-occurrence of a single heterozygous individual at both the PEP-1 (100, 95 heterozygote) and PGM locus (100, 89 heterozygote). While significant the data should be treated with caution, as a single statistically significant result could be due to chance alone. The data could be corrected for a potential significance bias using a *posteriori* method such as a Bonferoni correction (Rice, 1989). Although after correction the test was not significant (P=0.027 compared to Bonferoni level of significance P=0.0009) and the result appears to be by chance alone.

Eight of the eleven populations sampled were in Hardy Weinberg equilibrium and deviations in the remaining three. The first, in Malham Tarn (P=0.0127), was associated with a single homozygote at the GAPDH locus (95 allele) and no heterozygotes (F_{IS} = +1). The second, in the Fleshwick Bay sample (P=0.0169), was again attributed to the presence of a single GAPDH homozygote (108 allele, F_{IS} = +1). The third deviation from expectations was in the Malham Village sample at the MDH locus (P=0.0146) caused by a deficiency of heterozygotes (109 allele) and an absence of homozygotes at the 85 allele (F_{IS} = +0.38).

3.3.3 Intrapopulation variability

Overall levels of variation within samples were low, with heterozygosity values across all loci not exceeding 4.5% (Figure 3.2). The highest levels of heterozygosity were recorded in the Lowthorpe population sample (4.2%). The lowest, were recorded in the Horrabridge and Ivybridge population samples, both of which were monomorphic

at all loci. Two of the loci (PGM and MDH) showed significantly higher variation than the other loci screened, with variant alleles at frequencies above 5% (Table 3.2).

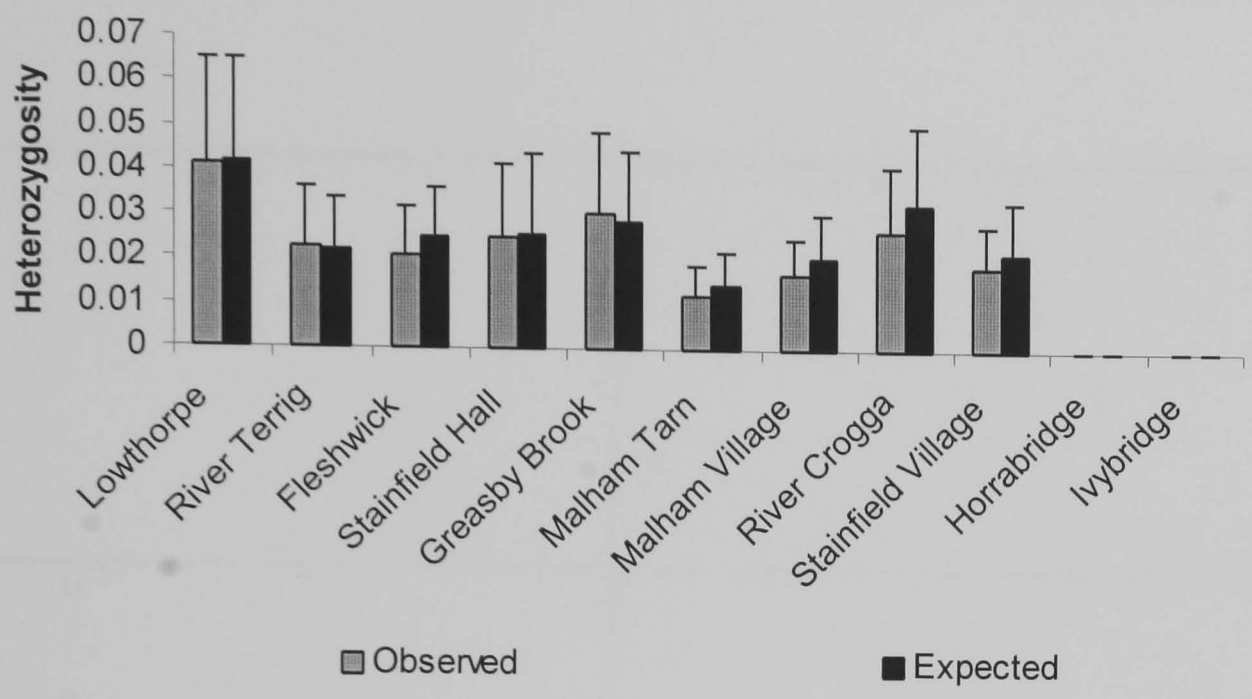


Figure 3.2: Observed and expected heterozygosity of the 11 populations of *G. pulex*.

3.3.4 Population differentiation

Estimates of population differentiation (genotypic, genic and F_{ST}) across all polymorphic loci were highly significant between populations (Table 3.2). Variation at the MDH and PGM gave significant F_{ST} and genotypic results between populations and were thought to be a strong influence in the overall differentiation of the populations sampled (Table 3.2). The significance difference between populations, and across all loci appeared to be driven by the MDH, PGM and the GADPH loci (where variant alleles were present). This was highlighted by the principal component analysis (PCA) which gave identical plots with either the three loci data (MDH, PGM & GADPH) or all seven polymorphic loci data used (Figure 3.3).

The PCA separated the 11 populations and the two axes accounted for 83 % and 92 % of the variation, for the seven and three loci respectively. In both cases only the first two axes were significant, with the remainder of the variation split between axes 3 to 8. From the plots, several patterns emerged: Lowthorpe was distinct from all other

populations; Malham Tarn, Horrabridge and Ivybridge clustered closely; and a separate cluster of River Terrig and Stainfield village. The remaining populations showed no strong clustering or clear disassociation with any of the other populations sampled.

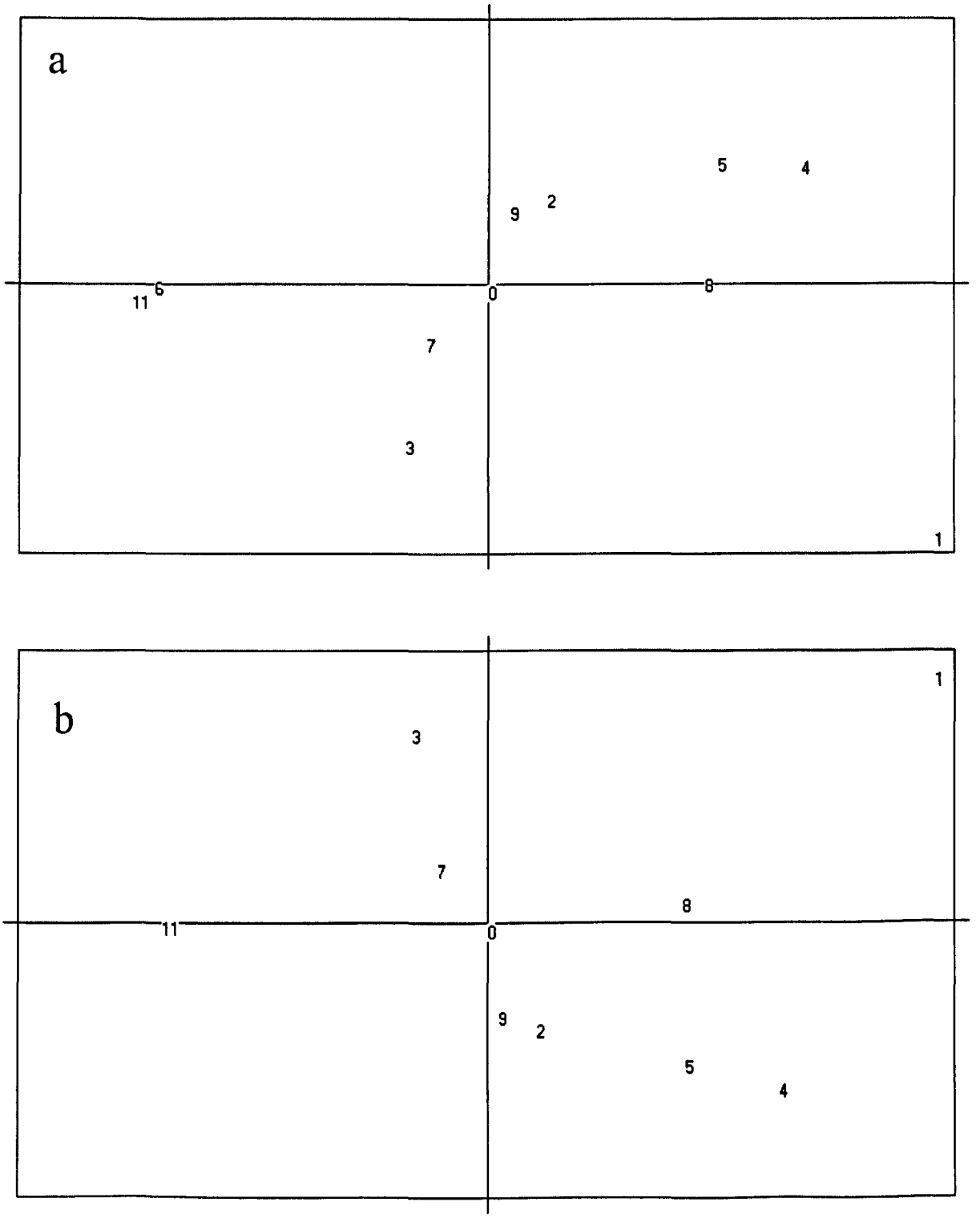


Figure 3.3: Principal component analysis plots based on (a) GAPDH, MDH and PGM loci and (b) all 7 polymorphic loci (for population numbers see Figure 3.1). Samples from population 10 are overlaid by samples from 11 at the same co-ordinates and in addition 10 and 11 in 3.3b overlie population 6.

Table 3.2: F_{ST} values (with significance levels given, ***: $p<0.001$) and levels of significance for population differentiation (genotypic and genic).

Locus	F_{ST}	Genic	Genotypic
GAPDH	-0.003	0.03	0.41
GOT	0.002	0.42	0.43
IDH	0.009	0.29	0.19
MDH	0.030***	0.00	0.00
PEP-1	-0.007	0.85	0.90
PGI	-0.001	0.27	0.29
PGM	0.055***	0.00	0.00
Overall	0.022***	0.00	0.00

Pair-wise comparisons between populations based on genotypic and genic differentiation (GENEPOP) agreed (Table 3.3). However the genic measure appeared more sensitive, with ten significant comparisons compared to eight at the genotypic level. All pairwise significant results occurred between either Lowthorpe or Horrabridge and another population. These two populations had the highest (Lowthorpe) and lowest (Horrabridge) levels of heterozygosity of all the sampled populations. Ivybridge also had no polymorphic loci and the lowest heterozygosity, yet showed no significant pair-wise differences (though the sample size was small).

Genetic distance data (Nei, 1972; Reynolds, 1983) was used to construct two unrooted trees (Figure 3.4), which both showed no strong clustering of populations by geographic distance. However Reynolds genetic distance tree appeared to separate the populations by levels of heterozygosity (Figure 3.4b) with the monomorphic samples from Horrabridge being most distant from the most heterozygous Lowthorpe samples.

Table 3.3: Pair-wise population differentiation, genotypic above the diagonal and genic below (* $p<0.05$ ** $p<0.01$).

Genotypic

	Low-thorpe	River Terrig	Fleshwick	Stainfield Hall	Greasby Brook	Malham Tarn	Malham Village	River Crogga	Stainfield Village	Horra-bridge	Ivybridge
Lowthorpe	-					**		*	*	**	
River Terrig		-									
Fleshwick			-								
Stainfield Hall				-						**	
Greasby Brook					-					**	
Malham Tarn	**					-					
Malham Village							-				
River Crogga	*							-		**	
Stainfield Village	*								-	*	
Horrabridge	**	*	*	**	**			**	**	-	
Ivy bridge											-

Genic

Table 3.4: Distance matrix, above the diagonal Nei's genetic distance (Nei, 1972) and Reynold's distance (Reynolds *et al*, 1983) below.

	Lowthorpe	River Terrig	Fleshwick Bay	Stainfield Hall	Greasby Brook	Malham Tarn	Malham Village	River Crogga	Stainfield Village	Horra-bridge	Ivybridge
Lowthorpe	-	0.0016	0.0012	0.0012	0.0013	0.0025	0.0013	0.0013	0.0016	0.0024	0.0024
River Terrig	0.0476	-	0.0007	0.0003	0.0002	0.0007	0.0003	0.0005	0.0001	0.0006	0.0006
Fleshwick Bay	0.0340	0.0279	-	0.0013	0.0011	0.0005	0.0003	0.0009	0.0007	0.0005	0.0005
Stainfield Hall	0.0352	0.0113	0.0488	-	0.0001	0.0016	0.0008	0.0006	0.0004	0.0015	0.0015
Greasby Brook	0.0357	0.0062	0.0376	0.0023	-	0.0012	0.0006	0.0006	0.0002	0.0011	0.0011
Malham Tarn	0.0829	0.0358	0.0269	0.0706	0.0506	-	0.0005	0.0014	0.0006	0.0002	0.0002
Malham Village	0.0393	0.0141	0.0123	0.0309	0.0218	0.0245	-	0.0006	0.0003	0.0003	0.0003
River Crogga	0.0340	0.0191	0.0318	0.0209	0.0177	0.0571	0.0234	-	0.0006	0.0013	0.0013
Stainfield Village	0.0477	0.0051	0.0271	0.0156	0.0078	0.0315	0.0145	0.0201	-	0.0005	0.0005
Horrabridge	0.1129	0.0570	0.0434	0.1059	0.0078	0.0283	0.0332	0.0806	0.0521	-	0.0000
Ivy bridge	0.1129	0.0570	0.0434	0.1059	0.0078	0.0283	0.0332	0.0806	0.0521	0.0000	-

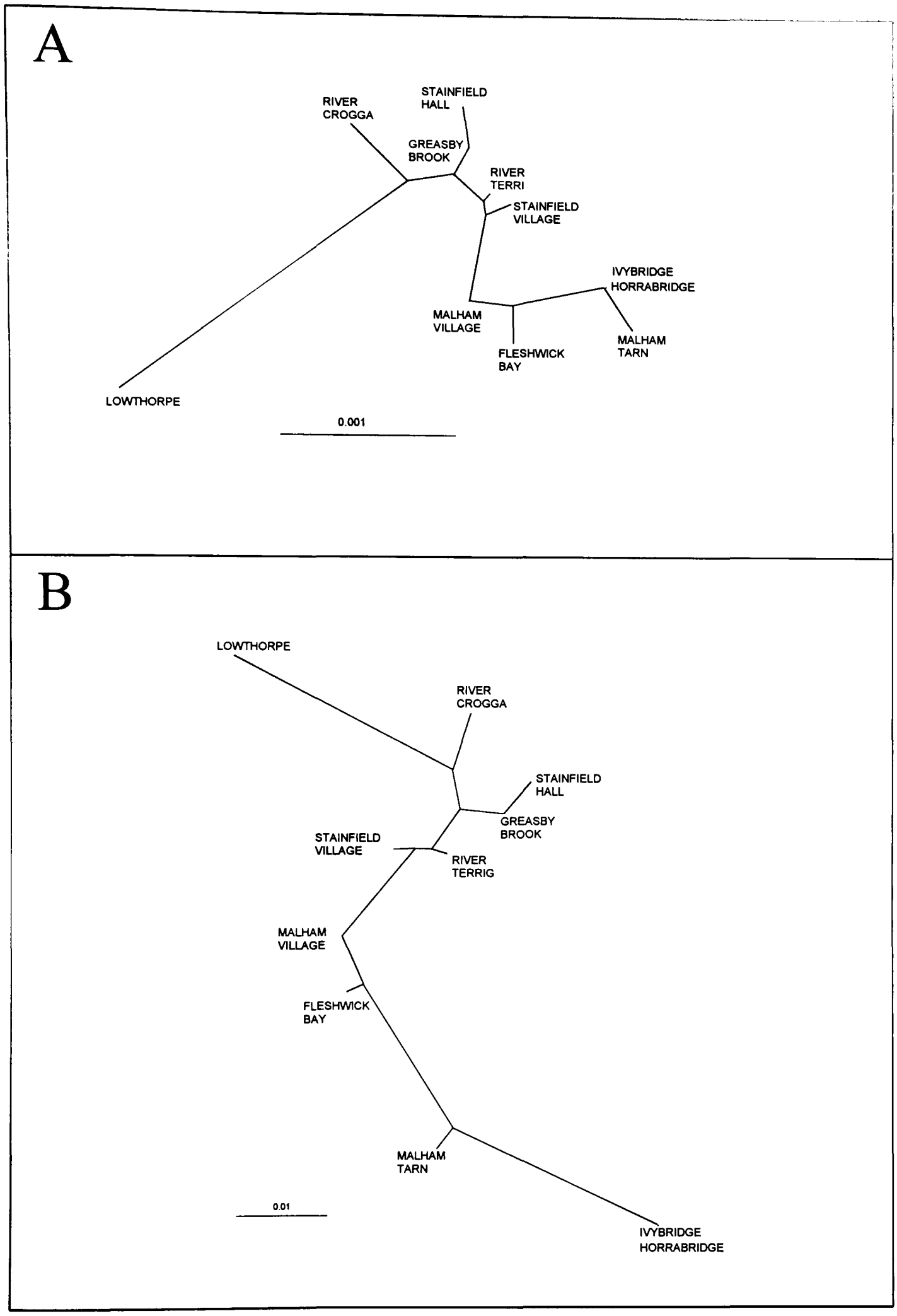


Figure 3.4: Neighbour joining trees of the 11 populations using (a) Nei's (1972) and (b) Reynolds' *et al* (1983) genetic distances

3.4 Discussion

3.4.1 Genetic diversity in *G. pulex*.

The expectation of low diversity and limited differentiation of *G. pulex* has been supported by the data presented here. There was no evidence to support differences in the levels of diversity between the UK and Europe, with similar mean population heterozygosities of between one and six percent recorded (see Scheepmaker & van Dalfsen, 1989; Scheepmaker, 1990). Such an observation was surprising, given that the loci scored in the European samples included two highly polymorphic loci, MPI (heterozygosity up to 45 %) and PEP-4 (heterozygosity up to 46 %). However, like the present study there was a large proportion of monomorphic loci scored.

In mainland Europe restricted niche range (caused by gammarid competition) and habitat fragmentation reduce the potential for gene flow between populations and increase population differentiation (Müller, 1998). However, within the UK there is less competition of gammarids and less habitat fragmentation leading to increased gene flow and less population structuring. Restricted gene flow has been observed in European *G. fossarum* (restricted to isolated headwaters) where greater differentiation was estimated compared to *G. roeseli,* which inhabits the lower reaches of rivers (Siegismund, 1988). There was no direct evidence to support the hypothesis that differences in the levels of genetic diversity were a consequence of habitat.

3.4.2 Genetic diversity in the British Isles.

Levels of heterozygosity in *G. pulex* within the British Isles estimated here are comparable to populations of *G. pulex* in mainland Europe, and as such are consistently low (Scheepmaker, 1989; Scheepmaker, 1990). However this study is the first to identify gammarid populations that show monomorphism across all scored allozyme

loci. Horrabridge and Ivybridge are both situated on isolated river systems that drain Dartmoor (an isolated moorland) directly into the English Channel. Additionally the isolation of this region is supported by the presence of another *Gammarus spp* (*G. duebeni*), which has only been found in restricted habitats in the west of the British Isles (Hynes, 1954). Cross-drainage movements are unlikely, as the distance between rivers is large and suitable seasonal habitat is unavailable. The large number of animals screened and the complete lack of heterozygosity in Horrabridge supports the hypothesis of extreme isolation and potential bottleneck effects at this site. However, the lack of heterozygosity and the inability to detect any rare variant alleles from Ivybridge could be a result of the limited sample size (n=10). With so few animals screened and the observed low polymorphism, even in the most heterozygous population (Lowthorpe), detection of rare variant alleles would be unlikely, even by chance.

The lack of strong population differentiation and low F_{ST} values are due to the lack of informative (strongly polymorphic) alleles at all loci and the presence of many rare alleles. Several processes, including colonisation by a small population (with little genetic diversity) and population bottlenecks can explain weak differentiation. As in the natterjack toad, there was limited population structuring of *G. pulex* in the UK. This could be a result of a similar lack of genetic diversity in the ancestral colonisers (Hitchings & Beebee, 1996). The occurrence of the same single allele in 6 loci of all the populations scored supports this hypothesis rather than the suggestion that a series of bottlenecks after colonisation could have been the cause (Hitchings & Beebee, 1996).

The historical evidence in Europe and the models of species colonisation (Hewitt, 1996) could support the hypothesis that colonisation was recent and by few animals and animals with little genetic diversity. During the last glacial period (~ 18,000 bp to ~ 8,000 bp) ice covered at least 60 % of the UK (Williams, 1969) and the

remaining land was permafrost. The environment at that time was unfavorable for most freshwater species, and *G. pulex* would be unlikely to survive. However once the ice melted, suitable habitat would be available for colonisation and it is at this time that *G. pulex* could have colonised the UK. Colonisation of the UK by species from European refugia after the retreat of the ice has been shown in several species, including the crested newts (Wallis & Arntzen, 1989).

Estimates for the speed at which colonisation occurred suggest rates of 300- 500 km per year for the grasshopper (Hewitt, 1990) and similar rates for other species (Hewitt, 1996). At this rapid speed of expansion the "leading edge" is expected to undergo a series of bottlenecks and an associated loss of alleles and homogeneity (Nei *et al*, 1975). If as suggested, there was a rapid colonisation of new (ice free) habitats in the UK, low levels of molecular variation could have been further eroded through a series of bottlenecks.

3.4.2.1 Population isolation and gene flow

The geologically isolated population of Malham Tarn clusters with the two monomorphic populations in the PCA plots, indicating a lack of diversity. Malham Tarn was formed from glacial moraine about 10,000 bp (Chapter 2) and has been separated from the village population ever since. Any gene flow between the two populations can only be unidirectional, from the Tarn to the village, due to geology and hydrology. The lack of significant differences is equivocal and was expected due to the low heterozygosities and cannot be used to support any hypothesis of unidirectional gene flow.

Although not significant, the two populations do show differences in the numbers of alleles present. All alleles present in the Tarn sample were also found in the village in addition to two private alleles at two loci (MDH and PGI)in the village

sample. These two rare alleles could have arisen by downstream migration from caves (that drains Malham moor) or upstream from the main river. Samples were not taken from either location, but the presence of two private alleles could indicate migration from a divergent source. The caves could be the source of any migration as selection (Fong, 1989) and marked genetic differentiation (Gooch & Henrick, 1979) has been shown in cave dwelling gammarids. To test this hypothesis more samples from the caves would be required in addition to a more polymorphic molecular marker.

3.4.2.2 Genetic diversity in an introduced population

Both the source (River Terrig) and the introduced population (Fleshwick bay) have very similar levels of heterozygosity and mean number of alleles per locus. All tests of population differentiation between the two populations are not significant. The limited number of founders (75) in the Fleshwick Bay population could be expected to act like a bottleneck and reduce the diversity. However from the data there appears to be no loss of genetic diversity between the source and introduced populations sampled.

There are two possible reasons for the lack of genetic erosion; first the number of founders could have been sufficient to maintain the allozyme diversity of the source population. This would assume that the effective and census population sizes of the founders are similar. If Hynes' (1954) introduced only mature animals this might be possible, though an initial mortality could be expected, due to colonisation of a new environment. The second reason could be that the current population is not descended from the introduction by Hynes', but from later introductions. Although unlikely, survey data (Dick *et al*, 1997) does show the absence of *G. pulex* from Fleshwick Bay between 1951 and 1952. This absence could be due to poor sampling of the small number of animals present or a real absence. Without further evidence, historical or molecular, the actual source(s) of Fleshwick Bay cannot be determined.

3.4.2.4 Small-scale geographic distances as barriers to gene-flow

The lack of genetic differentiation between Stainfield Hall and Stainfield Village was expected due to the small distance between the two populations (400m). The small geographic distance appears insufficient to prevent gene flow between the sample sites. Additionally the lack of differentiation among populations separated by larger geographic distances (e.g. Malham Tarn and Stainfield village) using allozyme loci would reduce the likelihood of differentiation at this fine scale.

3.4.3 Genetic diversity within populations.

Variation within populations is low and the heterozygosities are mainly dependent on the two most polymorphic loci, MDH and PGM. Most of variant alleles scored are rare and occur at less then 5%, and have little effect on the overall diversity of the populations.

Overall, the small isolated populations had the lowest diversity (Horrabridge, Ivybridge and Malham Tarn), and the large populations had the highest (Lowthorpe). The remaining populations were very similar in allele frequencies, accounting for the lack of differentiation. Population size has been shown to affect the amount of genetic variability maintained (Amos & Harwood, 1998) and would appear to be one of the main factors in maintaining the genetic diversity of *G. pulex* populations. Large stable populations have several characteristics that help maintain higher genetic diversities, including year round breeding, a large and varied habitat and the lack of seasonal bottlenecks. This could explain the higher diversity in Lowthorpe compared to all other sampled populations.

Population differentiation of *G. pulex* in the UK with allozymes is significant overall, but pairwise reveals no obvious structuring or geographic clustering. The selection of a weakly polymorphic molecular marker system is one likely reason why

little structuring, especially at the fine scale, was observed. Although allozymes have been shown to differentiate *Gammarus* at the species level (Müller, 1998; Scheepmaker & van Dalfsen, 1989; Scheepmaker, 1990; Siegismund *et al,* 1985) and at the population level (Siegismund *et al,* 1985; Siegismund, 1988), in this case the resolution of seven polymorphic allozyme loci was apparently insufficient. In the previous studies on population differentiation (mentioned above) sample sites were separated by large geographic distances; by strong barriers to gene flow; and restricted habitat utilisation. All of these factors in addition to the suite of allozyme loci utilised could have accounted for the lack of significant differentiation in this study.

3.5 Summary

Data from the initial screening of 30 allozyme loci provided 13 scorable loci for screening genetic diversity in eleven populations of *G. pulex*. Seven of the loci were polymorphic (99% level) and two populations were monomorphic across all loci (Horrabridge and Ivybridge). Observed heterozygosities were low (0-4.2%) and as such comparable to previous allozyme studies on *G. pulex* in European populations. The low genetic diversity precluded, to a statistical extent, population differentiation across the geographic range of populations sampled. There was no significant difference between the source (River Terrig) and the introduced population (Fleshwick Bay) used by Hynes (1954) in a transplantation experiment. Although not statistically significant, populations that had been known to be separated from each other by thousands of years (Malham Tarn and Malham village) did show differences in the alleles present. The difference in alleles and their frequencies could be a consequence of long-term uni-directional gene flow between the two populations.

Chapter 4

Environmental and genetic effects on the morphology of *Gammarus pulex* (L.)

4.1 Introduction

Morphological traits have been used throughout the animal kingdom for species classification and the investigation of phylogenetic relationships among species. The use of functional morphological markers (feeding and reproductive structures) can provide an insight into localised adaptation of species and their evolution (e.g. Fryer, 1991b). The different levels of character polymorphism found in morphological traits allow the study of adaptation and evolution across a wide taxonomic range, from the genus level to populations, and to families within populations.

Morphological characters have been used extensively in the classification and systematics of the freshwater *Gammarus* species. At present, morphological characters form the basis of the three major species groups of freshwater *Gammarus* species in Europe created by Karaman and Pinkster (1977a; 1977b; 1987). However the use of morphological characters in taxonomy of the freshwater *Gammarus* species has been questioned (Pinkster, 1983).

Karaman and Pinkster (1977a) identified a series of characteristics which they termed 'stable' within a species and used this for the definition of the *G. pulex* group. These characteristics previously regarded as 'stable' (i.e. constant within species and unaffected by environment) have more recently been shown to have significant variation within a species and a high degree of character overlap between closely related species (Müller *et al*, 2000; Pinkster, 1983). The considerable variation in morphological characters within a species identified by Pinkster (1983) clearly presents

a major drawback for the morphological taxonomy of the freshwater *Gammarus*. However on a smaller scale, significant variation in morphological characters within a species can be used to reveal potential reproductive isolation, restricted migration and localised adaptation between populations (Müller *et al*, 2000). Although the use of morphological characters alone in the classification of *Gammarus* species may be questioned, the same lack of 'stability' makes them useful population markers. It is this latter aspect of morphological variation that forms the subject of the current chapter.

Unlike molecular markers, morphological characters are generally not neutral and are subject to selection (sexual and natural), often displaying localised phenotypic plasticity in response to environmental conditions (Müller *et al*, 2000). In *G. pulex,* both natural and sexual selection on phenotypic traits have been shown (Ward, 1988; Culver *et al*, 1994). However, the effect of environmental variation on the phenotype and heritability of morphological characters of amphipods has been rarely studied (Culver *et al*, 1994; Jernigan *et al*, 1994; Jones *et al*, 1992). Strong (1972) found significant differences in the size and growth rate of *Hyalella azteca* (Amphipoda) individuals from different populations, containing either the large or small body size ecotype. When reared under uniform conditions in the laboratory, Strong (1972) concluded that the variation observed was not heritable, but was environmentally determined. The work done by Strong (1972) has been the only work to date to, excluding of the work on the cave gammarids, show that morphological characters within amphipod species show localised phenotypic plasticity in different environments.

Freshwater amphipod species are a good model system to investigate phenotypic variation within and between populations due to localised differences in adaptation and selection on morphological characters. This has been demonstrated in several morphological characters (within a species) including the eye (Culver *et al*, 1994;

Jernigan *et al*, 1994; Jones *et al*, 1992), the gnathopods (Strong, 1972; Wellborn, 2000) and body length (Ward, 1988). Additionally, freshwater amphipods have clearly defined morphological characters (Figure 4.1) that are relatively easy to measure.

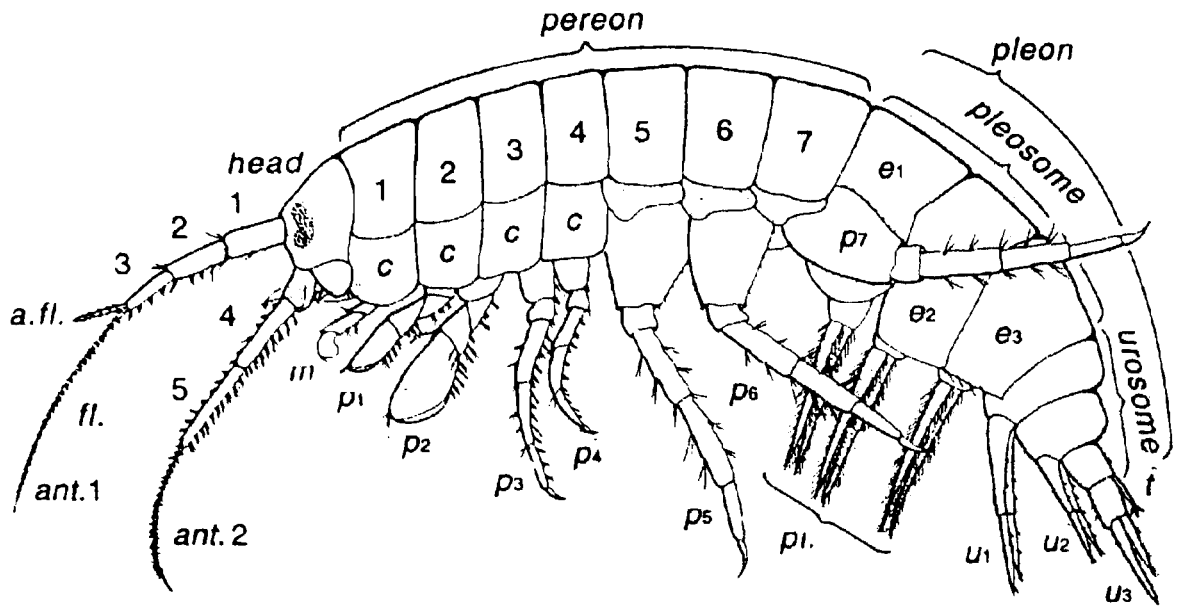


Fig. 8. *Gammarus*, *ant.* 1 and *ant.* 2 ; 1st and 2nd antennae; 1-3, peduncle articles on antenna 1, 4-5 peduncle articles on antenna 2; *a.fl.*, accessory flagellum; *c*, coxal plate; *m*, maxilliped; p_1-p_7, pereopods (p_1 is gnathopod 1 and p_2 is gnathopod 2); e_1-e_3, epimera; *pl*, pleopods; u_1-u_3, uropods; t, telson. (Note that there appear to be only seven thoracic segments (pereon 1-7) as the first is fused with the head.)

Figure 4.1: A schematic of the generalised body plan of the freshwater gammarids, taken from Gledhill *et al.* (1993).

The effect of the environment on the variability of each morphological character can be estimated by comparing animals collected from several natural populations and reared under standardised laboratory conditions. Differences in the phenotypic variance between animals from different populations reared in the lab are expected to be predominately determined by the genetic component of variance as the source of environmental variance has been minimised. Conversely, animals in the wild are expected to be heavily influenced by the local environment.

4.1.1 Aims

There were three main aims of the morphological analysis of *G. pulex* populations:

- To measure levels of morphological variation within *G. pulex* populations and examine the extent of population differentiation;
- To compare morphological characters in laboratory-reared and wild animals to estimate the possible genetic and environmental effects on individual morphological characters;
- To undertake a comparison of morphological and genetic differentiation using molecular markers

4.2 Methods

4.2.1 Samples

Samples from Lowthorpe, River Terrig and Fleshwick Bay (Table 2.1, Chapter 2) were collected during 1998. Morphological data were collected from the three populations in the wild. Precopula pairs from the same three wild populations were collected, and their offspring were born and reared in a controlled environment in the laboratory for morphological analysis (Table 4.1). Additionally, thirteen *G. pulex* were collected from Schendelbeke in Belgium in 2000 (chapter two) and used for comparison.

Only mature male animals were used for the collection of morphological data, due to a strong male sex bias in the animals reared in the laboratory. The presence of male genital papillae and absence of female ooestigites (Figure 4.2) were used to determine the sex of all animals. Both the genital papillae and the ooestigites were used for sex determination as inter-sex animals (both male and female secondary sexual characteristics) have been observed in *G. duebeni* (Dunn *et al*, 1996).

Table 4.1: Summary of the six groups of animals used, laboratory-reared animals were the offspring of animals collected in the wild.

Name	Population	Environment
LW	Lowthorpe	Wild
LL	Lowthorpe	Laboratory-reared
TW	River Terrig	Wild
TL	River Terrig	Laboratory-reared
FW	Fleshwick Bay	Wild
FL	Fleshwick Bay	Laboratory-reared

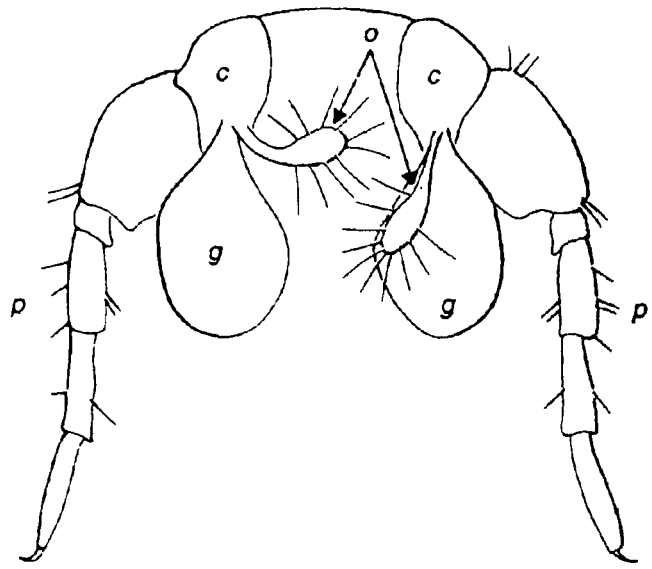


Fig. 9. Transverse section of female *Gammarus* (diagrammatic) showing the location of ooestigites (↗ *o*) and gills (*g*) on the inner base of the coxal plates (*c*), and the pereopods (*p*). (After LeRoux 1933).

Figure 4.2: Transverse section of the ooestigites (female secondary sexual characteristics) from Gledhill *et al*, 1993.

4.2.2 Laboratory rearing

Offspring from wild-caught precopula pairs from the three populations were reared in the laboratory for a single generation in a standardised environment. Variation in water quality, temperature (16.0°C ±1.0°C), food quantity (in excess) and light conditions (16h light, 8h dark) were minimised throughout the experiment to reduce environmental effects on the development of the animals. The final design for the rearing system consisted of 150 rearing chambers (Figures 4.3 & 4.4) on three shelves, each containing 500ml of water stocked with a single *G. pulex* family. Each rearing chamber was randomly stocked and positioned on the shelves to reduce any possible positional bias using a random ranking system based on the random number generator

in Excel 97 (Microsoft). Every two weeks the rearing chambers were re-positioned using a random block design.

Water temperature in all rearing chambers was maintained at 16°C ± 1°C, by using electric fans to increase air flow and by using reflected light, rather than direct light that caused localised hot spots (data not shown). Water quality was maintained by replacing 200ml of water from each of the rearing chamber once per week. An excess of food was maintained in all the rearing chambers at all times to exclude limitations of diet on growth rate. Animals were fed on a diet of partially decayed sycamore (*Acer pseudoplatanus*) leaves that were left to rot in river water for at least one week prior to feeding.

50 precopula pairs from each of the three populations were transported into the laboratory and placed in separate 500ml rearing chambers. Once the males had copulated with the females and separated, they were removed from the rearing chambers to reduce the risk of cannibalism. If the females could not be seen to contain eggs in the marsupial pouch two days after copulation, the same males were reintroduced for another round of mating. Developmental time of the eggs in the marsupial pouch was expected to take around 20 days (Sutcliffe, 1992), after which time juveniles were released into the water. All hatchlings were counted on the second day after hatching as the hatching process look longer than one day for some mothers. The removal of all mothers, once the juveniles had been counted, also reduced the risk of cannibalism. After one week the juveniles were culled to a maximum family size of eight to reduce the effect of crowding and competition between siblings. All 150 families were then maintained to the age of 170 days when they were preserved in a sucrose ethanol solution (chapter 2) for morphological analysis.

Figure 4.3: Photograph of the *Gammarus* rearing system comprising 150 rearing chambers.

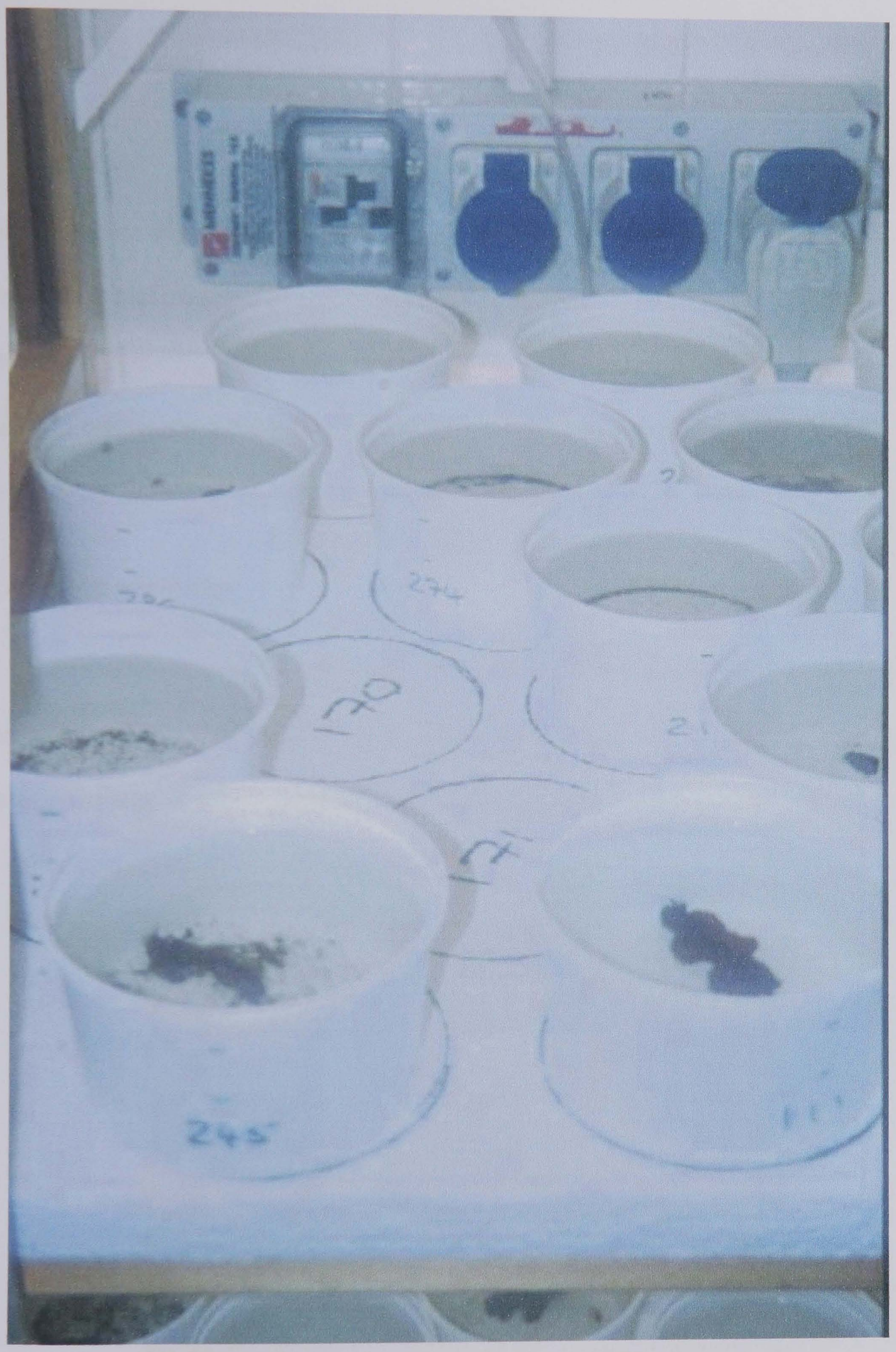


Figure 4.4: Photograph of the rearing system showing the shelf positions and rearing chambers that were randomly re-positioned every two weeks.

4.2.3 Morphological traits

In total, nine measurements and five counts were taken (Table 4.2) from the right hand side of 50 animals (from different families) in three laboratory-reared and wild populations. Figures 4.5- 4.10 show the position of some of the morphological character counts and measurements. All measurements were made using a binocular microscope to the nearest 0.1mm using a graticule slide. Additionally, the surface area of the eye, assuming the shape of an ellipse (Culver, 1987), and the density of ommatidia within the eye were calculated. An estimation of the surface area of an individual ommatidium was not made due to practical constraints (see Culver, 1987).

4.2.4 Repeatability

Repeatability of all the morphological characters was measured by re-measuring all morphological traits from ten randomly selected animals from individuals of the three populations already screened. A repeatability score and its standard error were then calculated using the method of Oosterhout (2000).

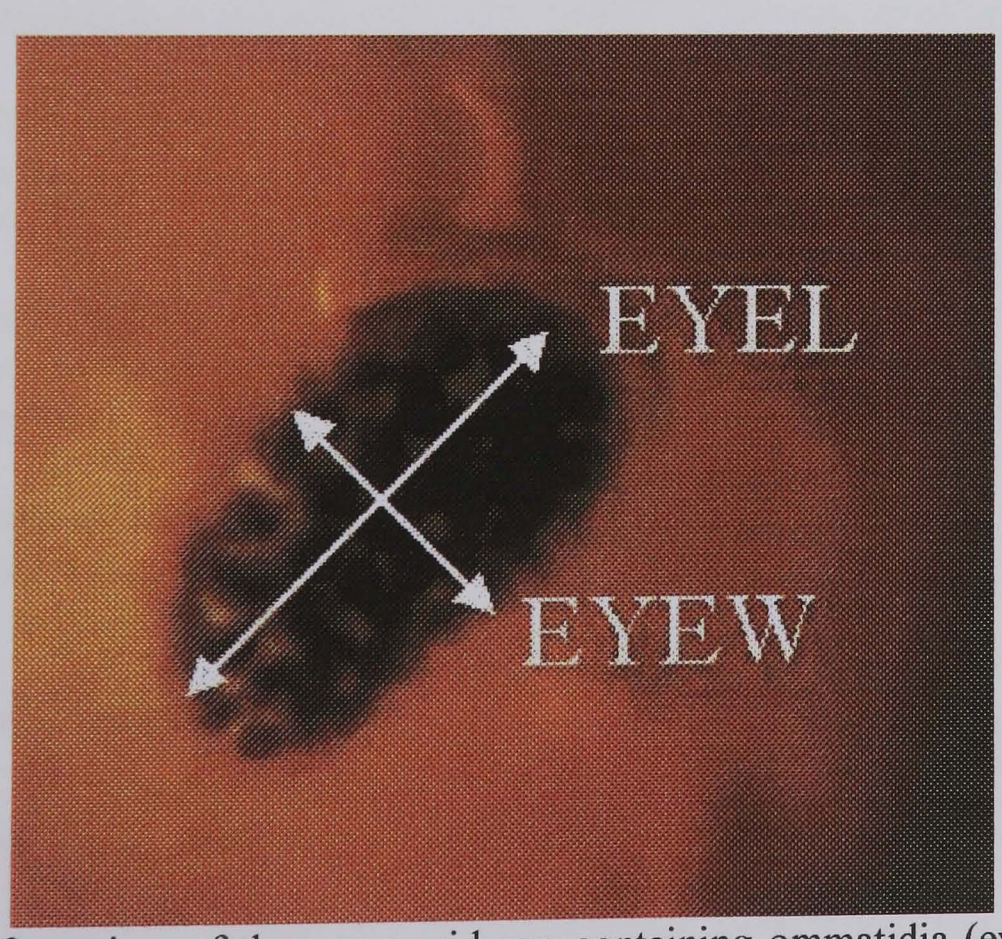


Figure 4.5: Surface view of the gammarid eye containing ommatidia (eye cells) with the position of the length (EYEL) and width of the eye (EYEW).

Table 4.2: Summary of the morphological character data collected from the right hand side of male *G. pulex*. Figures 4.5- 4.10 show the position of some of the morphological character counts and measurements

Morphological character	Abbreviation	Type of data
Length of the eye	EYEL	Measurement
Width of the eye	EYEW	Measurement
Length of the propodus of gnathopod one	GL	Measurement
Width of the propodus of gnathopod one	GW	Measurement
Length of the merus segment of the fifth pereopod	P5L	Measurement
Width of the merus segment of the fifth pereopod	P5W	Measurement
Length of pleopod one, not including the fringing setae	P0	Measurement
Length of pleopod one, including the fringing setae	P1	Measurement
Length of the body (from the anterior edge of the head section to the posterior tip of the third uropod	TL	Measurement
Number of ommatidia in the eye	EYEO	Count
Number of setae on the posterior surface of the basis segment of the fifth pereopod	P5BS	Count
Number of setae on the posterior surface of the basis segment of the seventh pereopod	P7BS	Count
Number of setae on the anterior surface of fourth peduncle article of antenna two	A2H	Count
Number of flagellar segments in antenna one	A1S	Count

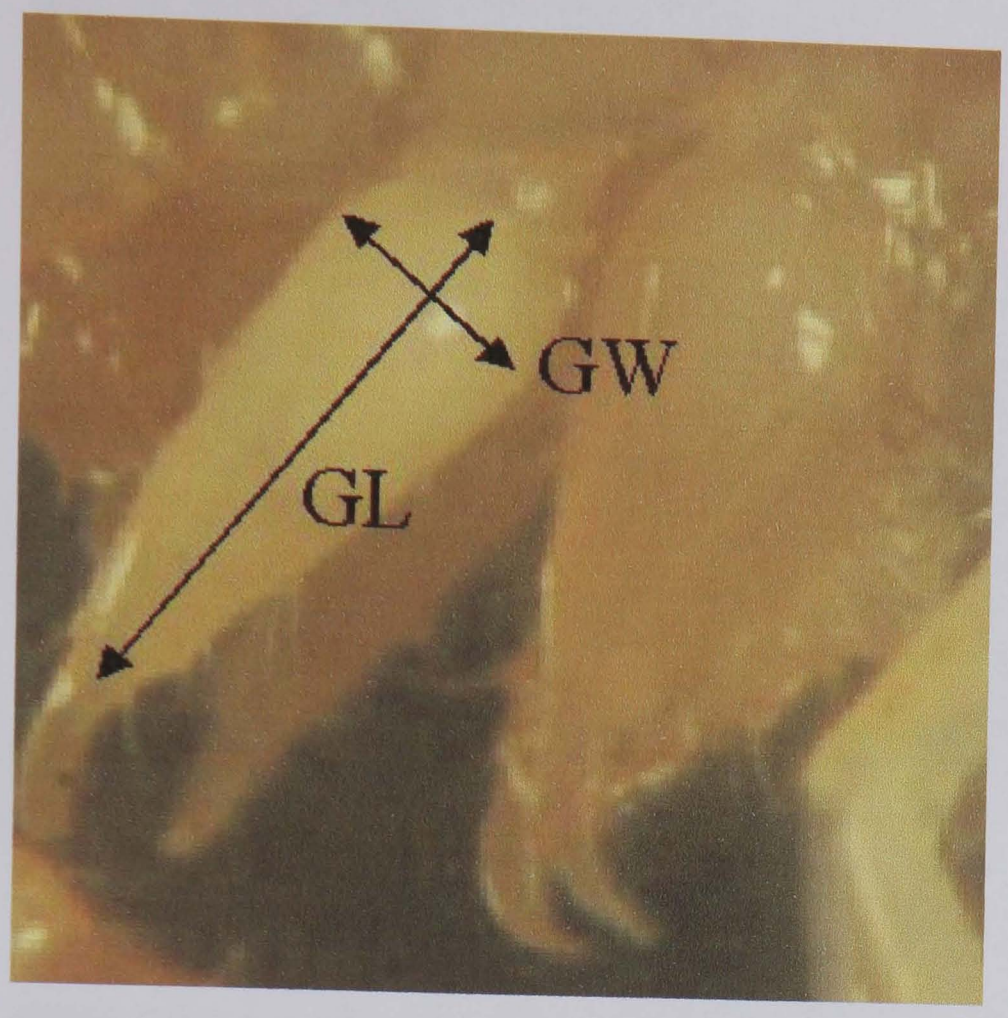


Figure 4.6: Surface view of the first gnathopod with the position of length (GL) and width (GW) measurements indicated.

Figure 4.7: Surface view of the basis section of the fifth pereopod, the arrows indicate the setae on the posterior surface that were counted (P5BS).

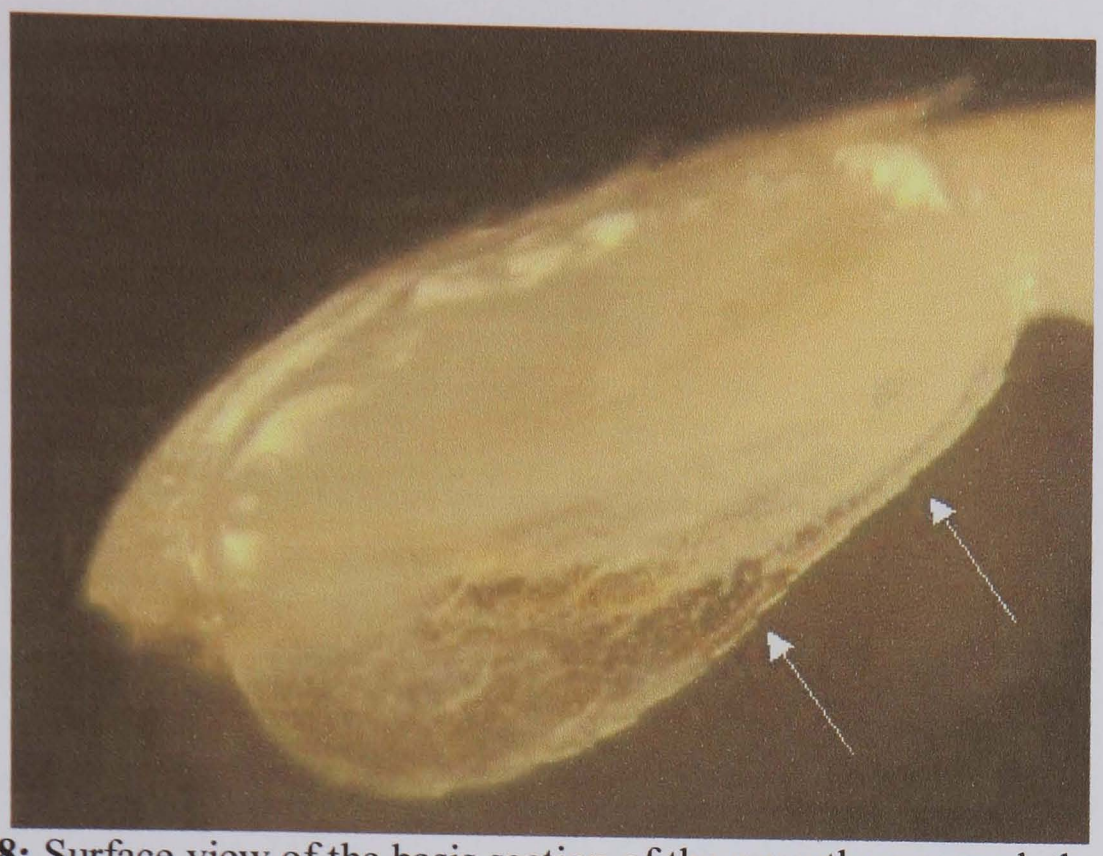


Figure 4.8: Surface view of the basis section of the seventh pereopod, the arrows indicate the setae on the posterior surface that were counted (P7BS).

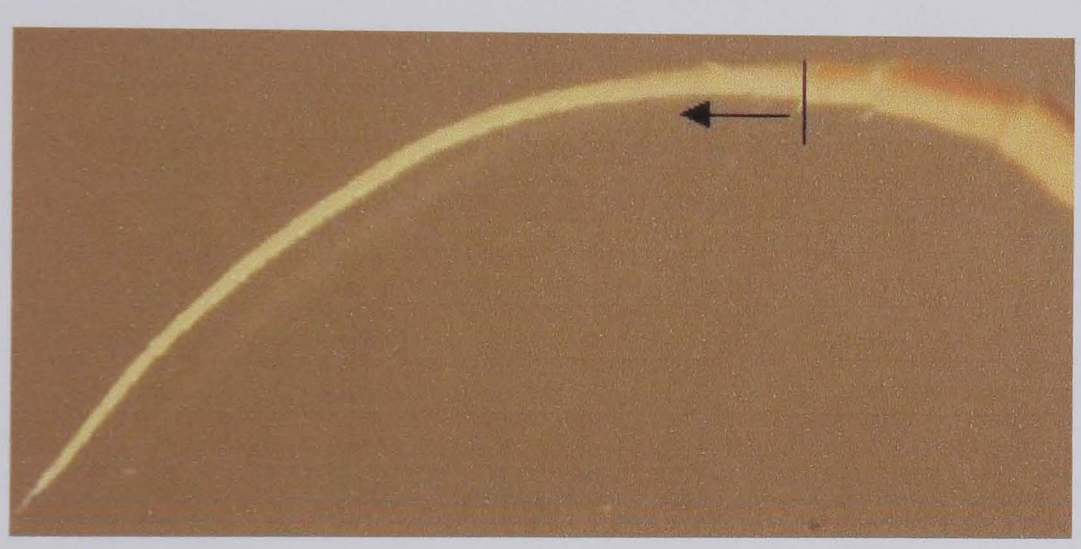


Figure 4.9: View of antenna one with the solid marking the point from which flagellar segments were counted (the junction between the third penducle segment and the first flagellar segment).

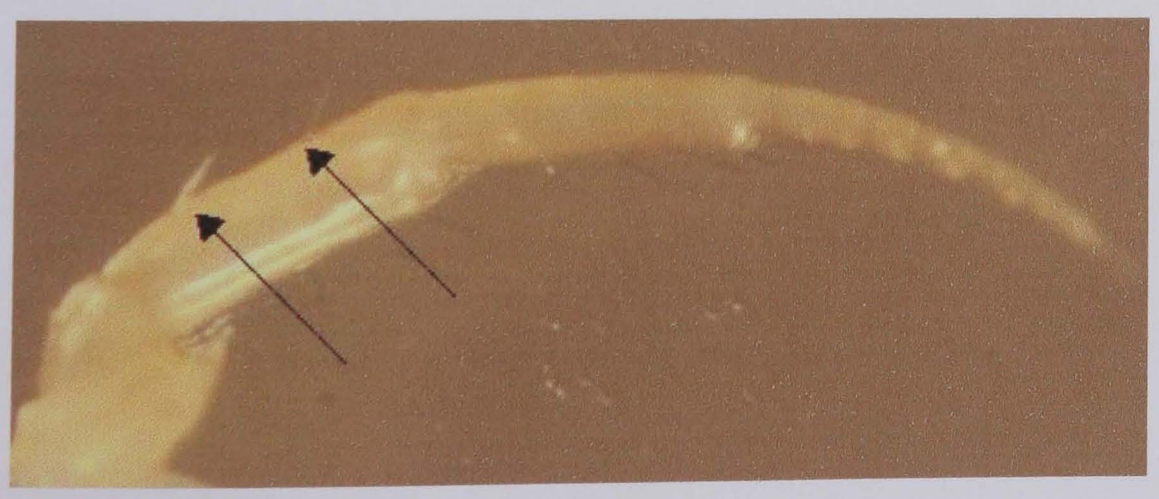


Figure 4.10: View of antenna two with the arrows highlighting the setae on the anterior surface of the forth peduncle segment (A2H).

4.2.4 Data analyses

4.2.4.1 Size adjustment for allometric growth

Samples of *G. pulex* previously examined from three populations, including Lowthorpe, showed a significant positive correlation between body size and most morphological traits measured (Farmer, 2000). The difference in growth, in terms of the size and shape of major legs relative to body size, enables a direct measurement of allometric growth in *Gammarus* to be made (Sutcliffe, 2000). Using a measure of body size, a correction for the effect of size variation on the total morphological variation measured can be carried out. One such measure used previously is head (cephalic) segment length (Goedmakers, 1981; Müller *et al*, 2000), which has been suggested to be more accurate than total length, due to the 'curvature' of the animals dorsal surface (Müller, *pers comm.*). However, local populations sampled by Farmer (2000) showed no significant correlation between body size and cephalic segment length, and so head length was rejected for use in the present study in favour of total body length.

A large proportion of morphological variance may be due to the effect of size, rather than shape variation in the data collected (Reist, 1985). Indeed, the significant differences in the size of animals taken from the wild and reared in the laboratory (Figure 4.11) could confound potential differences in shape variation. The original data required statistical transformation to separate size and shape variation allowing the analysis without the biased effect of size. There have been many methods proposed for size adjustment (Reist, 1985), all of which have limitations. Univariate approaches (ratios, regression techniques) have been favoured in the past to separate size and shape, but fail to take into account all measurements at the same time. Conversely, multivariate approaches use all measurements at once, but their ability to clearly separate size and shape has been questioned (Reist, 1985).

One univariate method was employed to correct for the effect of size variation on the total morphological variation in the 14 traits taken. The method used was proposed by Thorpe (1976) and allows for the effects of allometric growth:

$$M_{adj} = logM - b * (logSL - logSL_{avg})$$

Where:

M:	original measurement
M_{adj}:	size adjusted measurement
SL:	standard length
SL_{avg}:	overall mean of standard length
b:	pooled within-sample slope of regressions of logM against logSL

A second, multivariate, method used to correct for the effect of allometric growth was to use a principal component analysis (PCA) based approach, in which the first principal component was removed. The PCA approach assumes that the variance of the first principal component can be equated to the size from the original data (Rolf, 1967; Schnell, 1970). Removal of the first principal component should therefore eliminate the effect of size on the morphological shape variation. The model assumes that the main shape axis is perpendicular to the size axis. However, the model also assumes an isometric relationship between morphological traits and size, and that size is proportional to shape, neither of which may be correct.

The ability of both methods to separate size and shape was tested using the correlation between the first discriminant scores of the discriminant function analysis (section 4.2.4.3) and the total length from the original data. A significant correlation would indicate that there was still some size variation in the data.

4.2.4.2 Morphological variability

The coefficient of variation was calculated for all traits in all groups using SPSS ver 10.0.5 (SPSS Inc.) to estimate morphological variation within samples from populations with different means. The coefficient of variation can be calculated as the ratio of the standard deviation and mean, expressed as a percentage (Fowler & Cohen, 1993). A mean of the coefficient of variation across all traits for each group and across all groups for each trait were calculated and used as a comparative measure between groups, populations and traits.

4.2.4.3 Multivariate analysis

Data were corrected for size using both of the proposed methods (section 4.2.4.1) using SYSTAT ver 5.05 (SPSS Inc.) and SPSS ver 10.0.5 (SPSS Inc.). Once corrected, the data were analysed using a discriminant function analysis from SPSS ver 10.0.5 (SPSS Inc.). The discriminant function analysis produced five discriminant functions (equal to the number of groups or populations minus one), which can be plotted to give a visual map of individuals. Only the first two function scores were plotted to discriminate between the groups in a two-dimensional matrix. The discriminant function analysis was also used to build a predictive model of the group membership of individuals, based on the observed characteristics.

4.2.4.4 Univariate analysis

The first univariate approach to determining the environmental and genetic effects on morphological variation was based on an ANOVA. As the original data contained significant size variation, size adjusted (Thorpe (1976) method) data was used. The data were analysed using a general linear model ANOVA, using population and environment as the parameters of the model (without interaction effects) in Minitab ver 12.1. Additionally the original uncorrected data were used, but were corrected for the effect of size by using size as a covariate in the analysis (Farmer, 2000). Levels of

significance for each of the morphological characters were calculated for the effect of environment (lab or wild) and genotype (population).

Characters that displayed a significant difference for both environment and population were not analysed further due to the uncertainty of which factor was more important. Where characters displayed a significant difference for either population or environment, data were plotted to elucidate any patterns or observable trends in the data.

4.3 Results

4.3.1 Repeatability

Measurements and counts of the morphological characters were highly repeatable indicating that the size calibration used was virtually identical between the measuring sessions (Table 4.3). All of the initial counts recorded were identical when recounted in the ten individuals that were resampled and measured from each of the initial population samples. The lowest repeatability score was for the length of the first pleopod with fringing setae (P1), which was often damaged during the first measuring session, but the range of variance was unlikely to bias among sample comparisons.

4.3.2 Size adjustment for allometric growth

As predicted from the data of Farmer (2000) there was a significant difference in total body length between the wild populations sampled, with Lowthorpe containing the largest animals (Figure 4.11). Animals bred in the laboratory were significantly smaller than those sampled in the wild populations (Figure 4.11; T-test, $P<0.001$), with a similar level of variation (Table 4.4), though the age of the animals from the wild was unknown. There was a significant correlation between all of the original morphological

characters measured and total length, with the exception of the number of setae on the second antenna (Table 4.6).

Table 4.3: Repeatability and standard error (SE) of all morphological characters of a sub-sample of ten animals from each population.

Morphological character	Abbreviation	Repeatability (SE)
Length of the eye	**EYEL**	0.996(0.002)
Width of the eye	**EYEW**	0.993(0.004)
Length of the propodus of gnathopod one	**GL**	0.998(0.001)
Width of the propodus of gnathopod one	**GW**	0.999(0.001)
Length of the merus segment of the fifth pereopod	**P5L**	0.995(0.003)
Width of the merus segment of the fifth pereopod	**P5W**	0.994(0.004)
Length of pleopod one, not including the fringing setae	**P0**	0.997(0.002)
Length of pleopod one, including the fringing setae	**P1**	0.896(0.028)
Length of the body (anterior edge of the head section to the posterior of the third uropod)	**TL**	0.998(0.001)
Number of ommatidia in the eye	**EYEO**	0.991(0.006)
Number of setae on the posterior surface of the basis segment of the fifth pereopod	**P5BS**	1.000(0.000)
Number of setae on the posterior surface of the basis segment of the seventh pereopod	**P7BS**	1.000(0.000)
Number of setae on the anterior surface of fourth peduncle article of antenna two	**A2H**	1.000(0.000)
Number of flagellar segments in antenna one	**A1S**	1.000(0.000)

Differentiation of the populations using raw data would include a significant bias due to size and therefore the use of a size correction factor appears justified. The effectiveness of the two methods (Thorpe and PCA) at separating size and shape variation was difficult to estimate. Data corrected using the method proposed by Thorpe (1976) had a significant positive correlation between the first discriminant function score and the original total length (Figure 4.12, $r^2=0.043$, $P=0.000$). The significant relationship between the original data and function scores (second and third functions were also significant $P=0.000$) suggested that some effect of size remains in the corrected data.

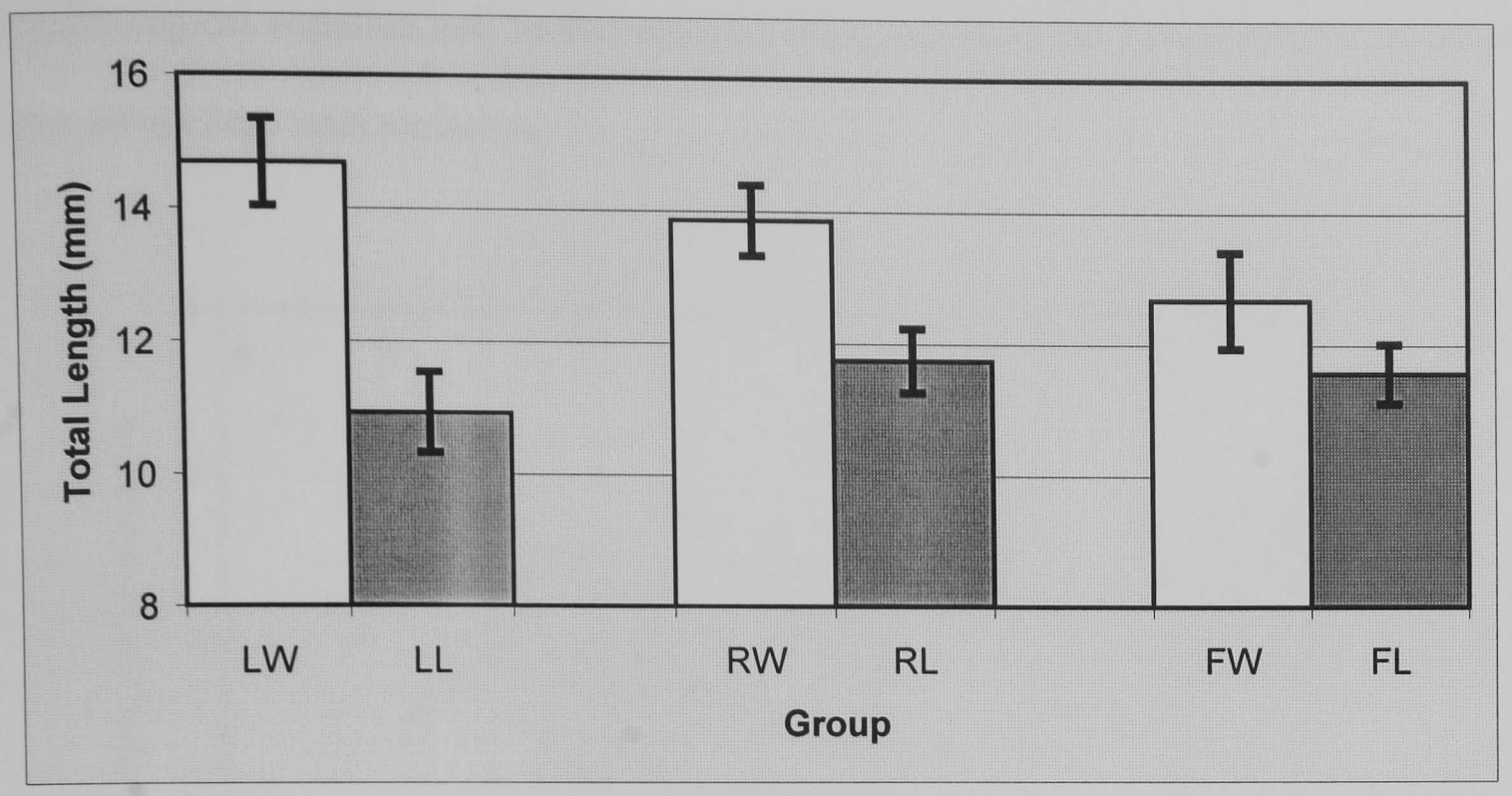


Figure 4.11: Mean total length of *G. pulex* from the three populations in wild (LW, RTW & FW) and reared in the laboratory (LL, RTL & FL), with errors bars indicating the 95% confidence interval of the mean.

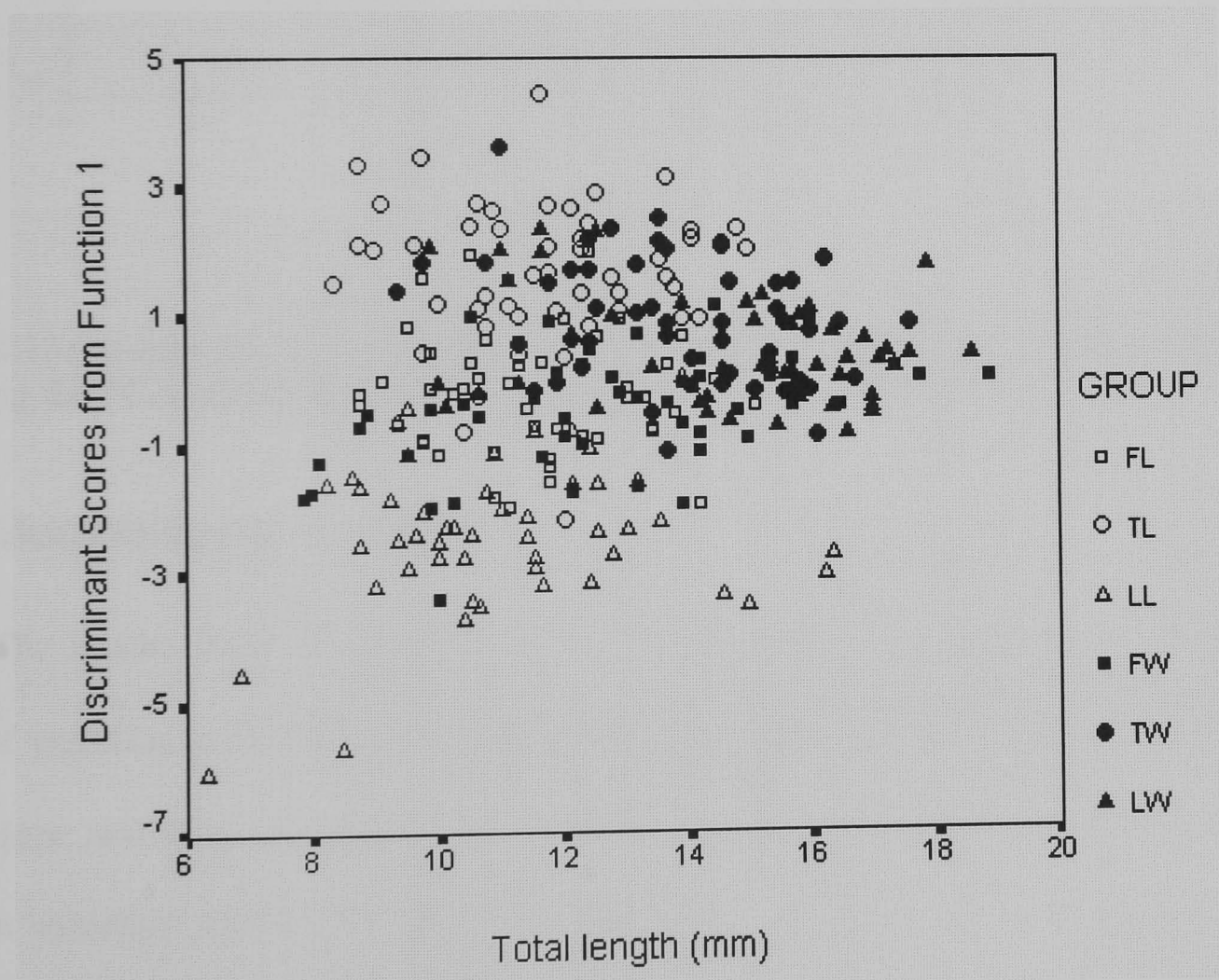


Figure 4.12: Scatter plot of the discriminant score of the first axis (data corrected using the method of Thorpe (1976)) against the original total length for all groups

Data corrected using the PCA method showed no significant correlation between the first discriminant function score and the original total length (Figure 4.13, *P=0.435*). The first principal component (~ size) accounted for nearly 60% of the total

morphological variation and further analyses (discriminant function) would be based on only 40% of the total variation.

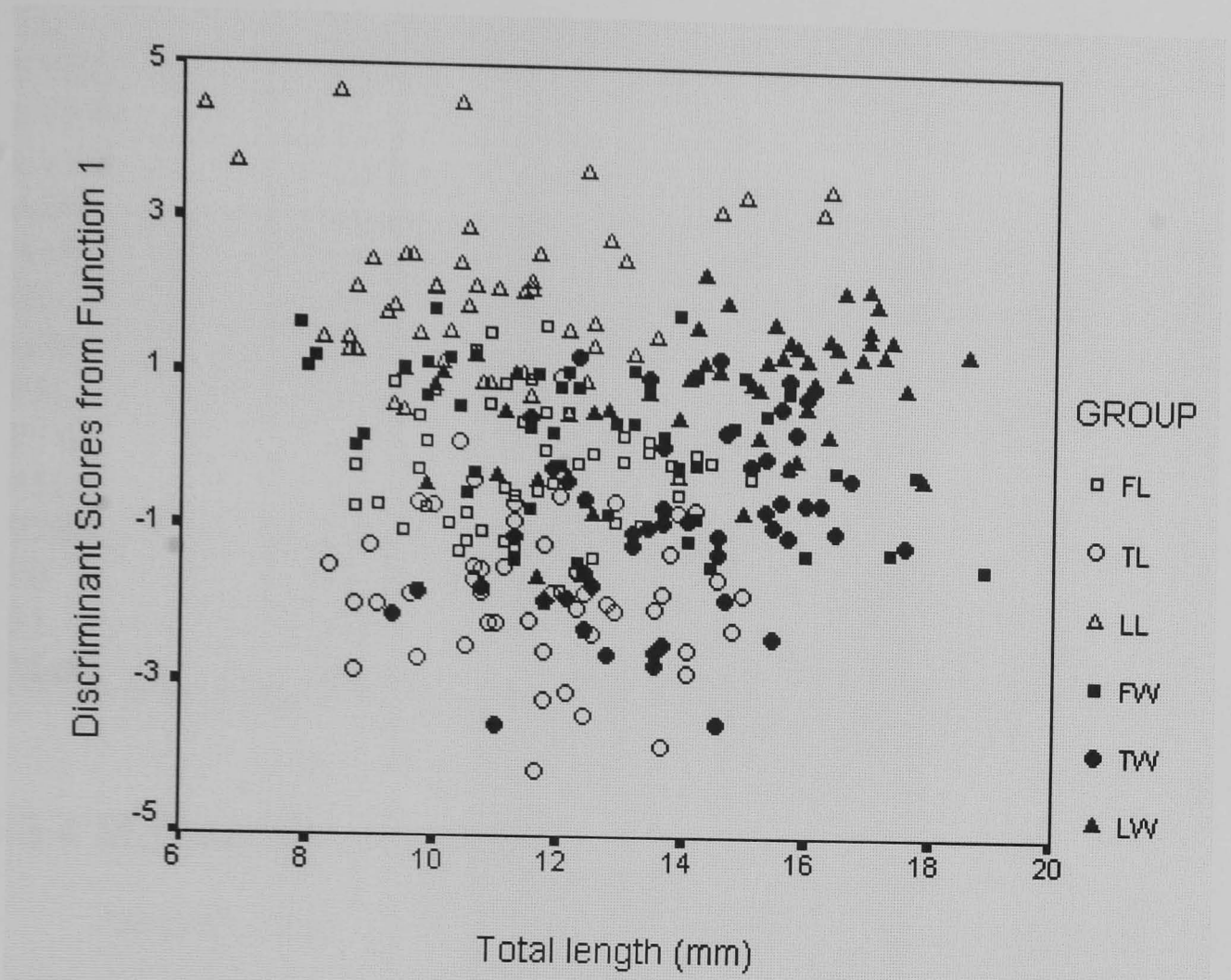


Figure 4.13: Scatter plot of the discriminant score of the first axis (data corrected for size using the PCA method) against the original total length for all groups

4.3.3 Morphological variation

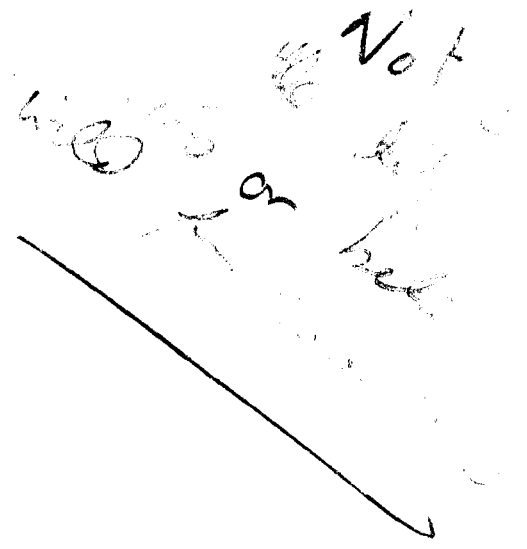
The results from the coefficient of variation (table 4.4) show that the overall levels of variation recorded at the 14 morphological characters were similar in all six groups (see raw data, Appendix 4.1). There was no clear pattern in the levels of variation recorded between the laboratory-reared and wild groups. Animals from the lab-reared groups did not show any consistent trends in the levels of variation, when compared to their wild comparators. Data for the variation measured in the number of setae on the second antenna was greatest in the Lowthorpe groups, with very high variation (compared to all other groups) recorded in the wild samples (60.38%).

Table 4.4: Mean coefficient of variation percentages for the sampled groups (mean across all 14 morphological characters and across groups). Abbreviations of populations and morphological characters defined in Tables 4.1 & 4.2.

Morphological character	LW	LL	TW	TL	FW	FL
TL	15.75	19.64	13.56	14.44	20.28	13.72
EYEL	10.66	19.35	8.33	9.82	17.85	10.65
EYEW	12.12	15.14	11.93	10.88	17.93	11.31
EYEO	9.90	21.42	13.64	15.98	17.43	14.93
A2H	60.38	35.91	22.30	14.43	15.05	19.26
A1S	12.77	10.79	13.51	13.99	21.28	12.12
GL	17.18	18.50	19.97	20.64	20.09	18.56
GW	17.07	16.01	19.11	22.26	19.66	18.75
P5L	14.47	13.16	13.32	17.27	19.03	14.07
P5W	14.43	16.43	12.94	17.62	17.72	14.72
P5BS	12.55	10.93	10.30	15.03	12.49	14.15
P7BS	10.73	15.84	12.20	14.98	13.79	14.08
P0	13.80	15.09	12.76	12.89	20.18	13.26
P1	12.84	14.59	12.12	13.47	17.64	12.55
Mean	**16.76**	**17.34**	**14.00**	**15.26**	**17.89**	**14.44**

4.3.4 Multivariate analysis

Analysis of all morphological data produced clear separation of some groups between the populations and environments (Figures 4.14 & 4.15). Separation between groups from the same population was clear in the case of Lowthorpe, where there was little overlap between wild and laboratory-reared animals. Conversely, separation between wild and lab-reared animals from Fleshwick Bay and River Terrig were weak, with little difference in their distributions on the discriminant analysis plots (Figures 4.14 & 4.15). Animals from Fleshwick Bay had a greater distributional overlap with the animals from the River Terrig than with those from Lowthorpe.



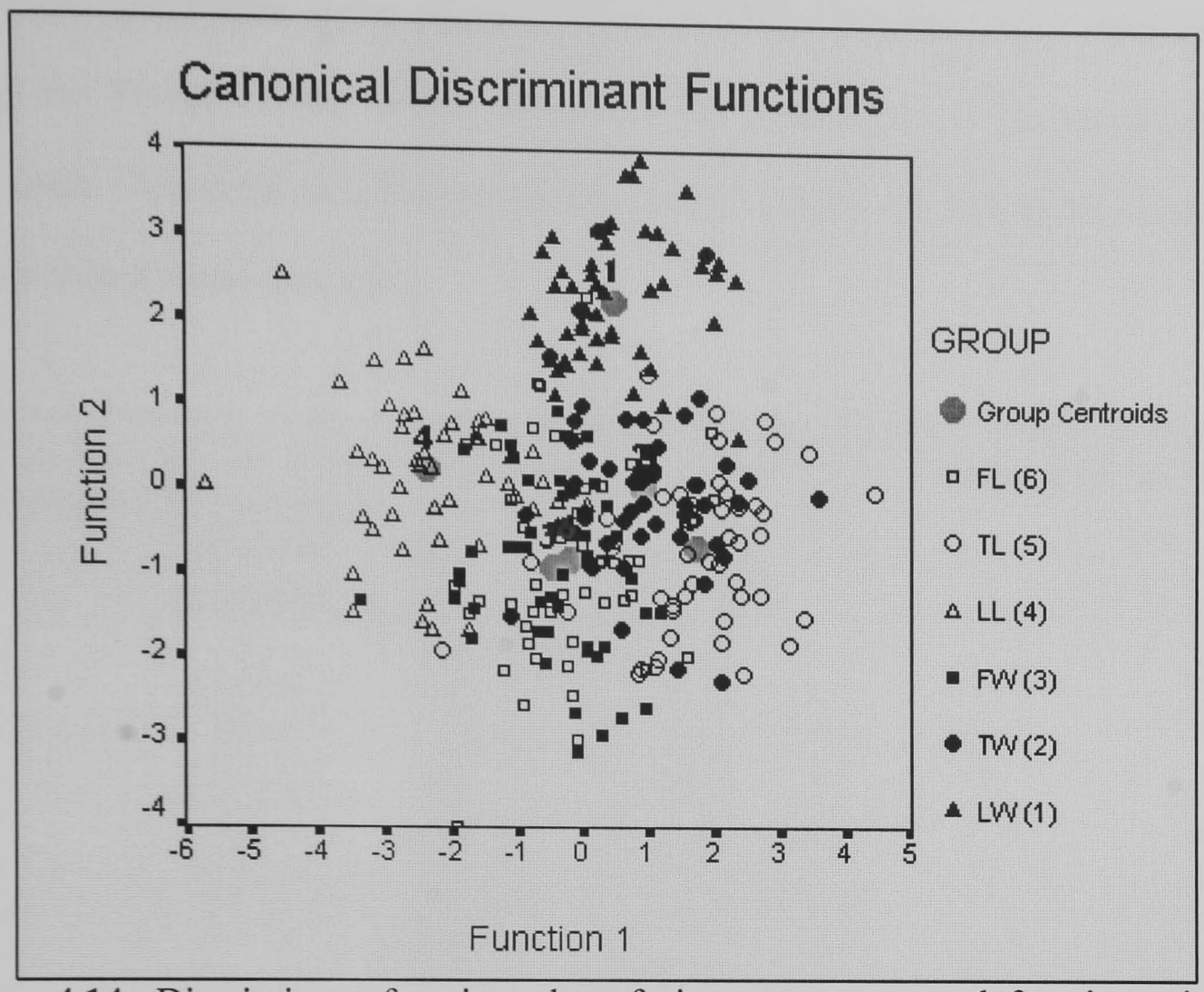


Figure 4.14: Discriminant function plot of six groups corrected for size using the method of Thorpe (1976).

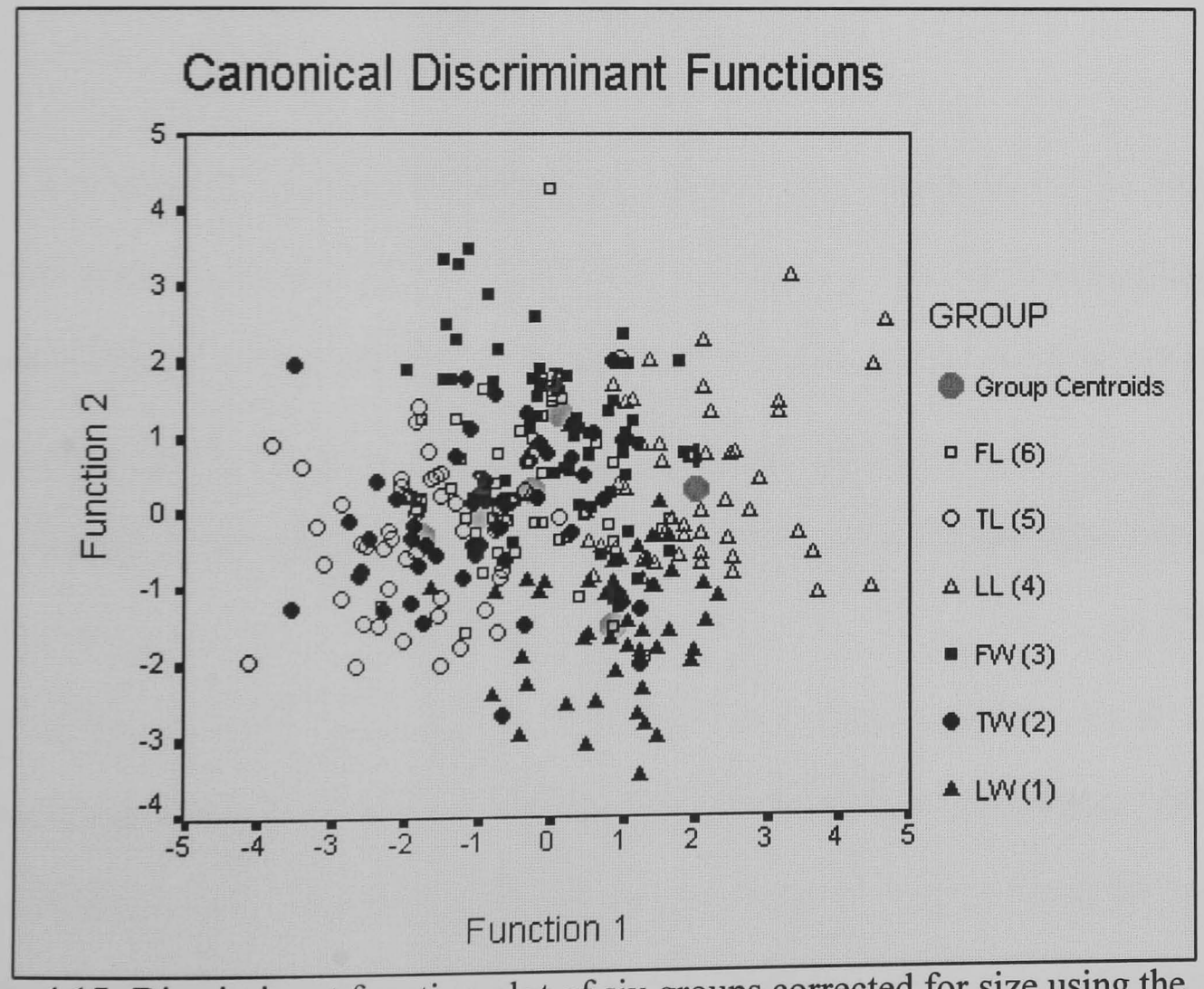


Figure 4.15: Discriminant function plot of six groups corrected for size using the principal component (PCA) method.

The assignment scores using both size correction methods were very similar, though the Thorpe (1976) method always assigned individuals to the correct groups more often. The difference between the two sets of scores was 5% or less, except for animals from Lowthorpe (26%).

Table 4.5: Summary of the overall assignment scores of individuals to the correct group based on discriminant function analyses, using data corrected for size with the Thorpe (1976) and the PCA methods.

Groups analysed	Data type	% Group membership
All samples	Thorpe	79.9
	PCA	77.0
Wild samples	Thorpe	88.3
	PCA	83.3
Lab samples	Thorpe	87.0
	PCA	85.3
Lowthorpe	Thorpe	100.0
	PCA	74.0
River Terrig	Thorpe	85.9
	PCA	82.0
Fleshwick Bay	Thorpe	93.0
	PCA	91.0

Separation of the Belgian and the UK samples on morphological data was not clear with significant overlap in the distribution of animals in the discriminant function analysis (Figure 4.16). The lack of separation in the populations was reflected in the lowest assignment score for four sampled populations of 73.9%.

The ANOVA results based on environment and population are shown in Table 4.6. Overall there was strong agreement between the two sets of data used (Thorpe correction and size as a covariate), though the Thorpe data appeared more sensitive. All traits were significant for the effects of either population and/or environment. In total, seven characters were significant for population (genetic effect), three for rearing condition (environmental effect) and five were significant for both population and environment. Table 4.7 summarises the effect of environment and population on the morphological characters recorded

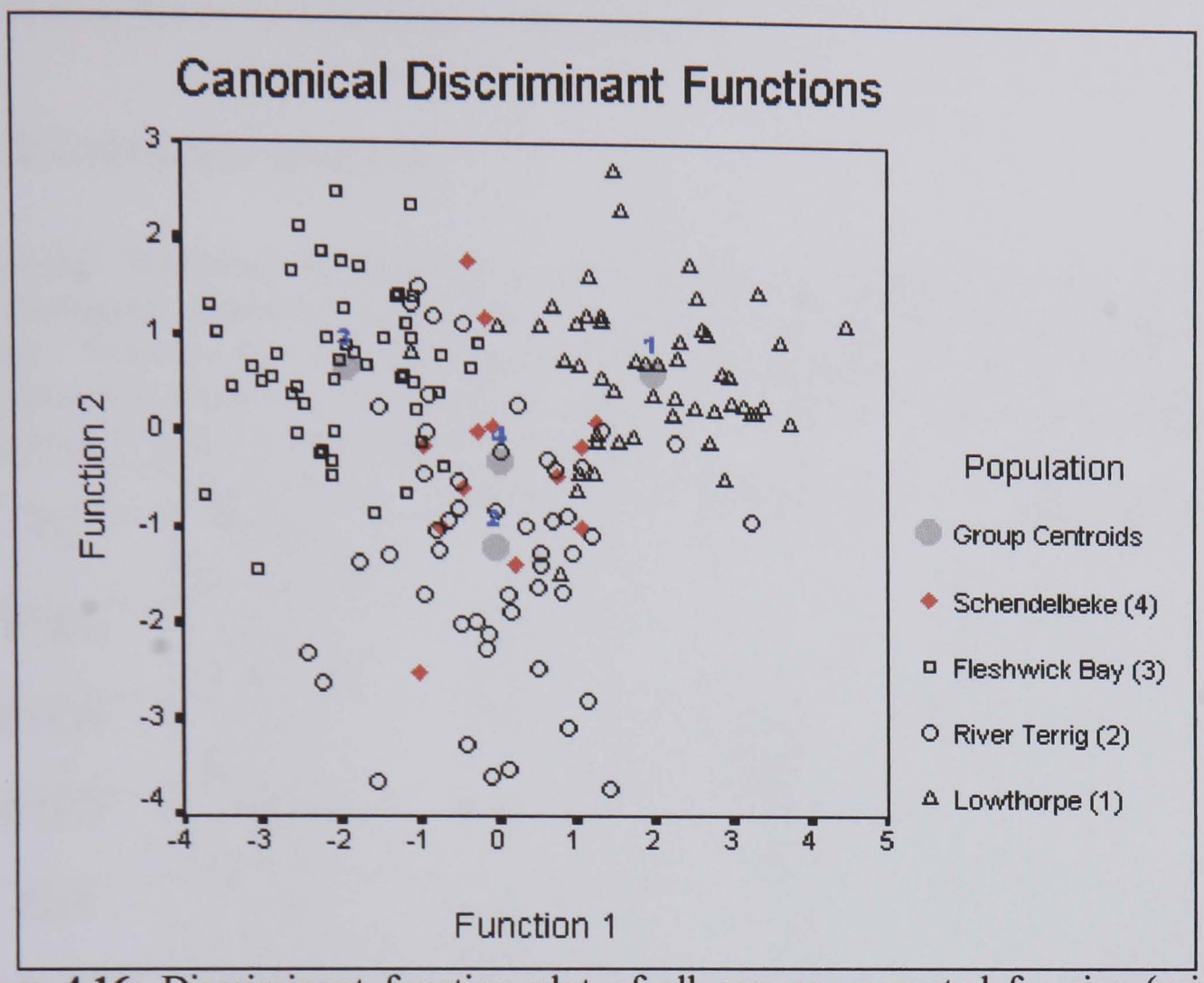


Figure 4.16: Discriminant function plot of all groups, corrected for size (using the method of Thorpe (1976)), including 13 animals from Schendelbeke, Belgium

4.3.5.1 Pleopod size

The ANOVA results showed significant differences between populations for the corrected length of the pleopods, with and without fringing setae, but not between environments and were significant for the interaction term. Figure 4.17 shows the difference in the size of pleopods between the three populations in both environments. There was no clear trend in the data with animals from Lowthorpe having a reduced pleopod length in the lab, the River Terrig having an increased length, and Fleshwick Bay showing little change between the environments. Figure 4.17 shows visually the interaction between population and environment, with each population responding differently to the switch in environment. The length of the fringing setae on the

pleopods was however highly conserved between populations, with a mean length of 20.5% (± 4.4%) of the total length of the pleopods.

4.3.5 Univariate analysis

Table 4.6: Statistical significance of univariate general linear model ANOVAs for all morphological characters using population and environment (lab or wild) as fixed factors. Tests for data corrected for size (Thorpe) using the formula of Thorpe (1976) and corrected using total length as a covariate in the general linear model (GLM-Cov) are given. (*:p<0.05; **:p<0.01; ***:p<0.001).

	Correction	Length	Population	Environment
TL	Thorpe		*	***
	GLM-Cov			
EYEL	Thorpe	NS	***	***
	GLM-Cov	***	***	***
EYEW	Thorpe	NS	NS	**
	GLM-Cov	***	NS	*
EYEO	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
A2H	Thorpe	NS	***	**
	GLM-Cov	NS	***	NS
A1S	Thorpe	*	NS	***
	GLM-Cov	***	NS	***
GL	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
GW	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
P5L	Thorpe	**	***	***
	GLM-Cov	***	***	***
P5W	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
P5BS	Thorpe	NS	**	NS
	GLM-Cov	***	**	NS
P7BS	Thorpe	NS	***	**
	GLM-Cov	**	***	*
P0	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
P1	Thorpe	NS	***	NS
	GLM-Cov	***	***	NS
Eye-Area	Thorpe	*	***	***
	GLM-Cov	***	***	***
Density	Thorpe	NS	NS	***
	GLM-Cov	***	NS	***

Table 4.7: Summary of Thorpe size corrected data, indicating significance of environment, population or a combination of both for the morphological characters.

Environment	Population	Population and Environment
Eye width Antenna 1 Eye density	Ommatidia Gnathopod length Gnathopod width Pereopod 5 width Pereopod 5 setation Pleopod P1 Pleopod P0	Eye length Antenna 2 Pereopod 5 length Pereopod 7 setation Eye area

4.3.5.2 Flagellar segments of antenna one

The number of segments in antenna one was significantly different between the two environments (wild and laboratory reared), but not significantly different between populations (Table 4.6). Figure 4.18 shows a regression plot of body length and flagellar segments and the associated variance between the two characters observed. However, even with the high level of variance, the laboratory-reared animals had a higher number of flagellar segments for the same body size as wild animals, as indicated by the regression slopes.

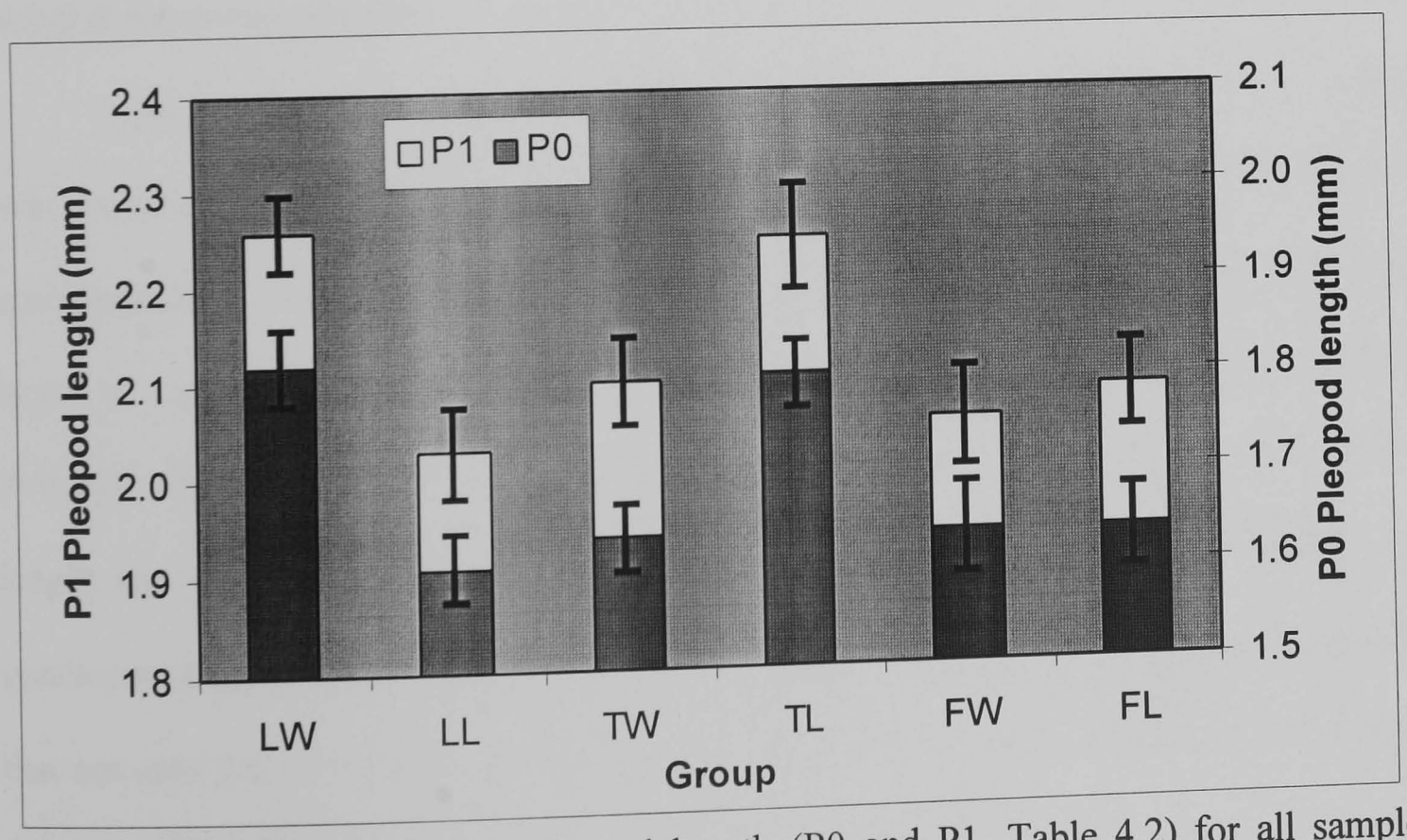


Figure 4.17: Mean corrected pleopod length (P0 and P1, Table 4.2) for all samples (wild and lab) from the three populations (defined in Table 4.1). Y-axis error bars display the 95% confidence interval of the mean.

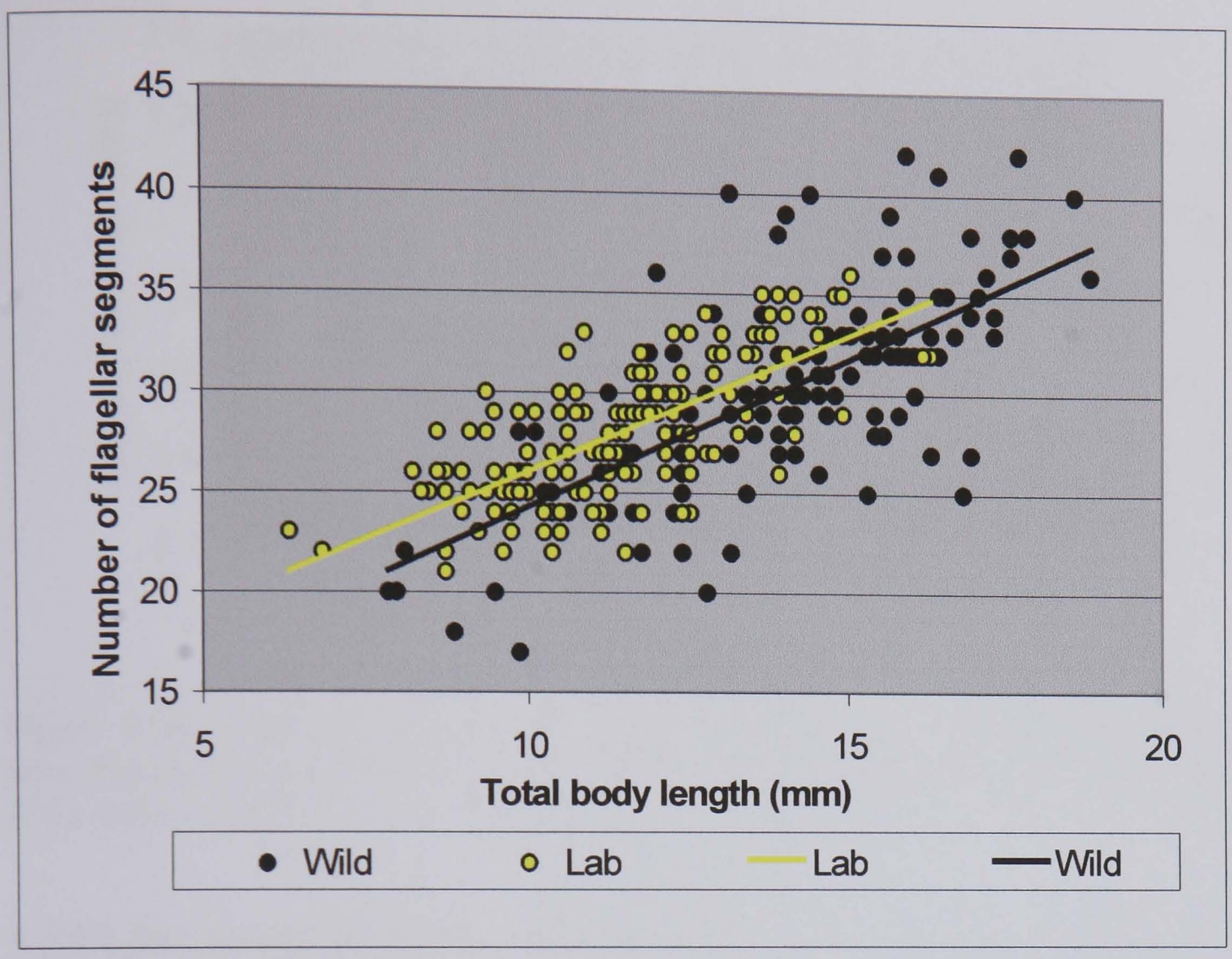


Figure 4.18: Plot of body size versus the number of flagellar segments in antenna one for wild and laboratory-reared animals. Lines indicate best-fit regression slopes.

4.3.5.3 Gnathopod one

Like the length of the pleopods, the size of gnathopod one (length and width) was significantly different between populations, but not between the different environments. Both the mean length and width of gnathopod one are significantly larger in Lowthorpe than both the River Terrig and Fleshwick Bay samples (T-test $P<0.01$). Figure 4.19 shows clearly the significant difference in the mean corrected length and width of gnathopod one between the three populations. Differences in gnathopod length, between the groups, were greater than in width, which would indicate that not only the length but also the shape of the gnathopods are different between the groups.

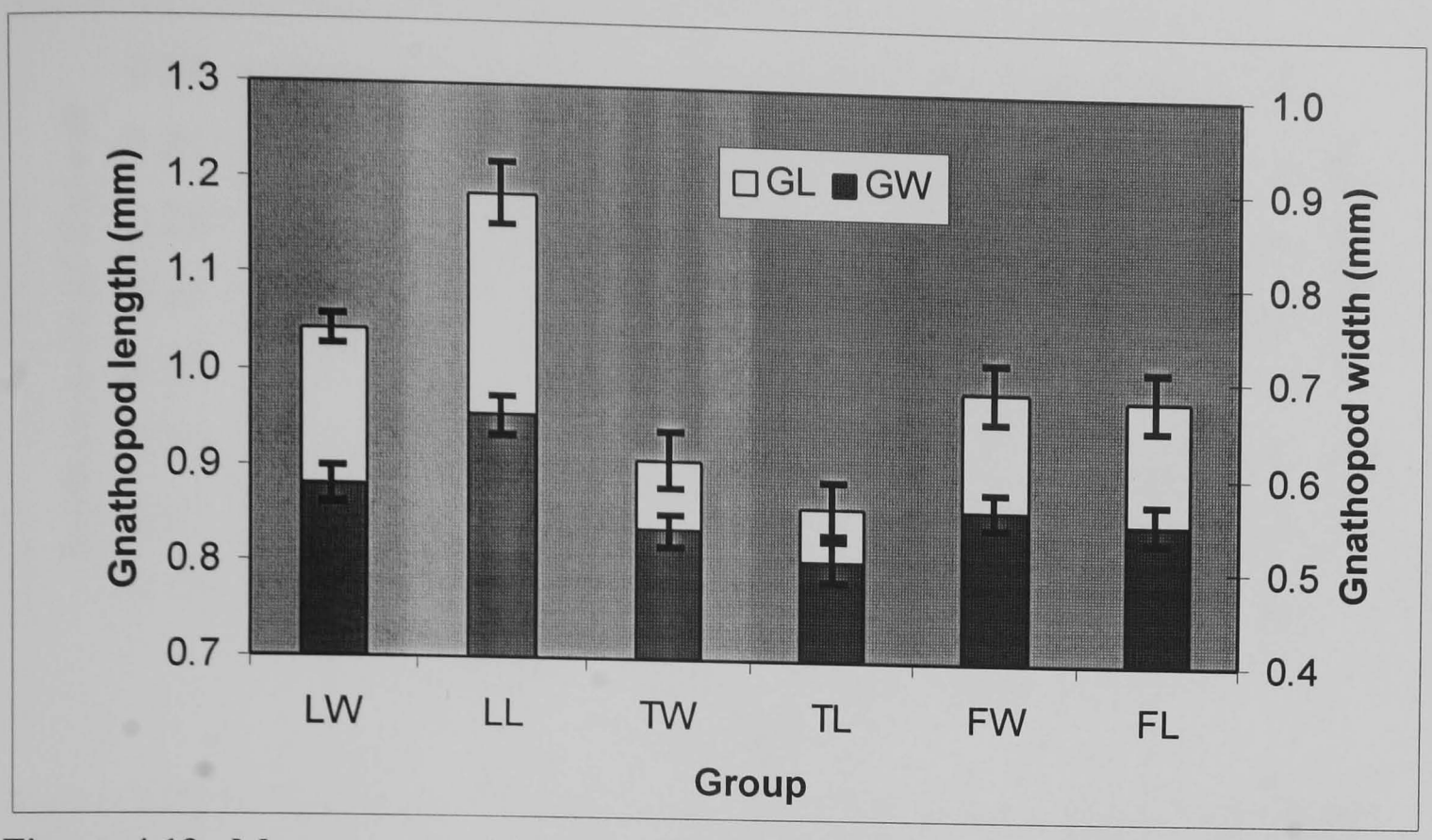


Figure 4.19: Mean corrected length (GL) and width (GW) of gnathopod one of the three populations (wild and lab). Y -axis error bars display the 95% confidence interval of the mean. Population abbreviations given in Table 4.1.

4.3.5.4 Eye area and density of ommatidia

The surface area of the eye was significantly different between populations and between environments (Table 4.6). The relative eye surface area was larger in animals reared in the lab compared to those animals sampled from the wild. Individuals from Lowthorpe had the smallest relative eye area and River Terrig the largest (Figure 4.20). Unlike the area of the eye, the density of ommatidia was significantly different only between environments, with animals in the lab always having a lower mean corrected eye density than animals from corresponding populations taken from the wild (Figure 4.21).

The different relationships between the density of ommatidia and total length for the six groups can be more clearly observed in figure 4.22. Significant differences in the slope and intercept of the plots were recorded for the Lowthorpe and Fleshwick Bay populations, but not the River Terrig.

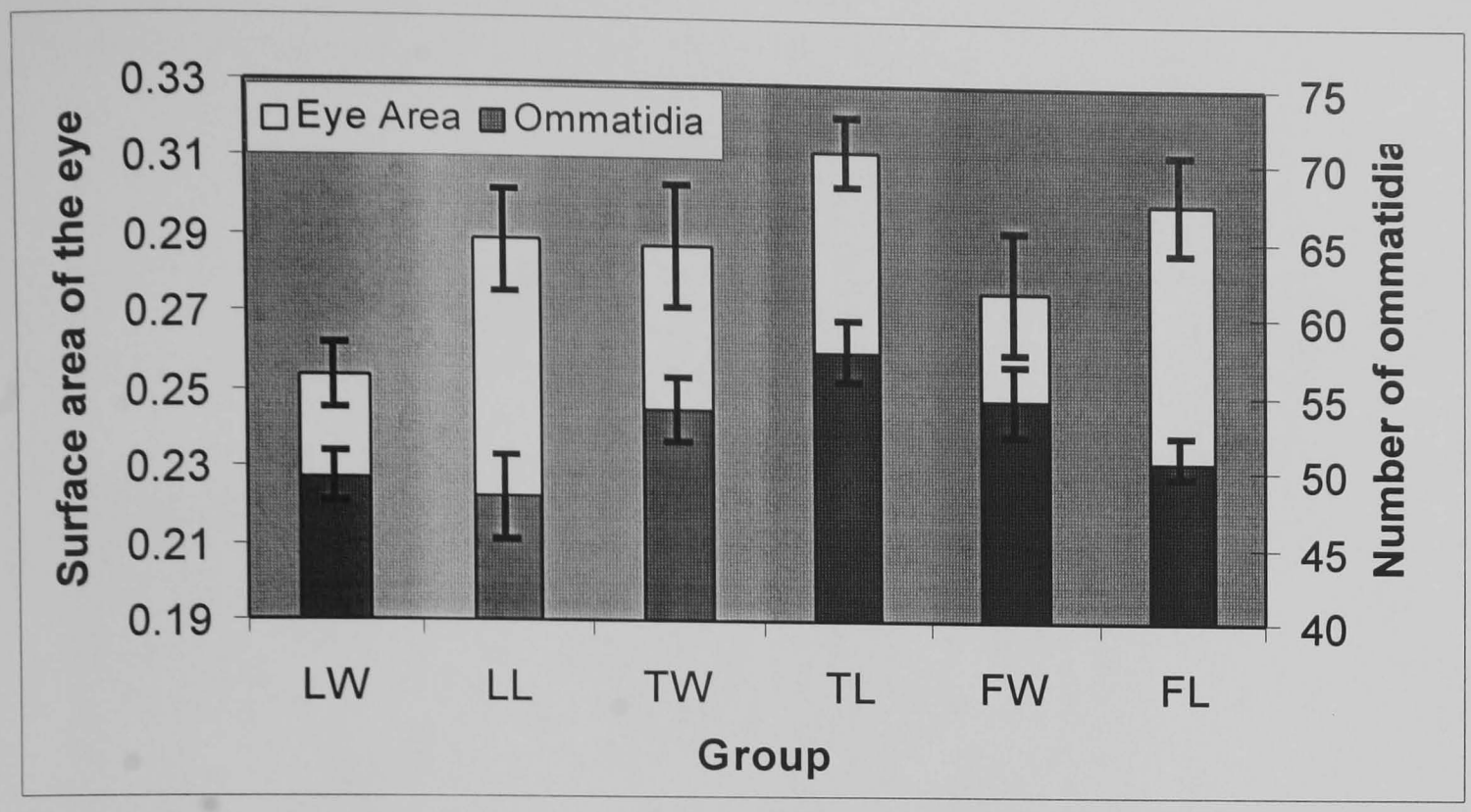


Figure 4.20: Histogram of the mean corrected eye area and number of ommatidia for wild and laboratory-reared animals for all populations. Y -axis error bars display the 95% confidence interval of the mean. Population abbreviations defined in Table 4.1.

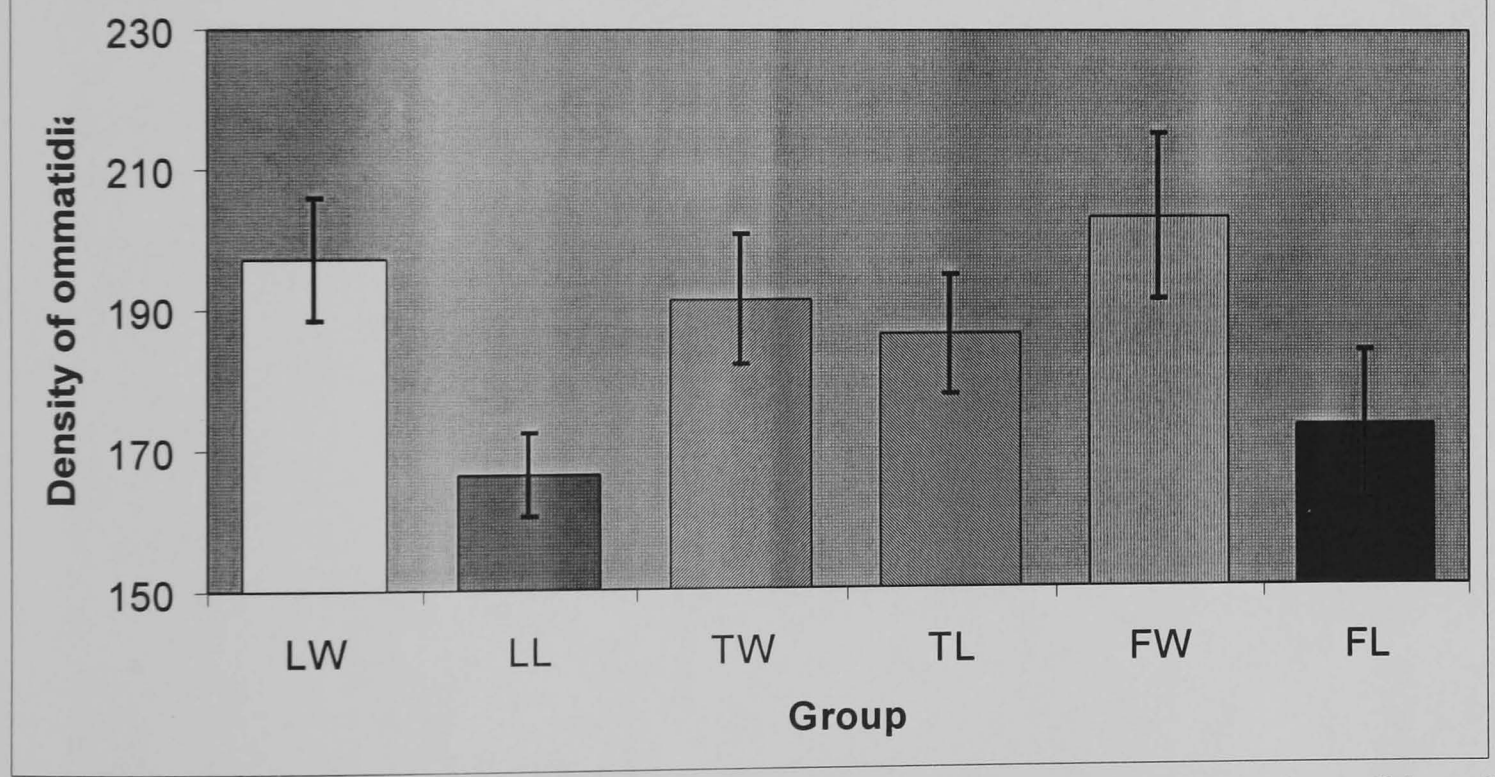


Figure 4.21: Histogram of the mean corrected density of ommatidia for wild and laboratory-reared animals for all populations. Y -axis error bars display the 95% confidence interval of the mean. Population abbreviations defined in Table 4.1.

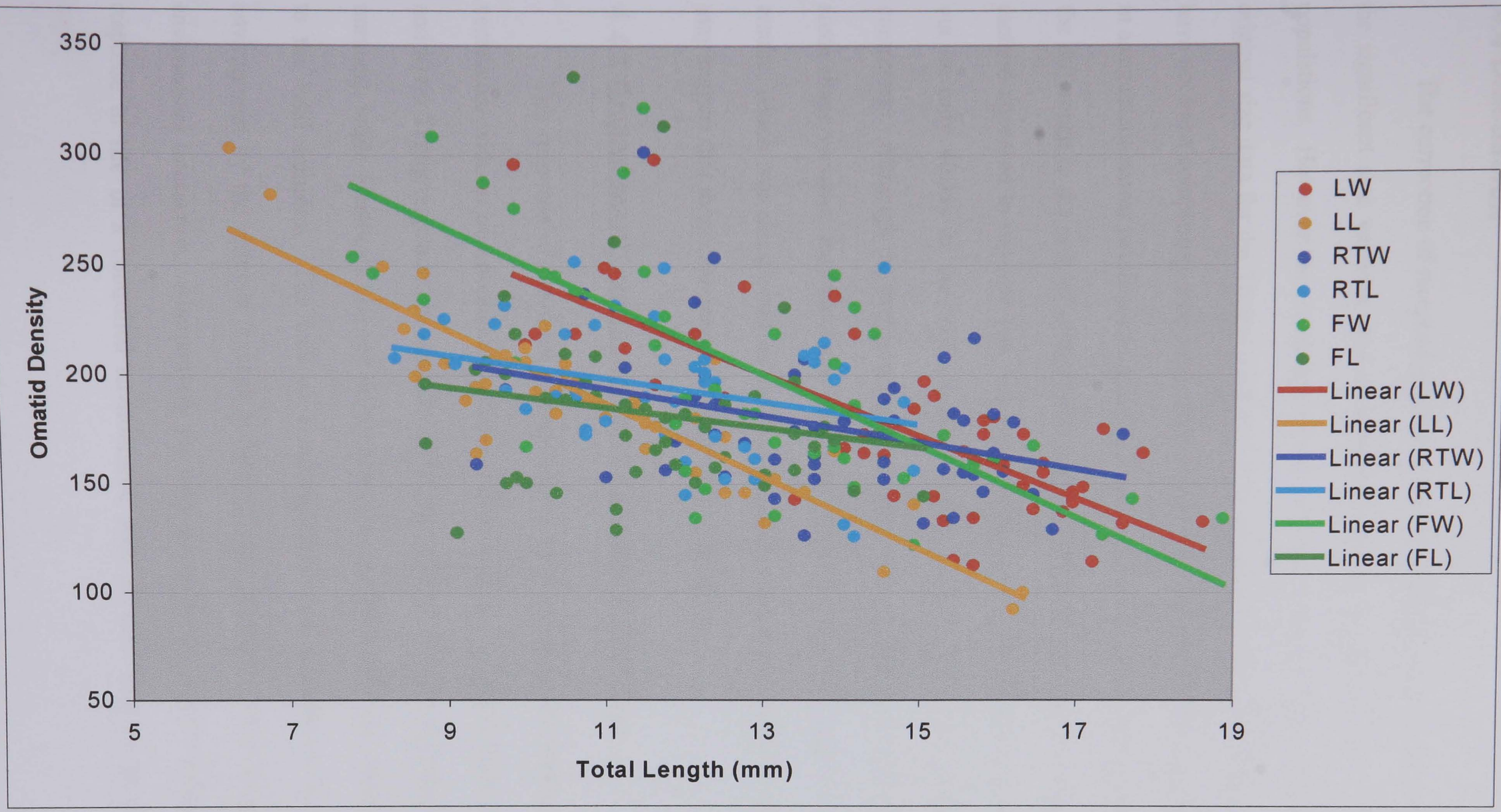


Figure 4.22: Regression plot of ommatidia density (cells cm^{-2}) and length.

4.4 Discussion

The correction of morphological data for the effect of size was necessary due to the significant size variation and correlation between size and all other traits in the populations. However the correlation between the discriminant function scores and the original size data for the Thorpe method (1976) suggested that size and shape variation have not been completely separated. This was further supported by the large difference in assignment scores between the correction methods for the Lowthorpe animals, where the difference in the size of animals was the most marked. Correction using the PCA method appeared to separate size and shape variation, though the analysis was carried out on only 40.3% of the total variation due to the removal of the first principal component. Although the first principal component was equated to the size component, some shape variation may have also been lost. Overall both methods gave comparable results, which was unexpected as the PCA method assumes an isometric growth, and may suggest that most characters indeed grew isometrically.

4.4.1 Environment, population and separation of groups

The response of the three populations to the controlled environment was not correlated, with no one population behaving like the other. Animals from Lowthorpe and River Terrig responded in opposite directions (relative increase in character size or number), while Fleshwick showed little response to the lab conditions, when compared to the wild samples. The lack of a correspondence in response to a controlled environment by the animals (except in antenna one) suggests a possible genotype - environment interaction. Alternatively the three populations could be distinct in their response to life in a controlled environment, with possible bias to the Lowthorpe

population, where environmental conditions (conductivity and levels of available calcium) were more similar to the laboratory than the other two.

Morphological differences between the three populations supported the history of the three populations and the relationship between Fleshwick Bay and River Terrig. The separation of Lowthorpe samples (both wild and lab reared) from the other two populations supports the predicted lack of recent gene flow. Additionally, the recent introduction in Fleshwick Bay from the River Terrig population may be reflected in the overlapping distribution in the multivariate approach used here. However, the lack of separation of samples from Belgium was very surprising. Molecular genetic analysis of the same samples indicated 10% nucleotide divergence between the UK and Belgian samples, which was proposed to differentiate subspecies (chapter 5). The morphological data did not support the molecular data in this case, though sample sizes from the Belgian site were small.

The variation in the morphological characters used revealed significant differences between both populations and environments (wild and laboratory reared). Two sets of traits (gnathopod one and pleopod one) showed significant differences between the wild caught individuals and those reared in the laboratory, indicating that some traits studied were under environmental, rather than genetic control. Conversely two other traits displayed significant differences between animals from the wild and those reared in the laboratory (eye and antenna one), indicating a possible phenotypic plasticity in those traits. These four sets of traits are discussed further in the following sections. The remaining traits that showed significant differences for both environment and population limited any further analysis of the genetic and environmental influences.

4.4.2 Oxygen demand and pleopod size

There are two main functions of the pleopods in freshwater amphipods, the first is to provide a propulsive force for swimming, and the second is to ventilate the gills (Boudrais, 1991; Sutcliffe, 1984). Swimming is used to migrate to new habitats and to escape predators, by producing short bursts of rapid swimming (Boudrais, 1991). The morphology (size and nature of the setation) and beat frequency of the pleopods will determine the speed at which animals can swim, and would therefore be expected to influence fitness and hence subject to natural selection.

Beating of the pleopods also creates a turbulent water flow in the gill cavity and increases the rate of oxygen diffusion across the surface of the gills (Sutcliffe, 1984). The ability to sustain a high metabolic energy demand of the animal, by replenishing water in the gill cavity, will be directly affected by the size and beat frequency of the pleopods. During exposure to periods of hypoxia, freshwater *G. minus* responds by hyperventilation in an attempt to maintain the oxygen supply (Hervant *et al*, 1999). This response to temporary hypoxic conditions has also been observed in other hypogean species, such as *G. roeseli* (Henry & Danielopol, 1999). In contrast, hypoxia tolerant species have adapted to continual hypoxic conditions by reducing their metabolic rate (and the associated oxygen demand) and therefore the need for hyperventilation (Hervant *et al*, 1999).

The significance of the lower mean pleopod length (with and without setae) found in the Fleshwick Bay population (wild samples only) could be due to a reduction in the ability of animals in the population to respond to periods of high metabolic energy demand (swimming and predator avoidance) or periodic hypoxic water conditions. In the three populations sampled there was no evidence to suggest that hypoxic conditions (anaerobic substrate) existed continually, though transitory or localised hypoxia cannot be dismissed.

The reduced pleopod size could be a local adaptation to the predation regime in Fleshwick Bay as *G. pulex* escapes predators with short bursts of rapid swimming. One of the main predators of *G. pulex*, the bullhead (*Cottus gobio*), was absent from Fleshwick Bay, but present in the two other populations (personal observations). The absence of one of the main predators of *G. pulex* in Fleshwick Bay and the correlated smaller pleopod size is not conclusive proof of an adaptive response to a reduced predation pressure. However the similarity in pleopod size between animals reared in the laboratory, without predators and hypoxic conditions, and wild animals adds further support to the predation hypothesis. There were no such similarities in the Lowthorpe and River Terrig populations, with the former having smaller mean pleopod size in the laboratory and larger mean pleopod size recorded in the latter.

4.4.3 Flagellar segments of the first antenna

The smaller number of flagellar segments (per unit body length) recorded in all wild populations (compared to the lab) suggests that conditions in the laboratory were more favourable to antennal growth. One of the factors that could influence the favourable antennal growth could be temperature, which could increase the overall growth rate of laboratory-reared animals compared to the wild (where temperatures were recorded as low as 8°C in winter). The number of flagellar segments produced at each moult can depend on the season and the nutritional status of the animal (Gee, 1988). Unlike the animals in the wild the nutritional status of the animals in the laboratory was known, with all animals having an excess of food available. Sutcliffe and Carrick (1981) found that the amount of available food, water temperature and other environmental variables were positively correlated to growth rate and the number of flagellar segments in the first antenna. Without separating individual environmental variables it is impossible from the present study to identify which variable(s) are

determining the growth rate of the first antenna, though clearly the laboratory environment had higher growth rates than in the wild.

4.4.4 Gnathopod size and mate choice

One of the main functions of the gnathopods in male gammarids is to grasp and carry potential mates in a precopulatory amplexus (where the male physically carries the female below him) to secure a successful copulation. Male *Gammarus* secure mates by inserting the dactylii of the gnathopods between the segments along the dorsal surface (Robinson & Doyle, 1985; Sutcliffe, 1992). The grasp of the males' gnathopods have to be strong as a female can 'kick' and escape by flexing her body and free herself to find a new mate (Ward, 1984). One of the rewards for the male securing the largest mate possible is the higher fecundity of larger females. Predictably, sexual selection for both larger body and gnathopod size has been observed in wild populations (Ward, 1988; Wellborn, 2000). However an increased body size increases the potential risk of predation (Ward, 1988), and similarly an increase in conspicuous traits, such as the gnathopods has also been suggested to affect the risk of predation (Wellborn, 2000).

The significant difference in corrected gnathopod size between the three populations appears to be due to larger relative gnathopod size in the Lowthorpe population. Males sampled from the Lowthorpe population were also significantly larger (body size) than the two other populations (Figure 4.12). A similar pattern was also observed by Wellborn (2000), with larger relative gnathopod size occurring in male *Hyalella azteca* from populations of the large ecotype. Wellborn concluded that a balance between the ability to reproduce and the ability to avoid predation maintained the relative size of male gnathopods. Where predators were absent, males had the highest relative gnathopod size. Unlike the *H. azteca* population (Wellborn, 2000),

predation of *G. pulex* does occur at the Lowthorpe site, and the size of conspicuous traits could be constrained by an increased mortality (Wellborn, 2000).

The presence of predators and the large body size of males from this site contradicts the concept of predation (visual) as a constraining force on both body and gnathopod size in the Lowthorpe site. An alternative hypothesis is that the apparent male sex bias in the Lowthorpe population (chapter 2) and the potential increase in competition for mates could favour individuals with larger gnathopods (and body) and override the constraints of predation. In addition to using the gnathopods to grasp a mate, they could also be used in intrasexual contests during precopulatory guarding (Strong, 1973) and intraspecific territorial interactions (Mattson & Cedhagen, 1989). Either of these proposed functions could explain the energetically expensive production of enlarged gnathopods.

4.4.5 Ommatidia density

The significant difference in the relative density of ommatidia between environments was a consequence of higher densities recorded in the wild animals compared to those reared in the laboratory. The preferred habitat of *G. pulex* in streams is within leaf masses, under surface vegetation or under rocks and stones (Dahl & Greenberg, 1996). All of the preferred habitats are dark and provide shelter from predation. In such sheltered locations the ability to detect potential mates and competitors will be highly important for the survival and reproduction of animals. Although chemical cues (pheromones) are used for mate attraction in *G. pulex* (Sutcliffe, 1992), vision plays a dominant role (Wellborn, 2000). In poorly illuminated habitats the ability and resolution to see will depend upon the number of ommatidia and their density. However in the laboratory system where the clarity of the water and the incident light levels were high, there was no need for a large energy investment in the

production of high ommatidia density eyes. A similar change in eye shape and structure has been shown in cave and spring environments (Culver, 1987).

The ability of freshwater amphipods to adapt to new habitats has been best studied in *G. minus* in the extremely fragmented environment of submerged cave systems in the USA. Animals that have migrated into the caves have reduced their eyes (size and structure) to the point of near blindness, while increasing the relative size of their sensory antennae (Culver, 1987). The same species is also present in springs around the caves, where animals have fully formed eyes and small antennae. Although the environments examined in this study were not as extreme as that in the caves highlighted above, a similar trend of reduced eye structure (ommatidia density) was apparent. Unlike the caves and springs, the variation between environments (laboratory and wild) presented here suggests that the variation in eye morphology has taken place over a single generation and could be due to phenotypic plasticity rather than local adaptation observed in *G. minus*.

4.5 Summary

Morphological data collected from three populations (Lowthorpe, River Terrig & Fleshwick Bay) showed a significant correlation between all the traits and total body length. All data was corrected for the effect of size using Thorpe's (1976) method and a PCA approach, both correction methods gave comparable results and indicated that growth in G. pulex could be isometric.

Animals reared in the laboratory were compared to wild samples and allowed the estimation of the affect of the environment on the variation in morphological characters. Of the 14 morphological traits recorded, only three (eye width, segments in antenna 1 and density of ommatidia) were significantly affected by the environment. The significant differences between environments for the three traits was thought to be a

consequence of greater food availability and increased light intensity, compared to conditions in the wild. Despite seven of the morphological traits being significantly different between populations, separation of the populations using a discriminant function analysis wasn't possible in all cases and there was overlapping distributions.

The morphological data did not support the separation of UK and Belgian samples that had been observed using molecular markers and suggested that morphological traits do not track molecular genetic changes.

Chapter 5

Phylogeography of *Gammarus pulex* in north west Europe: Colonisation and speciation

5.1 Introduction

There has been much interest in the relationship between the geographic distributions of species and their genealogical lineages, and the study of this relationship is in essence, phylogeography (Avise, 1994). Phylogeography has now become one of the most studied areas of molecular ecology since its conception in the 1980's (Avise *et al*, 1987). The increased attention has occurred as a result of improved molecular methods (mainly sequencing) and greater understanding of historical dispersal patterns (Avise, 2000). Much of the research effort has focused on two continents, Europe and America, and there is now extensive literature on the relationship between the geological history and the distribution of present day species (Avise, 2000; Hewitt, 2000).

Many authors consider the Pleistocene epoch to be one of the most influential geological periods on the current distribution of species (and the process of speciation) around the globe, especially in the present temperate regions (Hewitt, 1996; 1999; Taberlet *et al*, 1998). Fragmentation of species distributions during the violent climate fluctuations associated with the glacial cycles of the Pleistocene have been proposed as a mechanism promoting speciation (Hewitt, 2000). Although the frequency of the glacial cycles declined (from every 23 ky to 100 ky) during the Pleistocene (the last 700 ky) their intensity increased and temperatures may have changed by ~7°C in less than a decade (Hewitt, 2000). During this period of rapid temperature change there was a

southern advance of the polar icecaps into the temperate regions, such as the North American continent and parts of northern Europe and Scandinavia (Hewitt, 1996).

The repeated glacial cycles have been thought not only to promote speciation but also to reduce genetic variability in colonising populations, by serial bottlenecking, and causing species extinction in some refugia when temperatures increased (Hewitt, 1996; 1999; 2000; Taberlet *et al*, 1998). As the ice sheets advanced from the poles the area of suitable habitat for temperate species was reduced by ice coverage and permafrost conditions. Species that were able to migrate moved south to ice free areas (refugia), such as the northern slopes of the Pyrenees or into the Balkans (Figure 5.1). Once the ice retreated colonisation from refugia into ice-free habitats was possible, and temperate species could move north. The movement of some species into new habitats was rapid and occurred over large geographical distances (e.g. the grasshopper spread at 300-500m/year, Hewitt, 1990), causing repeated bottlenecks and a loss of genetic diversity (Hewitt, 1996). Such processes contrast with refugia that could have provided suitable habitat and variation in topology over several glacial periods helping to preserve high genetic diversity and species richness. However some species present in refugia during warm interglacial periods may have disappeared and become extinct during the rapid cooling of the next glacial period (Taberlet *et al*, 1998).

The foundations of the present day river systems in northern Europe were laid in the Miocene epoch of the Tertiary period when earth movements associated with the opening of the North Atlantic were at their height (Gibbard, 1988). At this time the primitive rivers in Europe were located in shallow valleys. During the Pleistocene severe downcutting occurred and river valleys deepened with connections between drainages lost and diverted. It was also during the Pleistocene that the English Channel and the straits of Dover underwent massive changes, from a large drainage delta for most of Europe to part of the Atlantic Ocean. The timing and nature of river course

connections and the formation of the English Channel presented provided limited opportunities for freshwater species to colonise across systems and into new habitats.

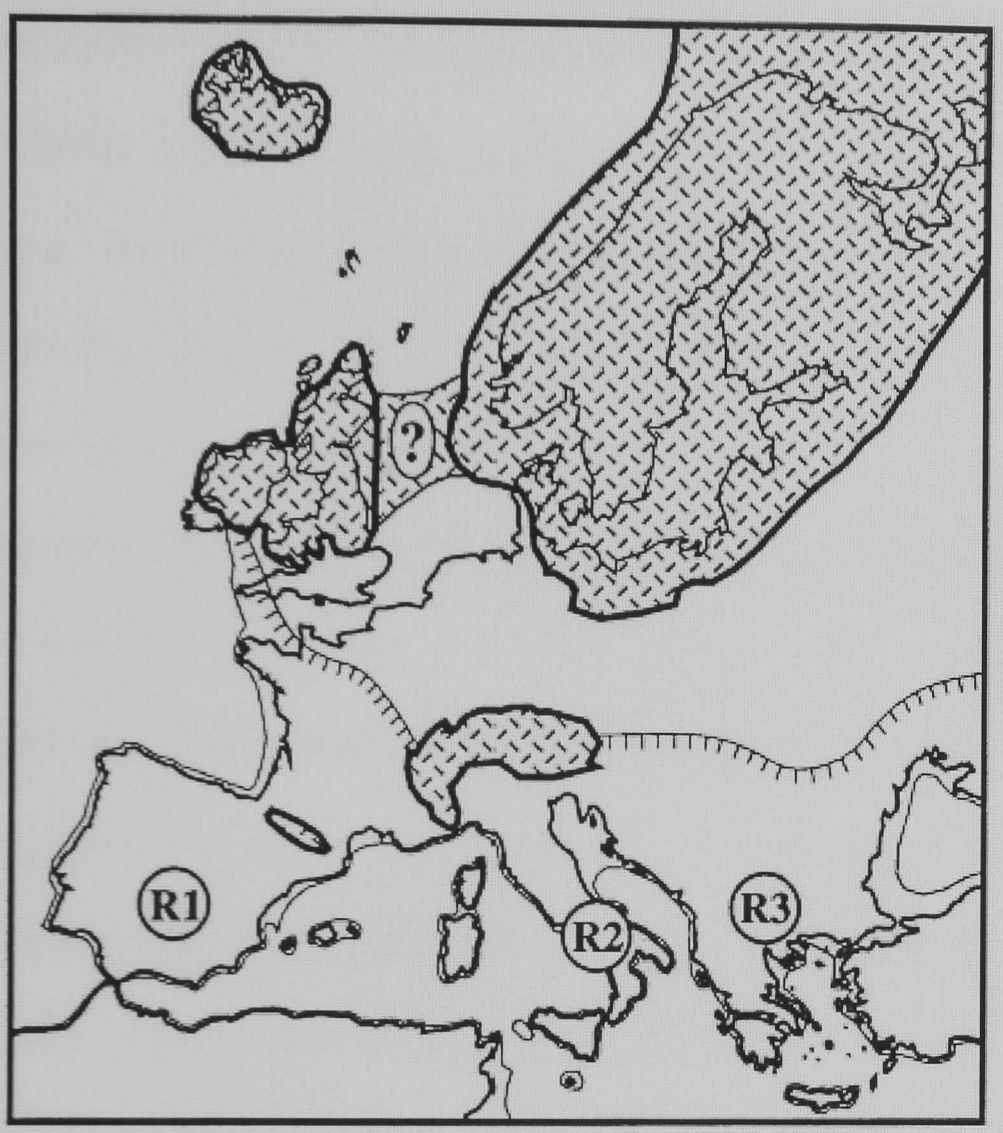


Figure 5.1 The maximum extent of the ice sheets and permafrost (scaled line) in Europe during the last glacial period (~18,000 years ago). R1, R2 and R3 highlight three possible refugia in Spain, Italy and the Balkans. Hatching area indicates the ice sheet with the limit of the permafrost indicated by the scaled line. The connection between the Scandinavian and UK ice sheets is equivocal as indicated by the '?'. (redrawn from Taberlet *et al.*, 1998).

Europe has now become one of the most studied areas with respect to the phylogenetic reconstruction of colonisation routes and the location of refugia. Though a large proportion of data has been based on plants (due to historical pollen records) there are now also many examples from freshwater species: *Cottus gobio* (Englebrecht *et* al, 2000); *Leuciscus cephalus* (Durand et al, 1999); *Perca fluviatilis* (Nesbø et al, 1999); *Triturus spp.* (Wallis and Arntzen, 1989); and *Gammarus fossarum* (Müller, 2000). These data form an important background to any new phylogenetic investigation and

highlight possible areas of hybridization, high genetic diversity (thought to be possible refugia) and probable colonisation routes.

Previous phylogenetic investigations in Europe using gammarids have been limited in their ability to resolve across small geographic ranges (Meyran *et al*, 1997; Müller, 1998; 2000) and have focused on the complex taxonomy and species identity within the group. In previous studies, large sequence divergence between species was found (11.5- 30.8%, Meyran *et al*, 1997; 11.1%, Müller, 2000) dating species splits back to the Miocene, prior to the dramatic climatic changes of the Pleistocene. In the case of *G. fossarum* two possible refugia have been proposed, either side of the Alps, that contain distinct sub-species of *G. fossarum*. These two forms colonised from their respective refugia and formed a contact zone in the upper Rhine (Müller, 2000).

Although areas of high diversity (possible refugia) and possible colonisation routes have been suggested for freshwater gammarids (Thienemann, 1950; Meyran et al., 1997; Müller, 2000), data are geographically limited and difficult to compare between species. In all previous cases, sampling has been confined to a few river systems with a limited improvement in the understanding of European historical patterns of gammarid species movements. The present study aims to increase the understanding of the historical processes that have created the present day distribution of *G. pulex* in Europe. A strategic sampling regime was designed to target possible regions of high genetic diversity (refugia) and trace historical colonisation routes (Chapter 2).

5.1.1 Aims

Within this study there were three specific aims that were based on previous molecular work on *G. pulex* (Chapter 3) and phylogenetic investigations using other *Gammarus* species (Meyran, 1997; Müller, 1998; 2000). The three aims were:

1. To estimate the level of genetic diversity in the UK using mitochondrial DNA sequencing. (Are the low levels of diversity revealed by allozymes supported by these data?)
2. To reconstruct the colonisation of the UK, the possible route and timing.
3. To reconstruct colonisation routes and potential refugia in north west Europe used by *Gammarus pulex* during the glacial periods.

5.2 Methods

5.2.1 Samples

Animals from all of the sampled populations were used (Chapter 2) with sample sizes ranging from one to eight (Table 5.1). Samples of *Gammarus pulex gallicus* from the Pyrenees (Scheepmaker, 1990, site 23) and *G. fossarum* from northern France (sites 30 and 32, present study) were used to compare patterns of genetic divergence and as outgroups for tree rooting procedures.

5.2.2 Mitochondrial DNA sequencing

DNA was extracted from the preserved animals using the InstaGene Matrix (Bio-Rad Laboratories). A single swimming leg (normally pereiopod 5, 6 or 7) of each animal was removed with forceps and added to 75µl of the InstaGene Matrix. The solution was vortexed and then heated on a Peltier Thermal Cycler (MJ Research), first at 56°C for 20 minutes then at 100°C for 10 minutes and stored at -20°C. Prior to use, all DNA extractions were spun at 13,000 rpm for 5 minutes to separate extracted DNA and cell debris. At this stage, the extracted DNA solution could be added directly to a PCR mixture.

Table 5.1: Haplotype distribution (with numbers in parenthesis) and sample size of the populations sequenced. (* G. fossarum; ** G. p. gallicus). Population locations given in Figures 2.1 and 2.2 (Chapter 2).

Population	No.	**Sample size**	Haplotypes present
Lowthorpe	1	5	Common (3), L2 (2)
River Terrig	2	4	Common (5)
Fleshwick Bay	3	4	Common (4)
Stainfield Hall	4	8	Common (1), SH1 (3), SH2 (2), SH3 (1) & SH5 (1)
Greasby Brook	5	4	Common (1), GB3 (3)
Malham Tarn	6	2	MT2 (2)
Malham Village	7	4	MV3 (4)
River Crogga	8	3	Common (2), CR22 (1)
Stainfield Village	9	Not sampled	N/A
Horrabridge	10	2	Common (2)
Ivybridge	11	3	IVY (3)
Cowside Beck	12	4	CB3 (2), CB4 (1) & CB5 (1)
Malham Spring	13	2	MI5 (1), MI6 (1)
Malham Outflow	14	5	MT2 (5)
River Alwen	15	4	AL1 (2), AL3 (1) & AL4 (1)
Driffield	16	2	L2 (3)
Pocklington	17	6	PC1 (5) & PC2 (1)
Pickmere	18	2	PM1 (1) & PM2 (1)
Norwich	19	3	Common (1) & N2 (2)
Oxford	20	1	Common (1)
Turo	21	4	Common (4)
Dunstanbrugh	22	4	Common (2) & DN1 (2)
Lennoxtown	23	4	CA1 (2), CA3 (1) & CA4 (1)
Hierden	24	8	AM1 (5), AM2 (2) & AM3 (1)
Hopfgarten	25	4	HOF (4)
Mainz	26	4	NOR1 (2), NOR12 (1) & NOR19 (1)
Kleinlutzel	27	4	KLEP (1), KLE2 (2) & KLE6 (1)
Schendelbeke	28	2	SCH2 (1) & SCH3 (1)
Correy-le Chateaux	29	3	COR1 (1), COR2 (1) & COR3 (1)
Berlaimont	30	5	BER1 (2), BER3 (1) & BER4 (2)
Foret de Mormal	31	3 + 1*	BERF1 (2) & BERF3 (1) *BERF2 (1)
St Georges	32	3*	*STG1 (3)
Dartmoor	33	4	DM2 (4)
Som	34	1	SOM (1)
Braunschweig	35	4	SH1 (2), BR6 (1) & BR0 (1)
Ramstein	36	5	RAM (5)
St. Hilaire	37	1	STH1 (1)
Fontain de Vauclus	38	1	H34 (1)
Iasr	39	3	ISAR4 (1) & ISAR5 (2)
Marching	40	1	MAR (1)
Nefiach	41	3**	**H28 (1), H29 (1) & H30 (1)

Figure 5.2 Sample site locations, including *G. p. gallicus* from the Pyrenees.

A fragment of a mitochondrial gene, cytochrome oxidase subunit I (COI), was amplified using universal primers (Folmer *et al*, 1994) LCO1490 (5' -GGT CAA CAA ATC ATA AAG ATA TTG G- 3') and HCO2198 (5' –TAA ACT TCA GGG TGA CCA AAA AAT CA- 3'). Each 10 µl PCR reaction mixture contained 1µl DNA extract (diluted 1:10), 200 ρM forward primer, 200 ρM reverse primer, 1X NH_4 reaction buffer (Bioline), 2.5 mM $MgCl_2$, 80 mM dNTPs, and 0.6 units of *Taq* polymerase (Bioline). Amplification was carried out on a Peltier Thermal Cycler (MJ Research) and consisted of the following steps: initial denaturation at 94°C for 3 min; 40 cycles of 15s at 94°C,

20s at 53°C and 1 min at 72°C; and a final extension of 3 min at 72°C. Products were checked for size (~ 710bp) and the presence of non-specific products using standard procedures on 2% agarose gels, visualised with ethidium bromide (Sambrook *et al*, 1989).

PCR products were sequenced directly using the Thermo Sequencenase™ fluorescent dye primer kit (Amersham Ref: RPN 2538). Electrophoresis was carried out on an *ALFexpress* ™ automated sequencer (Amersham). Forward and reverse primers were used in all animals. The electrophenograms were compared and consensus sequences were generated using the Alfwin ™ software (Amersham).

5.2.3 Data analysis

Mitochondrial DNA sequences were aligned by the program Clustal X based on the algorithm described in Thompson *et al.* (1994) and 647 bp were used for further analysis. The most-parsimonious trees of all present haplotypes were found using replicates of random tree addition sequences for heuristic searches with tree-bisection-reconnection (TBR) branch swapping algorithm using the PAUP program (Swofford, 1993). Support for the branching pattern was obtained using 1000 bootstrap replicates, again obtained using an heuristic search.

A haplotype cladogram displaying the absolute number of base pairs differences between haplotypes was generated using the TCS program (Clement *et al*, 2000). The TCS program estimated the cladogram based on the equations of Templeton *et al* (1992) and only resolved connections between haplotypes that were less than 11 base pairs (95% parsimony). Minimum estimates of branch lengths that were greater than 11 base pairs and possible roots of clades were made (where possible) using the most-parsimonious trees from PAUP (Swofford, 1993).

loci, estimated a slower rate of divergence of 1.4% per million years. Both molecular clock estimates were used in the calculation of the separation of clades and species.

A nested clade analysis (NCA) (Templeton, 1998) approach was used to discriminate between contemporary processes (gene flow and isolation by distance) and historical events (long distance colonisation, range expansion and past fragmentation) in the phylogeny of *G. pulex* in the UK. NCA uses haplotype frequency data and geographic locations to test the null hypothesis that the haplotype clades are randomly distributed within the nested cladogram.

A NCA analysis has four main steps: the construction of the most-parsimonious cladogram; the nested cladogram design; testing the null hypothesis (that all haplotypes are randomly arranged in the cladogram); and a biological inference of significant results. The nesting design of the most-parsimonious cladogram (see above) followed the procedure outlined by Templeton & Sing (1993). Nesting starts (first level) by grouping together haplotypes separated by one mutation from the tips of the cladogram. The nesting then continues by grouping together these first level clades separated by one mutation in to second level clades and so on until all clades in the cladogram are nested. Testing the null hypothesis requires clades to contain both variance at the geographic (multiple locations) and the genetic level (multiple haplotypes), all other clades were discarded at this point. The remaining "informative" clades were tested against the null hypothesis using the GeoDis program (Posada *et al*, 2000). The program calculates clade distances [D_c (X)] (the average distance of all individuals in clade X to the geographical centre of that clade) and the nested clade distances [D_n (X)] (the average distance of clade X individuals from the centre of the higher level clade in which clade X is nested). Distances that were significantly large or small (5% level) were determined using a permutation procedure with 100 re-samples. All significant results were interpreted following the key of Templeton (1998).

species pairs in the Caribbean and Pacific after the formation of the Isthmus of Panama (3.0 to 3.5 mya). Knowlton *et al* (1993) estimated rates of COI sequence divergence to be between 2.2 and 2.6% per million years. Knowlton & Weigt (1998), using multiple loci, estimated a slower rate of divergence of 1.4% per million years. Both molecular clock estimates were used in the calculation of the separation of clades and species.

A nested clade analysis (NCA) (Templeton, 1998) approach was used to discriminate between contemporary processes (gene flow and isolation by distance) and historical events (long distance colonisation, range expansion and past fragmentation) in the phylogeny of *G. pulex* in the UK. NCA uses haplotype frequency data and geographic locations to test the null hypothesis that the haplotype clades are randomly distributed within the nested cladogram.

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5.3 Results

Table 5.1: Haplotype distribution (with numbers in parenthesis) and sample size of the populations sequenced. (* G. fossarum; ** G. p. gallicus). Population locations given in Figures 2.1 and 2.2 (Chapter 2).

Population	No.	**Sample size**	Haplotypes present
Lowthorpe	1	5	Common (3), L2 (2)
River Terrig	2	4	Common (5)
Fleshwick Bay	3	4	Common (4)
Stainfield Hall	4	8	Common (1), SH1 (3), SH2 (2), SH3 (1) & SH5 (1)
Greasby Brook	5	4	Common (1), GB3 (3)
Malham Tarn	6	2	MT2 (2)
Malham Village	7	4	MV3 (4)
River Crogga	8	3	Common (2), CR22 (1)
Stainfield Village	9	Not sampled	N/A
Horrabridge	10	2	Common (2)
Ivybridge	11	3	IVY (3)
Cowside Beck	12	4	CB3 (2), CB4 (1) & CB5 (1)
Malham Spring	13	2	MI5 (1), MI6 (1)
Malham Outflow	14	5	MT2 (5)
River Alwen	15	4	AL1 (2), AL3 (1) & AL4 (1)
Driffield	16	2	L2 (3)
Pocklington	17	6	PC1 (5) & PC2 (1)
Pickmere	18	2	PM1 (1) & PM2 (1)
Norwich	19	3	Common (1) & N2 (2)
Oxford	20	1	Common (1)
Turo	21	4	Common (4)
Dunstanbrugh	22	4	Common (2) & DN1 (2)
Lennoxtown	23	4	CA1 (2), CA3 (1) & CA4 (1)
□Hierden	24	8	AM1 (5), AM2 (2) & AM3 (1)
Hopfgarten	25	4	HOF (4)
Mainz	26	4	NOR1 (2), NOR12 (1) & NOR19 (1)
Kleinl(tzel	27	4	KLEP (1), KLE2 (2) & KLE6 (1)
Schendelbeke	28	2	SCH2 (1) & SCH3 (1)
Correy-le Chateaux	29	3	COR1 (1), COR2 (1) & COR3 (1)
Berlaimont	30	5	BER1 (2), BER3 (1) & BER4 (2)
Foret de Mormal	31	3 + 1*	BERF1 (2) & BERF3 (1) *BERF2 (1)
St Georges	32	3*	*STG1 (3)
Dartmoor	33	4	DM2 (4)
Som	34	1	SOM (1)
Braunschweig	35	4	SH1 (2), BR6 (1) & BR0 (1)
Ramstein	36	5	RAM (5)
St. Hilaire	37	1	STH1 (1)
Fontain de Vauclus	38	1	H34 (1)
Iasr	39	3	ISAR4 (1) & ISAR5 (2)
Marching	40	1	MAR (1)
Nefiach	41	3**	**H28 (1), H29 (1) & H30 (1)

5.3 Results

5.3.1 Sequence variation

A total of 647 bp (after alignment) of the mitochondrial COI gene were compared for the 41 sampled populations. The total number of polymorphic sites scored was 217 (Table 5.2), over a third of the total aligned sequence. Despite the high level of polymorphism, the number of nonsynonymous site mutations was low and changes in amino acids were most readily observed in the comparison between *G. pulex* and *G. fossarum*.

In total, 143 animals (including *G. p. gallicus* and *G. fossarum*) were sequenced, revealing 65 unique haplotypes (Table 5.1). The two out-group species, *G. p. gallicus* and *G. fossarum* accounted for only three and two haplotypes, respectively. The highest number of haplotypes found in a single population was five (Stainfield Hall) and the lowest was one (in many populations).

Table 5.2: Descriptive data of the polymorphism of all haplotypes

Total polymorphic sites	217
Total number of site mutations	275
Total number of parsimoniously informative sites	189
Total number of monomorphic sites	430
Mean number of synonymous site mutations	37.7
Mean number of nonsynonymous site mutations	3.4

The pairwise comparisons of haplotype polymorphisms plotted against genetic distance (Figures 5.3 & 5.4) showed the increase in site mutations and the associated increase in genetic distance. The plots also showed the bias towards transitions and synonymous mutations.

5.3.2 Molecular trees and the geographical distribution of phylogenetic groups

The bootstrap consensus tree revealed four distinct and well supported *G. pulex* clades (not including *G. p. gallicus*) that all have bootstrap values above 90% (Figure 5.5). The four clades have been named the UK clade (green), the Rhine clade (red), the East-west clade (blue) and the Vendee clade (yellow). Haplotypes within the clades were exclusive to the populations within the clade, with the exception of two populations on the River Rhine (Kleinlützel and Mainz). The River Rhine was also the only area sampled that showed a geographic overlap of the clades, see Figure 5.6.

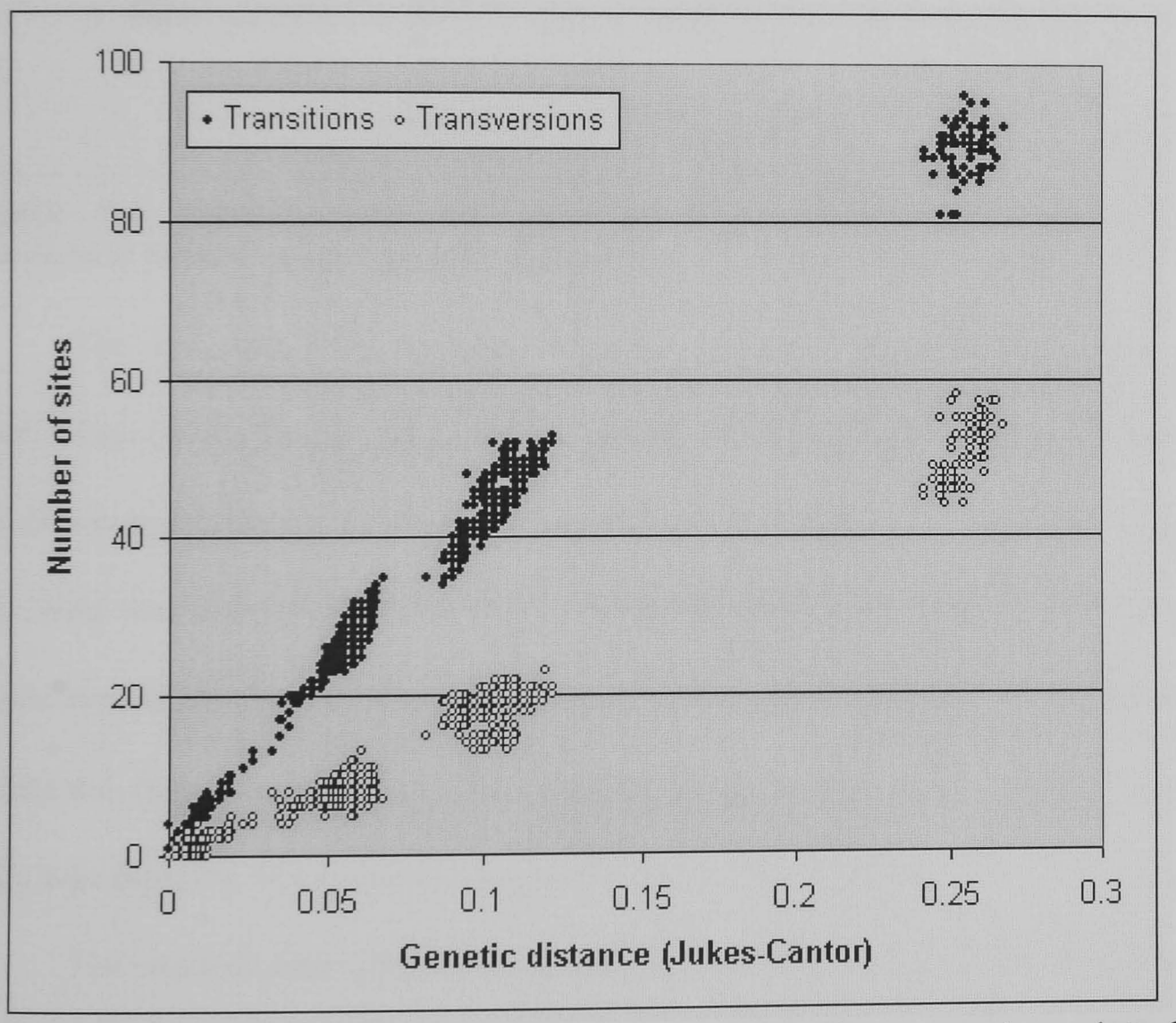

Figure 5.3: Transitions and transversions sites in pairwise comparison plotted against genetic distance

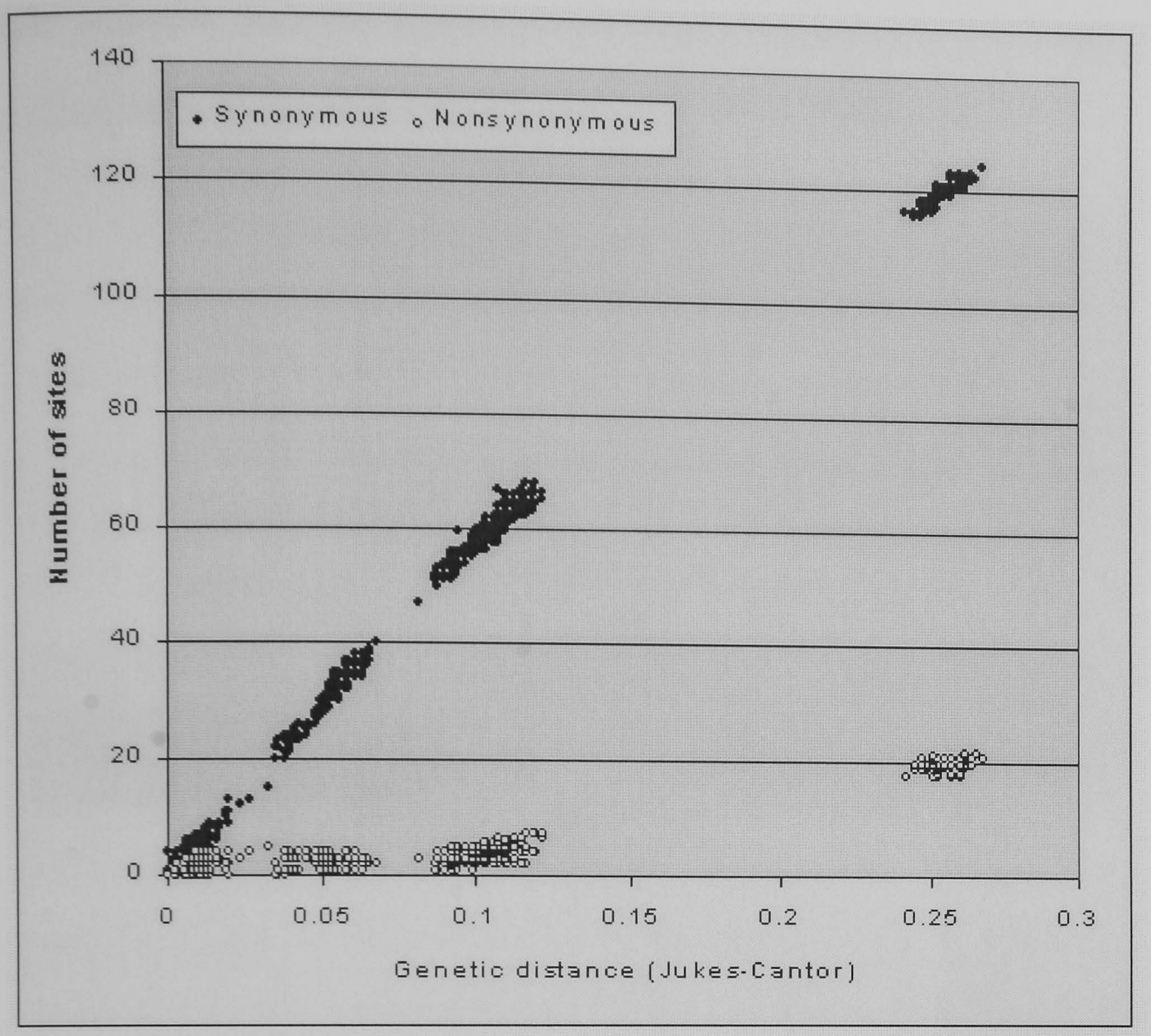

Figure 5.4: Synonymous and nonsynonymous polymorphic sites in pairwise comparison plotted against genetic distance

The first clade (UK) includes all haplotypes from the UK and populations on the rivers Maas (AM), Weser (BR), Meuse (SOM), Elbe (HOF) and Rhine (NOR & KLE). The two populations on the Rhine also contained haplotypes from the Rhine clade. The UK clade was characterised by a star like distribution of haplotypes with a frequent central type (Common) and many infrequent satellites separated by a maximum of three mutational steps (Figure 5.7). The most basal haplotype and the assumed root of the clade was from the Mainz population (NOR) on the River Rhine.

The second clade (Rhine) included haplotypes from three European rivers, the Rhine (NOR & KLE), Rhone (H34) and Danube (MAR). This clade was the only clade to exhibit a clear range overlap with other clades (UK and East-west). The overlap occurs in the middle and upper parts of the river Rhine, where the rivers Moselle, Main and Danube join (or have previously been joined). The most basal haplotype in this

clade was H34 from the Fontain de Vauclus population on the Rhone, which was geographically distant from the remaining populations in the clade.

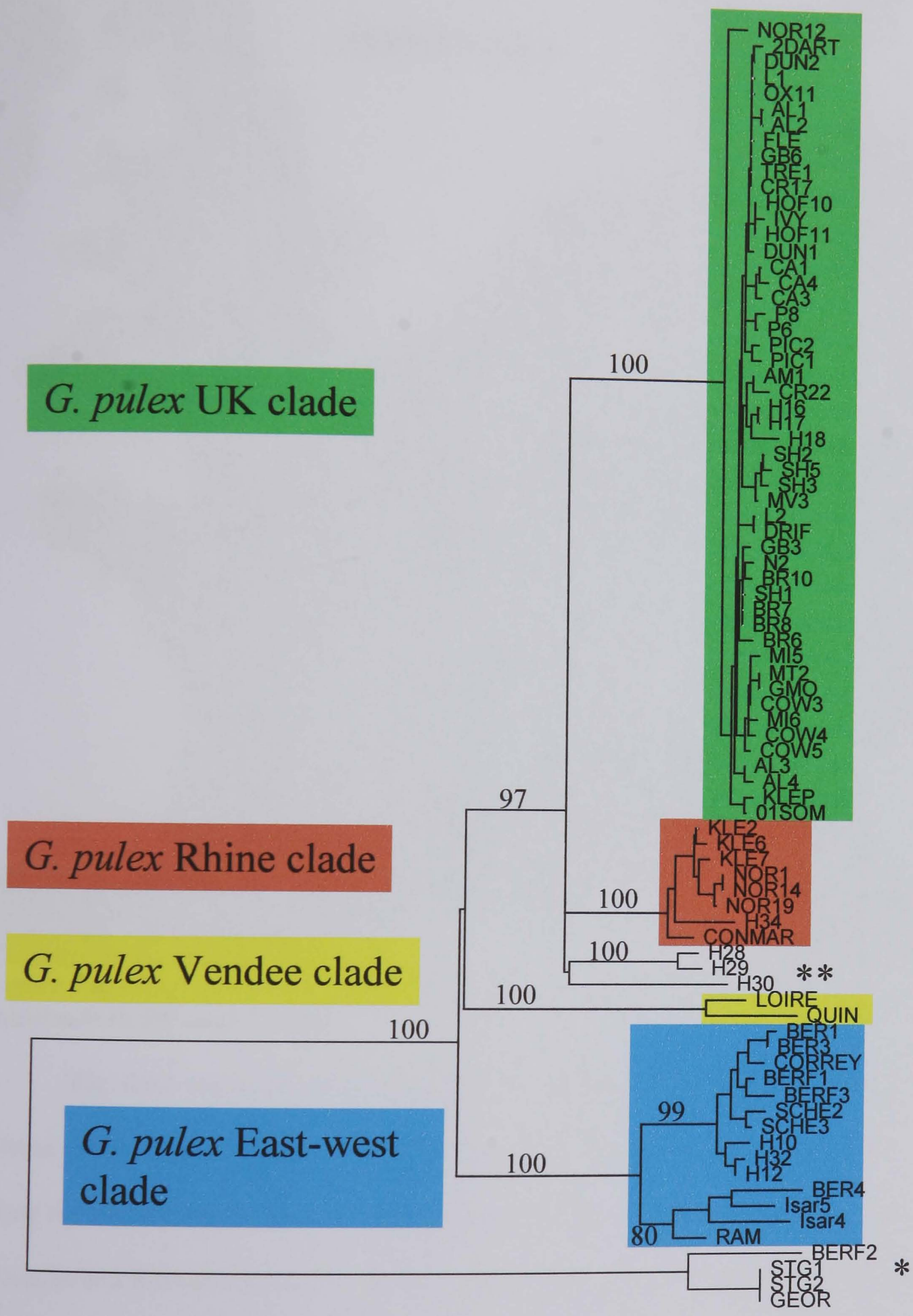

Figure 5.5: Kimura 2 parameter genetic distance tree constructed using 1000 bootstrap replicates with neighbour joining, tree routed with *G. fossarum* as an out-group. Support for each of the major clade nodes is shown as a percentage of the bootstrap replicates. (* *G. fossarum*. ** *G. pulex gallicus*.)

Figure 5.6: Geographic distribution of the four main *G. pulex* clades (*= *G. fossarum* samples from the same location)

The third clade (East-west) contains haplotypes from the rivers Olse (COR), Meuse (BER, BERF), Danube (ISAR), Rhine (RAM) and Loire (H32). Within the clade the haplotypes showed two geographical clusters of the populations from the east (Danube and Rhine) and the populations from the west (Olse, Loire and Meuse). There was one exception to this pattern with a single haplotype from a population on the Olse (BER4) clustering with the eastern samples. The western group appeared to be the

basal within this clade, though the branching support was lower (80% bootstrap, Figure 5.3) and the number of sample sites was smaller (Table 5.2).

The final clade (Vendee) contained two haplotypes from two sample sites on the river Vendee that were well supported as a separate clade (bootstrap support = 100%) and that are geographically isolated from all of the other sampled populations. This clade had no overlap with any other clades and showed no obvious structure within the clade, as a result of limited sample sites and numbers.

The relationship between the five *G. pulex* (including *G. p. gallicus*) clades was unresolved at the most basal level, with either the East-west or Vendee clades forming the root with *G. fossarum* (Figure 5.5). The remaining clades clustered more closely to each other compared to the more basal Vendee and East-west clades suggesting greater divergence (see 5.3.3).

5.3.3 Differentiation and diversity within clades

The low geographical density of samples prevented a detailed analysis (mis-match distribution plots and construction of a cladogram) of all the clades, though some general patterns could be resolved. The levels of diversity within the clades estimated using Nei's (1987) π showed values ranging from 0.5% to 3.6% (Table 5.3). The lowest level of diversity was found in the UK clade, which was the most intensively sampled of all the clades (Table 5.2). This contrasted with the diversity of the *G. p. gallicus* clade that had highest level of diversity, even though the clade consisted of a single population. The diversity of the east-west clade appeared to be due mainly to the divergence between the two groups within the clade (Figure 5.5). The same pattern appeared in the Vendee clade where there were only two haplotypes sampled. Within the Rhine clade diversity appears to be a consequence of samples from the Danube and Rhone drainages that form the basal haplotypes of the clade.

Table 5.3: Nucleotide diversity, π (Nei, 1987) within clades.

Clade	π
UK	0.005
Rhine	0.010
East-west	0.026
Vendee	0.023
G. p. gallicus	0.036
G. fossarum	0.016

The low diversity of haplotypes in the UK clade showed a very similar uni-modal pairwise differences distribution to the expectation of an expanding population (Figure 5.7). Similar to the total data set (Figure 5.8) the raggedness statistic (*r*, see Harpending, 1994, equation 1) of the UK plot was very small and suggested that the clade was recent and still expanding.

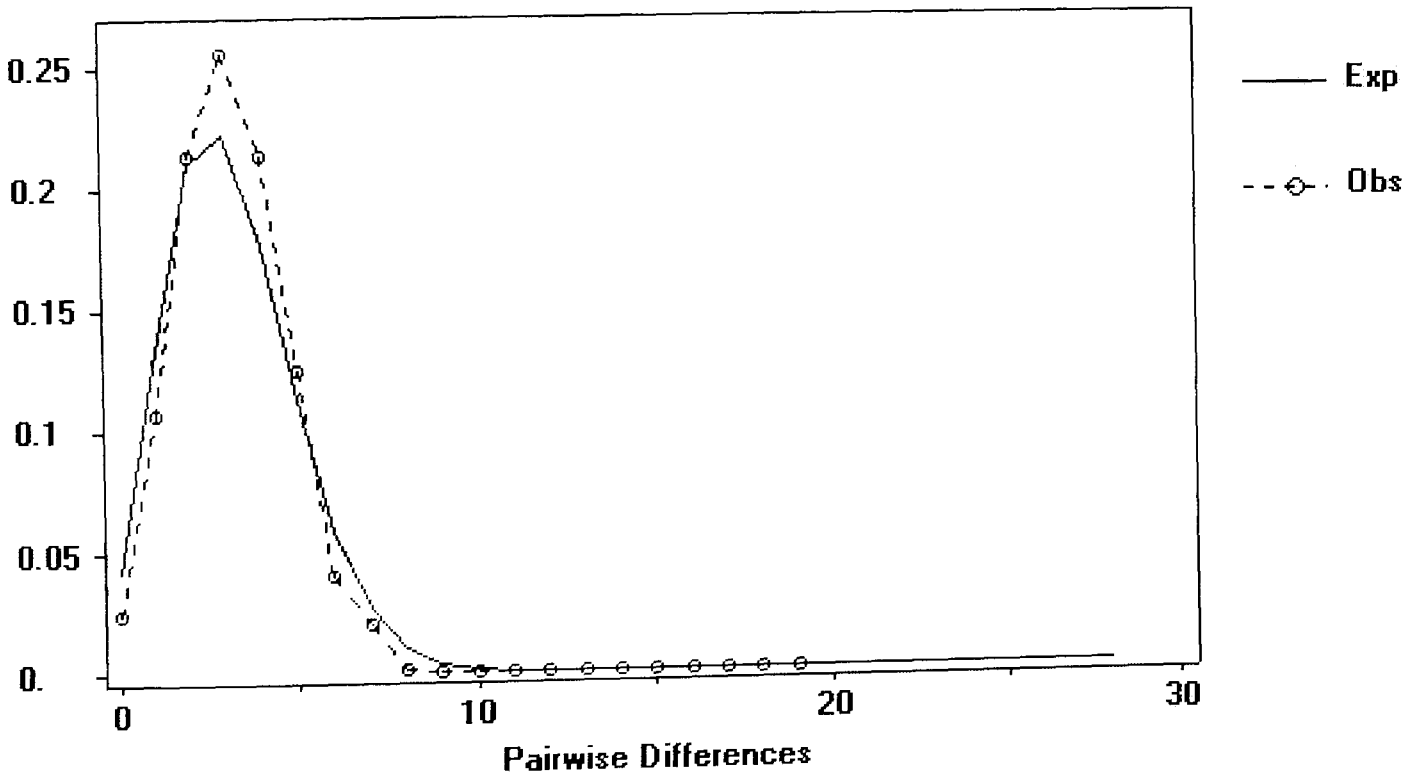

Figure 5.7: Frequency distribution of pairwise differences of haplotypes from the UK clade ($\tau = 3.177$, $\theta_0 = 0$, $\theta_1 = 1000$ and $r = 0.044$).

5.3.4 Divergence between the species and clades

Differentiation between *G. fossarum* and the *G. pulex* clades was very clear, with deep splits in the tree, supported by high bootstrap values (Figure 5.5). Divergence between *G. fossarum* and all *G. pulex* clades (including *G. p. gallicus*) was consistent

and ranged between 21.2% and 21.7% (Table 5.4). Assuming the calibration of COI molecular clocks by Knowlton *et al* (1993) and Knowlton and Weigt (1998), divergence between these two species would have taken place 8.3 to 15.5 million years ago (mya). Even with the high variance of the estimated time of divergence (inherent with all molecular clocks) the species split would have occurred in the Miocene epoch (~5.3-23.7 mya) of the Tertiary period (Appendix 5.1), prior to the strong climate fluctuations of the Quaternary period.

Table 5.4: The average number of substitutions per site between clades (Nei, 1987).

	UK	Rhine	East-west	Vendee	*G. p. gallicus*	*G. fossarum*
UK	-					
Rhine	0.057	-				
East-west	0.107	0.089	-			
Vendee	0.096	0.098	0.102	-		
G. p. gallicus	0.056	0.052	0.092	0.094	-	
G. fossarum	0.214	0.217	0.217	0.217	0.212	-

Divergence between the sub-species of *G. pulex* was more recent than the split of *G. fossarum* and *G. pulex*, as would be expected. However the sequence divergence between *G. p. gallicus* and the four main clades (section 5.3.1) varied between 5.2% and 9.4% (Table 5.4). Both the East-west and Vendee clades are nearly twice as divergent from *G. p. gallicus* as the UK and Rhine are. Assuming the same molecular clocks as before, divergence times between *G. p. gallicus* and the UK and Rhine clades would be between two and four mya. This split would have occurred sometime between the mid-Pliocene and the early Pleistocene (Appendix 5.1). Divergence between *G. p. gallicus* and the East-west and Vendee clades appears older, between 3.5 and 6.7 mya, occurring in the Pliocene epoch.

Divergence times between the four clades of *G. p. pulex* sampled here appear to have occurred at the same time as the divergence between the two subspecies. The level

of sequence divergence between the clades (Table 5.4) indicates again that the Vendee and East-west clades split from the other clades much earlier than the divergence between the UK, Rhine and *G. p. gallicus* clades. Assuming the same molecular clocks as before, the split of the Vendee clade (~3.7 to 7.3 mya) and the East-west clade (~3.4 to 7.6 mya) from the other clades occurred during the Pliocene. Such timing appears to pre-date the divergence between the Rhine, UK and *G. p. gallicus* clades which occurred between two and four million years ago, during the Pliocene/ Pleistocene boundary (Appendix 5.1).

The overall pattern of divergence between the clades and species was clearly shown by the plot of pairwise haplotype differences (Figure 5.8). The data did not fit the expectation of an expanding population (uni-modal), with a multi-modal distribution plot. The multi-modal distribution corresponded to the sequence divergence between haplotypes of ~ 5, 10 and 20%, the same as the divergences between clades and species previously described. The data suggested that there have been at least three separate expansions within *G. pulex* clade.

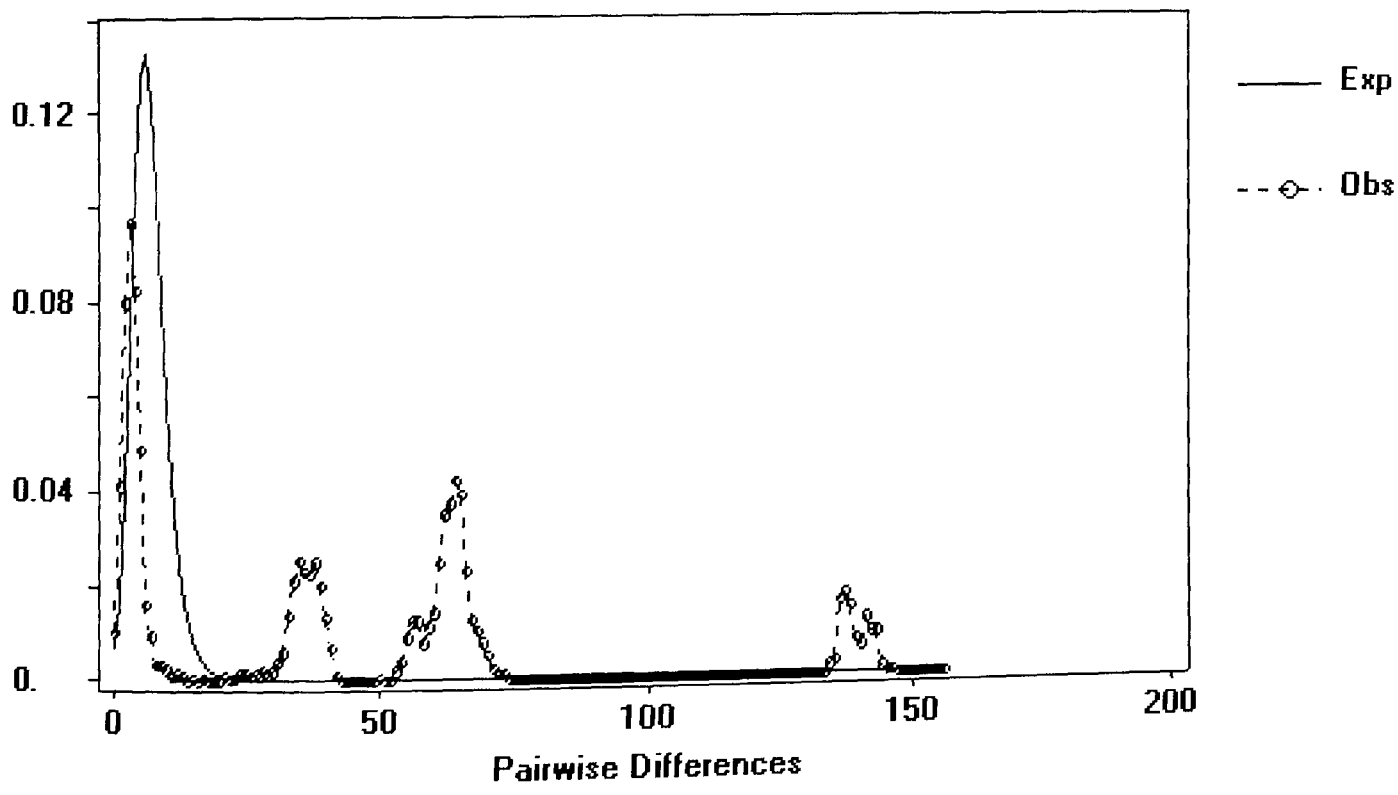

Figure 5.8: Frequency distribution of pairwise differences of all haplotypes across Europe ($\tau = 5.000$, $\theta_0 = 2$, $\theta_1 = 200$ and $r = 0.006$).

5.3.5 Nested Clade Analysis

The final nested clade design comprised of three levels of nesting, with a total of 21 clades (16 first level, four second level and one third level), including the total cladogam (Figure 5.9). Of the 21 nested clades identified, seven contained both geographical and genetic variance and were tested against the null hypothesis. Of the seven testable clades, six clades rejected significantly the null hypothesis that the haplotype clades are randomly distributed within the nested cladogram (Table 5.5). The biological interpretation of the significant clades revealed both recent and historical processes (Table 5.5). Each of the inferences was displayed as a series of arrows on a map of the area (Figure 5.10).

Table 5.5 NCA inference of significant clades using the Templeton (1998) key.

Clade	Reference number	Biological Inference
1-1	1	Restricted geneflow with isolation by distance
1-4	2	Contiguous range expansion
1-14	3	Past fragmentation
1-16	4	Long distance colonisation
2-1	5	Past fragmentation
Total cladogram	6	Allopatric fragmentation

The clade 1-1 includes samples from only the Malham area, which has an unusual hydrography with water systems connected by submerged caves (Chapter 2). The inference from the NCA suggests that there is limited movement between the populations within this isolated area.

All the remaining inferences by the NCA are historical processes that appear to have shaped the population structuring in the UK clade (Table 5.5). The absence of haplotypes from the UK clade in northern France and Belgium, but not in Holland and Germany, suggests that there was a break in the distribution of the UK haplotypes and so generates the overall inference of past allopatric fragmentation.

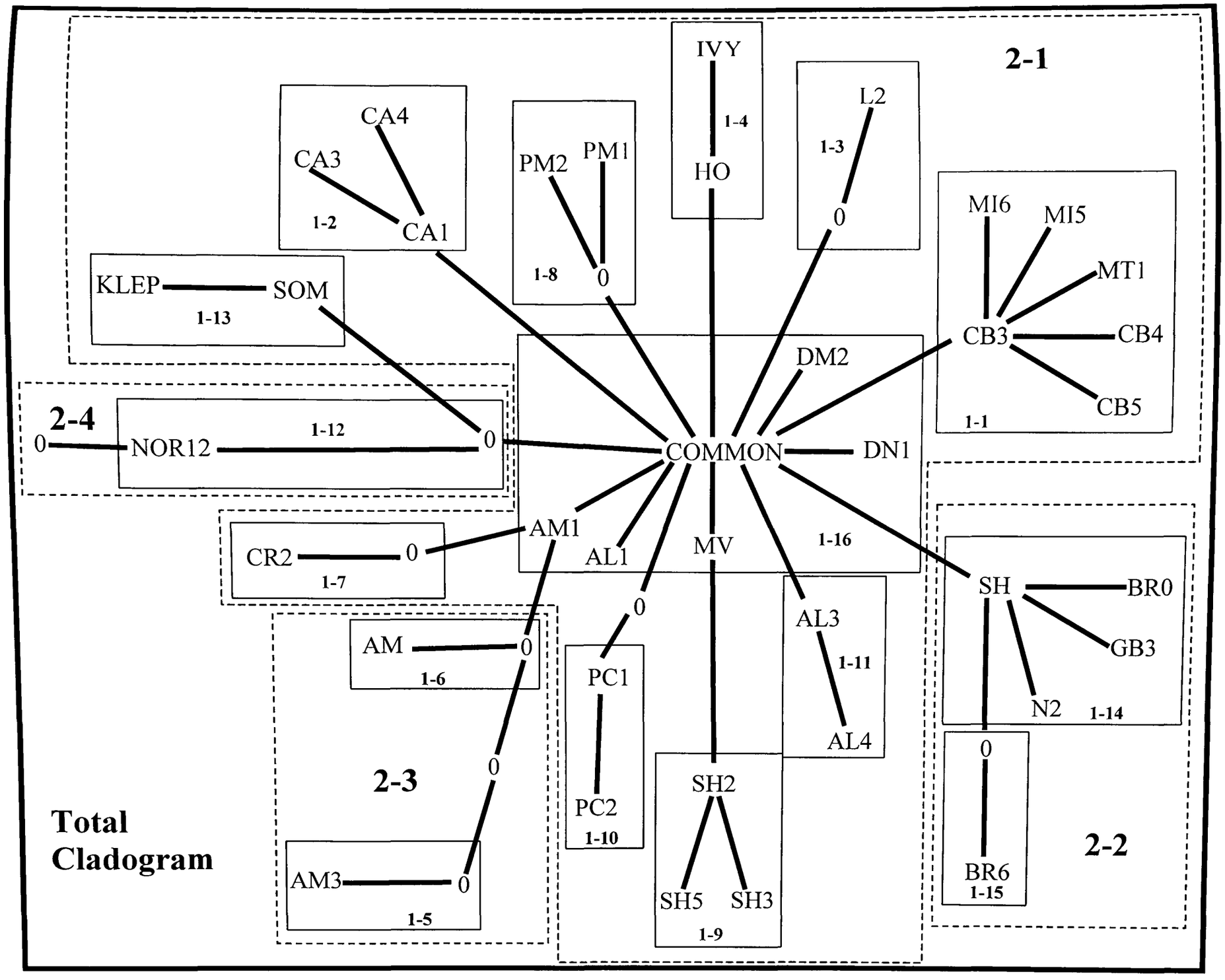

Figure 5.9: Nested clade design for the UK clade. All branches linking haplotypes are a single mutation with zeros representing un-sampled haplotypes. Thin solid lines (-) represent first level clades e.g. 1-16. Dotted lines (----) represent second levels clades e.g. 2-1. The solid bold line (—) represents the third levels of nesting, the total cladogram.

Figure 5.10: Biological inferences Nested Clade Analysis based on the key by Templeton (1998) for samples within the UK (green) clade. Numbers refer to those in Table 5.5.

1. Restricted gene flow within the Malham samples
2. Contiguous range expansion between mainland Europe and the UK
3. Past fragmentation between the mainland European sample and the UK
4. Long distance colonisation of the UK from the sample in Holland
5. Past fragmentation between the mainland European samples and the UK

5.4 Discussion

The overall level of genetic diversity revealed by this study was greater than expected, as genetic variation of *Gammarus pulex* has often been regarded as lower than that of its close relatives e.g. *G. fossarum* (Müller, 1998; Scheepmaker, 1990). Until recently, the distribution of *G. pulex* sub-species (other than *G. pulex pulex*) was thought to be restricted to caves and isolated mountains (Karaman and Pinkster, 1977). However, the present data suggests that the distribution of several cryptic *G. pulex* subspecies were not as restricted as previously thought, and may result, in part, from the violent climatic changes of the Quaternary period.

All freshwater *Gammarus* species have no distinct life history stage associated with dispersal, and are limited in their ability to migrate over large distances by their swimming strength and downstream by seasonal spate (Litterick, 1973). Although swimming is the main active mode of migration, some species have the ability to move short distance out of water, especially in humid conditions (Müller *pers comm*). The majority of movements of freshwater *Gammarus* occur by passive processes, such as downstream flushing in flood conditions (Elliott, 2002a; 2002b). The actual limited dispersal ability of freshwater *Gammarus* and the intolerance of saline conditions (Bulnheim, 1987) make this group of animals a good model system to study the historical process of the colonisation of the UK across the English Channel and North Sea.

5.4.1 Comparative phylogeography in Europe

There have been many phylogeographic studies undertaken in Europe (reviewed in Hewitt, 1999), but no strong congruence between individual species patterns of colonisation have been found (Taberlet *et al*, 1998). However there are some broad

patterns that Hewitt (2000) has categorised into three groups, the grasshopper, the hedgehog and the bear. The closest colonisation routes to the data collected was the grasshopper pattern. Unlike both the bear and hedgehog, which have the colonisation of the UK from the Iberian Peninsula, the grasshopper and *G. pulex* show colonisation of the UK from a central European origin. The lack of expansion out of Italy by the grasshoppers, and the absence of *G. pulex* in Italy (thought to be replaced by *G. italicus*, Karaman & Pinkster, 1977a), further support the grasshopper pattern.

5.4.2 Post-glacial expansion into new habitats and the British Isles

The low diversity of mitochondrial haplotypes in the UK clade vindicated the previous allozyme findings of limited differentiation between populations and overall low diversity. Additionally, the pairwise difference plot of the UK clade supported the expectation of an expanding population and suggests that the colonisation of the British Isles was recent. Being an island system, the formation and isolation of British Isles has been well studied over the last 20 years (Gibbard, 1988). The geological timing of the formation of a true marine English Channel could be used to calibrate the time of colonisation.

Until the early Pleistocene (~2.4 mya) the UK was isolated from Europe by the marine water of the Atlantic Ocean (Funnell, 1995; Gibbard, 1988; 1995). During the mid-Pleistocene a connection between the UK and Europe was formed, aided by the fall of sea levels and the deposition of large volume of material from the North German rivers (Gibbard, 1988). The net result was the formation of the Great European (Ur-Frisia) Delta in what is now the southern North Sea (Funnell, 1995), damming a proglacial lake to the north. The British Isles remained land-locked until the Anglian stage (~0.4 mya), when the proglacial lake breached the dam and flooded the delta, creating a channel between the North Sea and the English Channel. In the late

Pleistocene a connection to Europe was gained and lost with the changing sea levels and glacial melt water. The final connection (land bridge) was lost ~ 9500 ya when the English Channel was initially brackish and finally saline ~ 7000 ya.

The last chance for *G. pulex* to colonise the UK appears to have been during the late- Pleistocene prior to the loss of the land bridge. However, most of the UK was covered with either ice or permafrost during the last glacial period (~ 18,000 ya) leaving a period of ~ 8000 years for successful colonisation. During the glacial periods of the Pleistocene, *G. pulex* would have been very unlikely to survive the extreme conditions across the British Isles, suggesting that it was at this time (~ 8000 years ago) that there was the final chance for colonisation.

There are suggestions that some species could have survived the last glacial advance in regions of southwest Britain (e.g. the bullhead, Hänfling *pers comm*), which were largely free of permafrost (Williams, 1969). Data from the four populations in this region sampled for this study do not show higher levels diversity that are commonly associated with refugia and cannot support the hypothesis of a southern British refugia. One reason for rejecting the hypothesis could be the intolerance and cessation of breeding of *G. pulex* during cold periods. This would also explain the survival of the bullhead (*Cottus gobio*) in tundra regions, which appears to have adapted to breeding in cold water conditions (Englbrecht *et al*, 2000).

The source of the colonisation of the UK appears to originate in Europe and follows the routes of the drainages emptying into the North Sea (Rhine, Elbe and Schelde). As previously mentioned, this agrees with the high volume of material transported down the rivers prior to the isolation of the UK. The proposed colonisation route could also be supported by the absence of "UK" like *G. pulex* in the Seine, Loire and Schelde, which also drained into the English Channel during the Pleistocene (Gibbard, 1988). The location of potential refugia for the colonisation has not been

resolved and could have been from any of the proposed eastern refugia (see Taberlet *et al*, 1998).

5.4.3 Pre-Pliocene refugia and colonisation in Europe

The sequence variation between the four *G. pulex* clades suggests that there have been two waves of divergence within the species. The first of these appears to have occurred during the Miocene, splitting the East-west, Vendee and remaining clades. The age of this split agrees with the divergence between *G. fossarum* subspecies from refugia either side of the Alps (Müller, 2000). The location of the populations from the two clades supports Müller's (2000) suggestion of refugia either side of the Alps.

The East-west clade contains two subgroups of which the eastern (Danube) group appears to be the older, more basal group. If the eastern group were older, it would suggest that the western group is derived from a westerly colonisation event of the eastern group. This hypothesis can be supported by the presence of an "ancestral" eastern haplotype in one of the western populations. The direction and location of the populations sampled in this clade suggests a colonisation route along the Danube and then the Rhine (Figure 5.11). This course has been proposed as a colonisation route for many freshwater species from a glacial refugium in the Balkans (Durand *et al*, 1999; Englebrecht *et al*, 2000; Nesbo *et al*, 1999; Wallis and Arntzen, 1989). Even now the Danube is a colonisation route for invasions of exotic *Gammarus* fauna from the Ponto-Caspian e.g. *Dikerogammarus* (Dick and Platvoet, 2000). Therefore a Balkan refugium for the East-west clade during glacial periods is the likely to generate the repeated westerly colonisation route proposed.

The small sample size in the Vendee clade limits the ability to locate possible colonisation routes from refugia. However, the lack of *G. pulex* from Spain suggest that the southernmost location for a refugium could be the northern slopes of the Pyrenees.

The Pyrenees has been suggested a refugium during several glacial cycles for some species including water voles (Taberlet *et al*, 1998) and shrews (Zima *et al*, 1996). (The Pyrenees could have been one possible location of refugia for *G. pulex* in the Vendee (Figure 5.11)). Colonisation from the refugia along the coast may have been possible during times of high glacial melt when coastal waters became brackish.

Figure 5.11: The location of two proposed Miocene refugia (R?) of *G. pulex* and the possible colonisation routes during the late tertiary.

5.4.4 Pliocene / Pleistocene colonisation

The second wave of divergences between the *G. pulex* clades (including *G. p. gallicus*) occurred sometime between the mid-Pliocene and the early Pleistocene. There are three lineage splits that occurred during this time: the splitting of the Rhine and UK

clades; the split of *G. p. gallicus* from *G. p. pulex*; and the split within the East-west clade. During this geological period the direction, size and routes of river drainages was subjected to great changes and the exact timing of these events is still much debated (Funnell, 1995).

Figure 5.12: The location of three Pliocene / Pleistocene refugia (R?) and proposed colonisation routes of *G. pulex*.

The division of the Rhine and UK clades appears to be in part derived from the connections between the Rhone and upper Rhine that formed in the Pliocene (Gibbard, 1995). During the mid- Pliocene the upper Rhine flowed south to join the tributaries of the Rhone. At this time, movement between the two drainages would have been possible. By the Pleistocene / Pliocene boundary the connection between the two had

been lost and the Rhine now flowed northwest to join the Meuse at the Maas estuary (Gibbard, 1988).

Within the Rhine clade the sample from the Rhone appears older and more basal than the rest suggesting movement from the Rhone to the Rhine was possible at some point during the formation of this clade. The proposed colonisation route of the Rhine clade is shown in Figure 5.12, originating from a possible refugium on the Mediterranean coast. The high diversity and endemism of gammarid fauna in the Mediterranean has been proposed as a possible refugium during the Pleistocene glaciation (Scheepmaker, 1990; Thienemann, 1950) and supports the refugial status proposed here. This refugial area could also have been used by the UK clade and *G. p. gallicus* clade and could explain the relationship between the three clades. If the same refugium was utilised by ancestors of the UK clade, the route of colonisation could have been via the Meuse, Rhine and Elbe systems.

The high diversity within the East-west clade suggests that gene□flow between the two locations was been restricted for several million years. Repeated colonisation from the Balkans up the Danube would have maintained the eastern populations. This would have been an unlikely scenario for the western populations, due to the long distance colonisation required after every retreat of the ice sheets, suggesting that the western populations survived the glacial periods in another refugium, possibly on the border between France and Belgium. There is no direct evidence for the location or existence of this refugium, but other authors have also found similar patterns (Nesbo *et al*, 1999; Hänfling *pers comm*) and suggested the location of a possible refugium in Belgium.

5.4.4.1 Evidence for a contact zone in the upper Rhine

Haplotypes from the UK and Rhine clades in the Mainz and Kleinlutzel populations add further support to the upper Rhine being a contact zone for a wide range of species, including *G. fossarum* (Müller, 1998). The presence of haplotypes from two separate *G. pulex* clades in two populations that are located in the *G. fossarum* contact zone strongly suggests that contact and possibly hybridization have occurred in *G. pulex* in this region of the Rhine like *G. fossarum*. The Mainz sample site was also the same site that Müller (2000) identified as being located in the centre of the contact zone (site NOR, Müller, 2000). As previously suggested, the Rhine was a major colonisation route and could have been utilised by both clades to expand or retreat during the Pliocene / Pleistocene.

5.4.4.2 Secondary contact in the upper Danube

The presence of two distinct haplotypes (MAR & ISAR) from separate clades within a 40 km stretch of the Danubian drainage could be evidence of colonisation of the upper Danube from two sources. The older (Miocene) colonisation from the route from potential Balkan refugia appears to have come into contact with a later (Pliocene) expansion of the Rhine clade. With the limited data collected here, and no evidence of secondary contact in this area observed in the literature to date, with all colonisation routes moving from the Danube into the Rhine (Durand *et al*, 1999; Englebrecht *et al*, 2000; Nesbo *et al*, 1999) the hypothesis of secondary contact needs further investigation.

5.4.5 Species status

The taxonomy of the freshwater *Gammarus* had been a source of argument long before the introduction of the artificial species groups by Karaman and Pinkster (1977a,

1977b & 1987) based on extensive morphological analysis. With the wider use of molecular markers, especially in the new field of phylogeography, the status of many of the freshwater *Gammarus* is now being questioned (Meyran *et al*, 1997). The previously identified 'classical' species are now being divided into sibling species groups and cryptic species forms (Meyran *et al*, 1997; Müller, 2000). In this study the *G. pulex* species group has been extensively investigated and revealed a more complex picture than previously identified (Karaman & Pinkster, 1977a; Meyran *et al*, 1997; Scheepmaker, 1990). The study has also confirmed the status of the *G. pulex* species group with a high sequence divergence (~21%) between all *G. pulex* types and its nearest known relative, *G. fossarum* (Table 5.4).

Divergence between the clades of *G. pulex* was greater than previously described within *G. pulex*, and suggests the possibility of at least four genetically divergent *G. pulex* clades. The taxonomic status of these four clades is unclear. Samples from the Pyrenees (*G. p. gallicus*) and *G. pulex* from Holland (Heirden) were previously identified based on morphology by Scheepmaker (1990). Assuming the identification by Scheepmaker (1990) was correct, then both the Vendee and East-west clades are more divergent than the described sub-species and should therefore warrant subspecies status. Data from Müller (2000) supports this claim, with an average sequence divergence of 6.8% between *G. fossarum* subspecies either side of a hybrid zone. The remaining clades (UK and Rhine) would also fall within the subspecies level of classification based on the identification by Scheepmaker (1990) and relative sequence divergence criteria of Müller (2000).

The late Tertiary and Quaternary climatic changes have had a greater effect than previously thought on the *G. pulex* species group by creating barriers to migration that have subsequently led to the formation of several distinct subspecies. Without the support of an extensive morphological survey and breeding experiments, the

reproductive isolation and true species identity of the clades resolved here remains uncertain and requires further study.

5.5 Summary

The phylogenetic analysis of 42 populations from north west Europe revealed a complex distribution within *G. pulex* consisting of four main clades. Divergence between the clades was between ten and five percent sequence divergence, indicating two separate 'splits' within *G. pulex*. The first split, occurring during the Pliocene/ Pleistocene, separated the east-west clade and the vendee clade from the remaining two clades, including one subspecies, *G. pulex gallicus*. The second split occurred during the Pleistocene separating the Rhine and UK clades, in addition to a further split of the east-west clade.

The data from *G. pulex* appears similar to that of the 'grasshopper' pattern identified by Hewitt (1996) and as such *G. pulex* could have used the same refugia (Pyrenees and Balkans). Another potential refugium that *G. pulex* could have utilised was one in Belgium proposed by Hänfling *et al.* (2002).

Chapter 6

Isolation of microsatellites in *Gammarus pulex* and an analysis of population genetic structure

6.1 Introduction

Microsatellites in eukaryotic organisms have been known since the early 1980s (Jarne & Lagoda, 1996). Indeed, every complete genome that has been sequenced to date has contained microsatellite repeat regions (Hancock, 1999). The term "microsatellite" refers to DNA sequence motifs between one and five bases long that are tandemly repeated in a genome. Microsatellite loci are generally unstable regions with one of the highest DNA mutation rates recorded for any part of a genome (between 10^{-5} and 10^{-2}), two to three orders of magnitude higher than allozymes, and four to seven orders of magnitude higher than point mutations (Hancock, 1999; Jarne & Lagoda, 1996). As a result of such high mutation rates, high levels of polymorphism are typically recorded, with the number of alleles per locus ranging between one and 50 (Jarne & Lagoda, 1996). The presence of microsatellite DNA in many genomes and their highly polymorphic nature make them ideal molecular marker loci for determining kinship (Queller *et al*, 1993), population structure (Carvalho & Hauser, 1998) and genome mapping (Dib *et al*, 1996; Dietrich *et al*, 1996).

Although microsatellites are one of the dominant molecular markers for estimating genetic diversity (Grimaldi & CrouauRoy, 1997), there are problems arising from the effects of size homoplasy (Angers & Bernatchez, 1997; Angers *et al*, 2000; Fagergerg *et al*, 2001; Garza & Freimer, 1996; Orti *et al*, 1997; Viard *et al*, 1998). Mutations in the flanking sequence of microsatellite loci have been reported in closely

related species to those from which the original loci were isolated and are thought to account for the presence of null alleles (Lehmann *et al*, 1996). At a population level, flanking site polymorphisms are more rarely reported (Estoup & Cornuet, 1999; Orti *et al*, 1997), and interruptions within the microsatellite repeat appear to be more common (Angers & Bernatchez, 1997; Angers *et al*, 2000; Grimaldi & CrouauRoy, 1997; Viard *et al*, 1998). Interruptions within microsatellite repeats that cannot be resolved by allele size scoring could reduce the mutation rate (Angers & Bernatchez, 1997; Kunst *et al*, 1997; Petes *et al*, 1997) and polymorphism (Weber, 1990). The detection of all types of size homoplasy at microsatellite loci has been possible only by sequencing (sometimes in combination with SSCP) many alleles (Angers & Bernatchez, 1997; Angers *et al*, 2000; Viard *et al*, 1998). However most microsatellite loci used in population genetic studies are derived from single colony isolates in genomic libraries that would fail to detect size homoplasy and its effect on estimates of mutation rate.

Identification and characterisation of microsatellite loci has been relatively simple in the few species where extensive genomic mapping has been undertaken, for example, in humans (Dib *et al*, 1996) and mice (Dietrich *et al*, 1996), where loci can be readily identified by screening genomic databases. Characterisation of microsatellites from species without extensive genome sequencing requires labour-intensive isolation processes of cloning, probing and sequencing to screen part of the total genome (Ender *et al*, 1996; Shaw, 1997). There are now numerous methods available for the isolation of microsatellites including enrichment protocols that have been developed to aid isolation from genomes with low microsatellite densities (Hammond *et al*, 1998; Kandpal *et al*, 1994; Ostrander *et al*, 1992).

Despite the obvious benefits of highly polymorphic microsatellites markers for estimating population differentiation in *Gammarus* species that are weakly divergent at allozyme loci (Scheepmaker, 1990; Siegismund *et al*, 1985), there are no published

microsatellites for any amphipod crustaceans. Microsatellite loci for some of the commercially important crustaceans, especially the shrimps, have, however, been developed (Baker *et al*, 2000; Ball *et al*, 1998; Xu *et al*, 1999). The highly polymorphic nature of microsatellite loci and the weak population differentiation estimated with allozymes and mtDNA in *Gammarus pulex* (Chapters 3 & 5) suggest that microsatellites would be a good candidate marker to reveal any population structuring in the UK.

6.1.1 Aims

There were three main aims of the the investigation in to the variation of microsatellite loci in *Gammarus pulex*:

- To isolate and optimise a minimum of five microsatellite loci for *Gammarus pulex*.
- To compare levels of genetic diversity at microsatellite loci in UK populations, with reference to estimated population abundances and population structure.
- To estimate any possible loss of genetic diversity (as measured by microsatellite loci) due to an introduction of animals to Fleshwick Bay from the River Terrig site.

6.2 Methods

6.2.1 Microsatellite Isolation

Two methodologies were utilised to locate microsatellites in *Gammarus pulex*, a standard "Shot-gun" isolation (Shaw, 1997) without enrichment, and isolation with enrichment for dinucleotide repeats (Hammond *et al.*, 1998).

6.2.1.1 Shot-gun isolation

The first method of isolation had no enrichment and was probed using standard radioactive hybridisation techniques. Five *G. pulex* from Pocklington and Malham Tarn

(Chapter 2) were cut in half and the posterior section was homogenised in 1.5 ml Eppendorff tubes using a pestle. DNA extraction was carried out using a standard phenol-chloroform technique (Sambrook *et al*, 1989) and checked on a 1% agarose minigel.

DNA from the ten *G. pulex* was completely digested overnight with Sau3A (Boehringer) endonuclease at 37°C (Appendix 6.7(i)). After checking the restriction products on a 2% agarose minigel, the 300-800 bp size region was excised from the gel and purified using a Prep-a-Gene matrix kit (Biorad). Size-selected DNA fragments (300-800bp) were ligated into the dephosporylated *BamHI* site of the pUC18 plasmid vector (Amersham) overnight at 13°C (Appendix 6.7(ii)). A plasmid vector to DNA fragment ratio of 2:1 was used to maximise the efficiency of the ligation procedure, as outlined in Sambrook *et al* (1989). Ligated plasmid vectors were cloned into DH5α library efficiency cells (Life-technologies) using a standard 42°C heat shock method and grown overnight on Luria-Bertani broth (Sambrook *et al*, 1989). Positive colonies (containing plasmid with size selected DNA inserts) were identified using the amphicillin blue/ white test (Sambrook *et al*, 1989). 2890 positive colonies were transferred to ten large gridded plates (17X17) and grown overnight at 37°C. These colonies were then blotted on to nylon filters (Hybond N+, Amersham) and dried for four hours at 65°C.

Prior to radioactive hybridisation, all nylon filters were washed for one hour, in a pre-hyb buffer (5X SSC, 0.1% SDS, 5X Denhardt's solution), which was discarded after use. Oligonucleotide sequences were end labelled with γ^{32}P ATP (Amersham) at 37°C using T4 kinase (Amersham) and checked for incorporation. The filters were probed three times with different labeled oligonucleotides: $[(AAT)_{10}\ (AAAT)_9]$, $[(AAAC)_6\ (AAAG)_6\ (GATA)_6\ (AAG)_8\ (AAC)_9]$ and $[(AC)_{12}\ (AG)_{12}]$ at 55°C (5X SSC), 55°C

(2.5X SSC) and 65°C (1.25X SSC), respectively. After probing, filters were washed at 5°C below the hybridisation temperature to remove excess labelled probes. Filters were then wrapped in clingfilm and exposed to X-ray films for 24-48 hours. X-ray films were developed and positive colonies scored for each of the three hybridisations.

A total of 27 positive colonies were identified, eleven tri and tetranucleotides and 16 dinucleotides. All of the positive colonies were sequenced using universal plasmid (M13) primers and run out on an *AlfExpress*™ automated sequencer (Amersham).

6.2.1.2 Dinucleotide enriched isolation

The second method followed the enrichment method outlined by Hammond *et al* (1998) with a modified probing procedure, which followed the PIMA (without RAPD enrichment) method of Lunt *et al* (1999). The initial genomic library was created in an identical manner to the shot-gun method, but with 24 animals from three populations (Lowthorpe, Fleshwick Bay & Stainfield Village, Chapter 2).

6.2.1.2.1 Library enrichment for dinucleotide repeat arrays

Prior to the enrichment, the quantity of DNA was increased to reduce any loss of potential microsatellite loci and to increase the efficiency during the stringent capture procedure (see below). All size-selected DNA in the library had GATC overhangs (from the Sau3A digest) at both ends of the fragments. These restriction site overhangs were used to ligate complementary linker oligonucleotide primers to the size selected DNA (SAULA & SAULB, see Hammond *et al*, 1998, Appendix 6.7 (iii)). The quantity of size selected DNA in the library was then increased using PCR with the attached primers. DNA quantity and concentration was estimated using *Genequant* (Amersham) and checked on a 2% agarose minigel for amplification.

5- 10μg of the first PCR (see above) was hybridised separately to 5μg of two biotinylated probes ($(TG)_{22}$ and $(GA)_{22}$ in 500μl of 0.5M sodium phosphate. After 15-

18 hours at 50°C, the biotinylated probes were bound to complementary DNA fragments in the library. 50mg of Vetrexavidin was then rehydrated (Hammond *et al*, 1998) and gently mixed with the hybridisation solution to remove any biotinylated probes/ genomic DNA hybrids from solution by binding to the Vetrexavidin matrix. After mixing, the solution was washed twice at room temperature and once at 55°C to remove non-repetitive DNA. The enriched DNA bound to the Vetrexavidin matrix was released and retained in solution using a "hot" wash (65°C). The retained solution was concentrated to ~ 30ng/ µl using Centricon-100 spin columns. A second round of capture was not undertaken in order to reduce the chance of isolating the same microsatellites many times as had been previously observed in other freshwater invertebrates (Wilcock *pers comm*).

Enriched DNA fragments with single "A" base overhangs (a consequence of PCR) in the library were ligated into the multiple restriction site of the pGEM®-T Easy vector (Promega) overnight at 13°C (as in Appendix 6.7(ii)). Ligated plasmid vectors were cloned into DH5α library efficiency cells (Life-technologies) using a standard 42°C heat shock method and grown overnight on Luria-Bertani broth (Sambrook *et al*, 1989). Positive colonies (containing plasmid with size selected DNA inserts) were identified using the amphicillin blue/ white test (Sambrook *et al*, 1989).

6.2.1.2.2 PIMA probing

432 successfully transformed colonies were transferred to three large gridded plates (12X12) and grown overnight at 37°C. Colonies were then screened for the size of the inserts using universal plasmid primers (M13). Additionally, all colonies were screened for dinucleotide (TG and GA) repeats using the PIMA technique of Lunt *et al.* (1999). The method uses the universal plasmid primers and an additional repeat primer in the same PCR mix. If a colony contains a repeat array a second band will appear (in

addition to a band the size of the insert). From the 432 colonies screened, 114 produced additional bands (normally one, but in some cases two or three (GPGT2 locus)) using the repeat primers (106 TG and 8 GA). 37 of the positive colonies were sequenced using an *AlfExpress*™ automated sequencer (Amersham). From these 37 colonies, three dinucleotide microsatellite loci were identified (two TG and one GA).

6.2.2 Optimisation of loci

Primers for all three loci were designed (Table 6.1) using the software program OLIGO™ Macintosh version 6.1 (National Biosciences Inc., USA), from the sequence flanking the microsatellite repeat region. Primers for the GPGT1 and GPGT2 locus gave inconsistent amplification or multiple bands at lower annealing temperatures (despite re-designed primers in both cases, Table 6.1). Only the GPGA1 locus yielded specific PCR products without additional non-specific amplification to allow routine population screening for variation.

Table 6.1: Microsatellite primers details for the three isolated loci

Locus	Primer name (F/R)	Primer Sequence	Number of repeats	Expected product size
GPGA 1	GP-GA1F GP-GA1R	TGA CGA CGA ATG GGG TGT CCT T TCT TCA CTC TCT TTG GCG AAC ACA	40	184
GPGT1	GP-GT1F GP-GT1R	AGG ATA ATC GTT TTA CCG CAC TTA CCT ACC TTC ACC GTC GCA AAC	22	144
GPGT1	GP-GT2F GP-GT2R	CAA ACA GCT CCG GCC CAT ATC TT GAG CAT CGG GTA TGT TGA GCT GCG TAA A	22	176
GPGT1	GP-GT3F GP-GT3R	CAA TTG CTA CGG TTG CCT ACC TTC GCG TAA ACC GTA GGG CCA TAC CAT	22	153
GPGT2	GP-AC1F GP-AC1R	CAA TGA GTA TGA GTT TTT ATG TKG AAT AAA AGG GTA AYC GGT TGC	22+23+22 *	~370
GPGT2	GP-AC2F GP-AC2R	TAA GTG TGG CAA CCG ATT ACC CTT CAT TTT GCC GCA ACT TAC A	22+23+22 *	~390

* Duplicate locus with three microsatellite repeats.

6.2.3 Population screening

The GPGA1 microsatellite locus was screened for variation on an *ALFexpress*™ (Amersham) automated sequencer with markers of known size (100, 154 and 259) in each gel lane to allow quantification of allele sizes using Fragment Manager 1.2 (Amersham). 10-40ng DNA was used in each 10µl PCR reaction, containing 10x NH4 buffer (Bioline), 2mM MgCl2, 0.2mM dNTPs, 0.375u Taq (Bioline), 200nM Cy5-labelled forward primer, 200nM reverse primer and ddH_20. Amplification of the locus was carried out on a Hybaid Omnigene thermal cycler according to the thermal profile: 94°C for one min. followed by 32 cycles of 94°C for 45s, 62°C for 30 s, 72°C for 30 s, then a single final step of 72°C for 3 min.

6.2.3.1 Preliminary population screening

40 individuals from six populations (Foret de Mormal (31), Stainfield Hall (9), Malham Tarn (6), Lowthorpe (1), River Terrig (2) and Fleshwick Bay (3)) were initially screened with the GPGA1 locus. Products from three animals from Foret de Mormal, Lowthorpe, River Terrig and Fleshwick Bay were sequenced, using the designed primers, to check that the amplified product was the GPGA1 locus. When products contained unknown DNA sequences their identity was checked against known sequences in the Genebank database.

6.2.3.2 Microsatellite diversity in three populations

Samples from three populations, Lowthorpe, River Terrig and Fleshwick were screened with the GPGA1 locus, with sample sizes ranging from 69 to 91 (Table 6.2). Allele sizes from different populations were compared on the same microsatellite gel to eliminate any potential differences in running conditions and mobility of alleles between different runs.

6.2.4 Statistical analysis

Allele frequencies, mean allele size and heterozygosities in all three populations were estimated using FSTAT V2.8 (Goudet, 1995). Deviations from Hardy Weinberg expectations and tests for heterozygote excesses and deficiencies were calculated with GENEPOP V3.2b (Raymond & Rousset, 1995). Differentiation between the populations was estimated by pairwise comparisons of F_{st} in FSTAT V2.8 (Goudet, 1995) and R_{st} in RST CALC (Goodman, 1997).

Differences in heterozygosity and the number of alleles between populations were calculated with a resampling protocol that corrected for unequal sample sizes. 50 individuals within each population were resampled randomly and the mean number of alleles and unbiased heterozygosity calculated with 95% confidence limits (Figures 6.2 & 6.3). The observed differences in heterozygosities and number of alleles between all pairwise comparisons were calculated. These values were compared to a mixed sample of two populations (e.g. Fleshwick Bay and River Terrig) comprising two groups of 50 animals resampled randomly1000 times (Monte Carlo analysis, PopTools, 2000). All test values (out of 1000) greater than or equal to the observed differences between populations were equal to the level of significance (P-value) divided by ten.

6.2.5 Phylogeny of colony GPGT1 alleles

From the sequenced clones containing the GPGT1 locus (Appendix 6.7), 16 had flanking sequences that could be scored reliably. These sequences were used to construct the most parsimonious phylogenetic tree of the colony GPGT1 allele flanking sequences using a heuristic search and a tree-bisection-reconnection (TBR) branch swapping algorithm with mid-point rooting in PAUP (Swofford, 1993).

The relationship between the number of repeats (at each sequenced colony allele) and the flanking sequence polymorphism, based on the position in the parsimony

tree was compared using a Kruskal- Wallis test. The average number of repeats in each clade was compared to a null hypothesis of a random distribution and tested using a ranking score for all alleles. If the size of alleles was not correlated to the flanking sequence the expectation should be no clustering of allele sizes in the topology of the tree, which could be tested against the above null hypothesis.

6.3 Results

6.3.1 Shot-gun isolation

No positive colonies sequenced contained microsatellite repeats, though some repetitive elements were observed (Appendix 6.1). All "false" positive sequences were compared for similarity to known sequences available on the Genebank database using a Blast search. No colonies matched any known sequences (apart from 20bp regions matching poorly to human chromosomes) in the database, and were assumed to be randomly distributed throughout the genome.

As no microsatellites were identified using the shot-gun method, estimating an exact density of microsatellites was impossible, though no loci were found in 1.6 Mbp (2890 colonies with mean DNA fragment length of 550bp). This result indicates that the density of microsatellite loci in the *G. pulex* genome was very low.

6.3.2 Dinucleotide enriched isolation

The dinucleotide enrichment method was more successful than the 'shot-gun' method, with three microsatellite loci identified and isolated (GPGA1, GPGT1 & GPGT2). It was impossible to estimate the density of microsatellite loci within the genome of *G. pulex* due to the unknown quantity of DNA assayed using the enrichment methodology. The majority of the sequenced colonies (29) contained the same microsatellite locus a dinucleotide GT repeat (GPGT1), with all colonies having almost

identical flanking sequences, though some polymorphism was observed (section 6.3.4). Repeat numbers found in sequenced colonies at this locus varied between eleven and 44. The structure of this locus also varied within the sequenced colonies with both perfect (Appendix 6.2) and interrupted (Appendix 6.3) alleles identified.

The second microsatellite locus (GPGA1), a $(GA)_{40}$ repeat, was isolated from a single colony and had three interruptions (Appendix 6.4). The third locus (GPGT2) was the most complex locus isolated, consisting of three copies of $(GT)_n$ microsatellite arrays joined (Appendix 6.5), and was found in three positive colonies. The flanking region of this locus was identical to that of the GPGT1 locus for 20bp either side of the microsatellite repeat array. The flanking regions of the two loci were, however different after the first 20bp (one in every five bases were different between the two loci), suggesting that the GPGT2 microsatellite could be a different locus from the GPGT1. However, some of the primers designed for the GPGT1 locus would either amplify GPGT1, GPGT2 or sometimes both loci depending upon the individual used and the method of DNA extraction. Indeed, both loci could still be part of a larger multi gene complex or family of microsatellites due to the RAPD like amplification patterns observed from primers designed for the two putative loci (Figure 6.1)

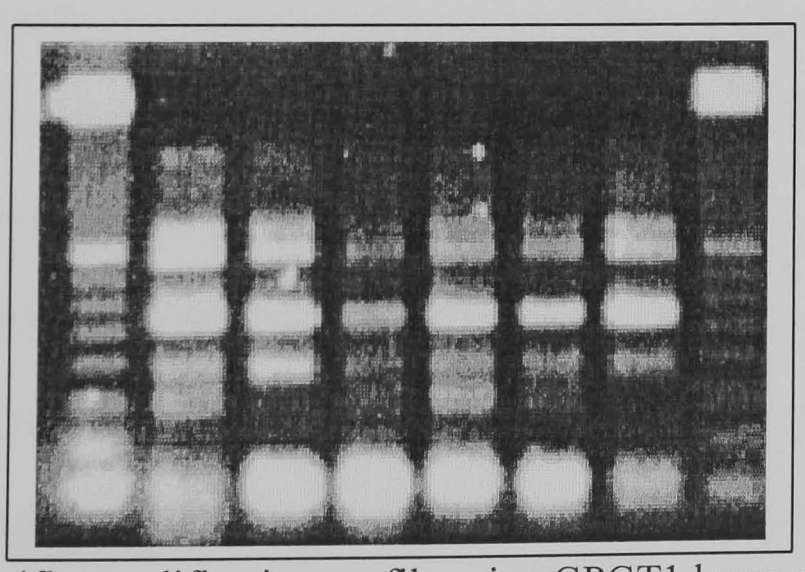

Figure 6.1: Non-specific amplification profile using GPGT1 locus specific primers (GP-GT2F and GP-GT2R).

The structure of the GPGT2 locus appeared more conserved than that of GPGT1 and the number of repeats in each the three adjoining microsatellite arrays were very similar (22, 23, 22 repeats respectively, Appendix 6.5). At the end of the isolation procedure a single microsatellite locus had been isolated (GPGA1) and could be scored consistently and reliably.

6.3.3 Population screening

All of the populations were screened only with the GPGA1 locus due to the poor amplification of size specific products from the other two loci isolated. Although the screening of populations with a single microsatellite locus would not always be advisable (due to locus specific patterns of population differentiation and structuring) it was decided screening should take place as locus specific differentiation could still add to the clarity of the molecular data already collected.

6.3.3.1 Preliminary screening

From the six populations used in the preliminary screening for variability at the GPGA1 locus, five had more than one allele. Samples from the French population appeared monomorphic, and the size of the PCR product was larger than expected (~500bp compared with the expected size of ~200bp). Similar sized non-specific products were also observed in samples from UK populations, but were weaker than the amplified microsatellite locus (Figure 6.2).

6.3.3.2 Microsatellite diversity in three populations

The three populations screened all showed high levels of polymorphism at the GPGA1 locus, all exceeding 85% heterozygosity (direct count, Table 6.2 and corrected for sample size, Figure 6.4). The highest heterozygosity occurred in the River Terrig population, but the highest allelic range and number of alleles occurred in the Lowthorpe population (which had the highest allozyme variability (Chapter 3)). The

contrasting allele distributions between the three populations are clearly shown in Figure 6.3. Both the lowest heterozygosity and number of alleles occurs in the Fleshwick population.

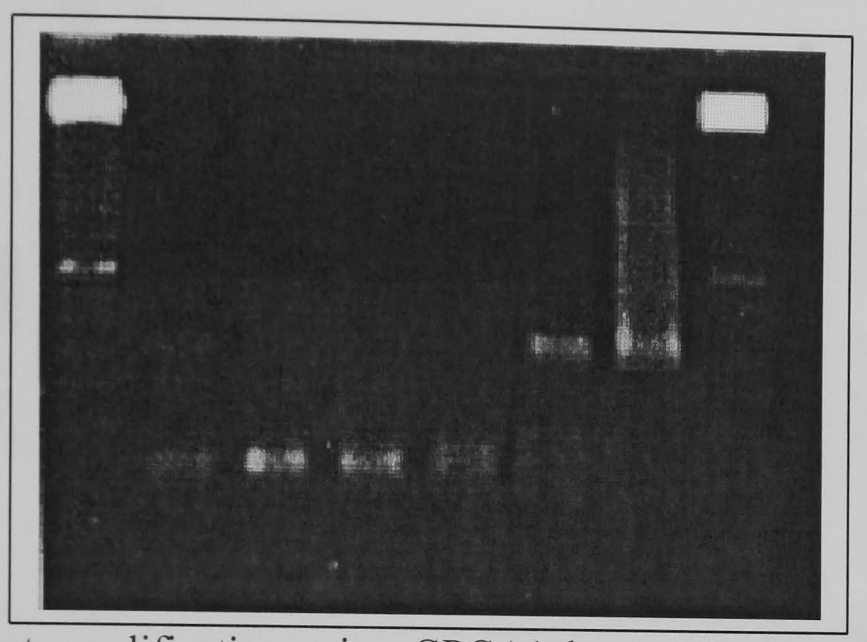

Figure 6.2: Product amplification using GPGA1 locus primers. The two end lanes show 100bp DNA ladder, lanes 2-5 (form the left) are individuals from Lowthorpe, 6 & 7 are individuals from Foret de Mormal, France.

There were no observed deviations from Hardy Weinberg expectations and tests for heterozygote excesses and deficiencies were not significant.

Table 6.2: Descriptive data of GPGA1 locus

Population	**Sample size**	**Number of alleles**	**Median allele size**	**Allele range**	**Heterozygosity *Ho***
Lowthorpe (1)	73	16	200	190-222	0.877
River Terrig (2)	69	12	198	188-210	0.898
Fleshwick Bay (3)	91	11	200	188-210	0.861

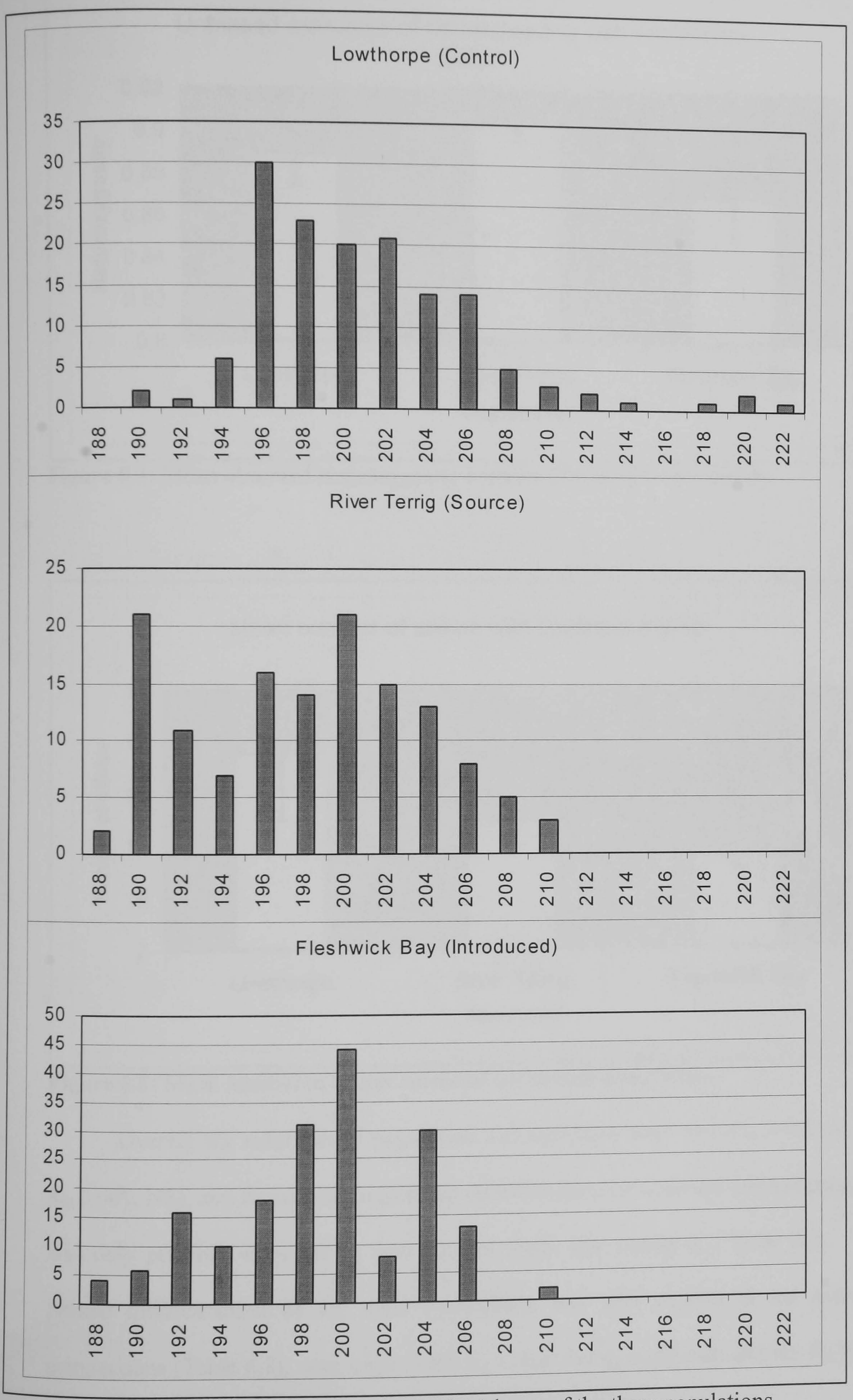

Figure 6.3: Allele distributions at the GPGA1 locus of the three populations.

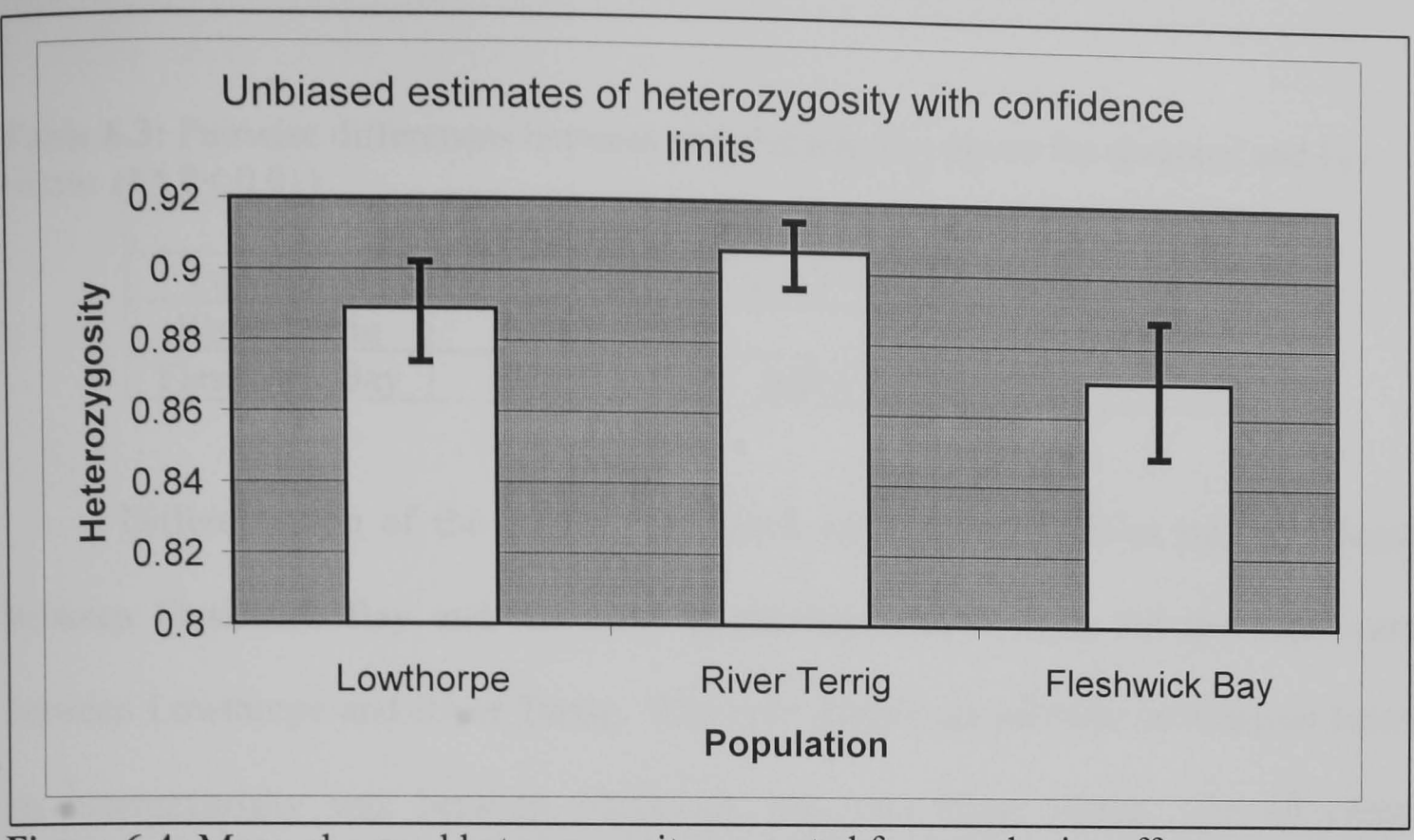

Figure 6.4: Mean observed heterozygosity corrected for sample size effects.

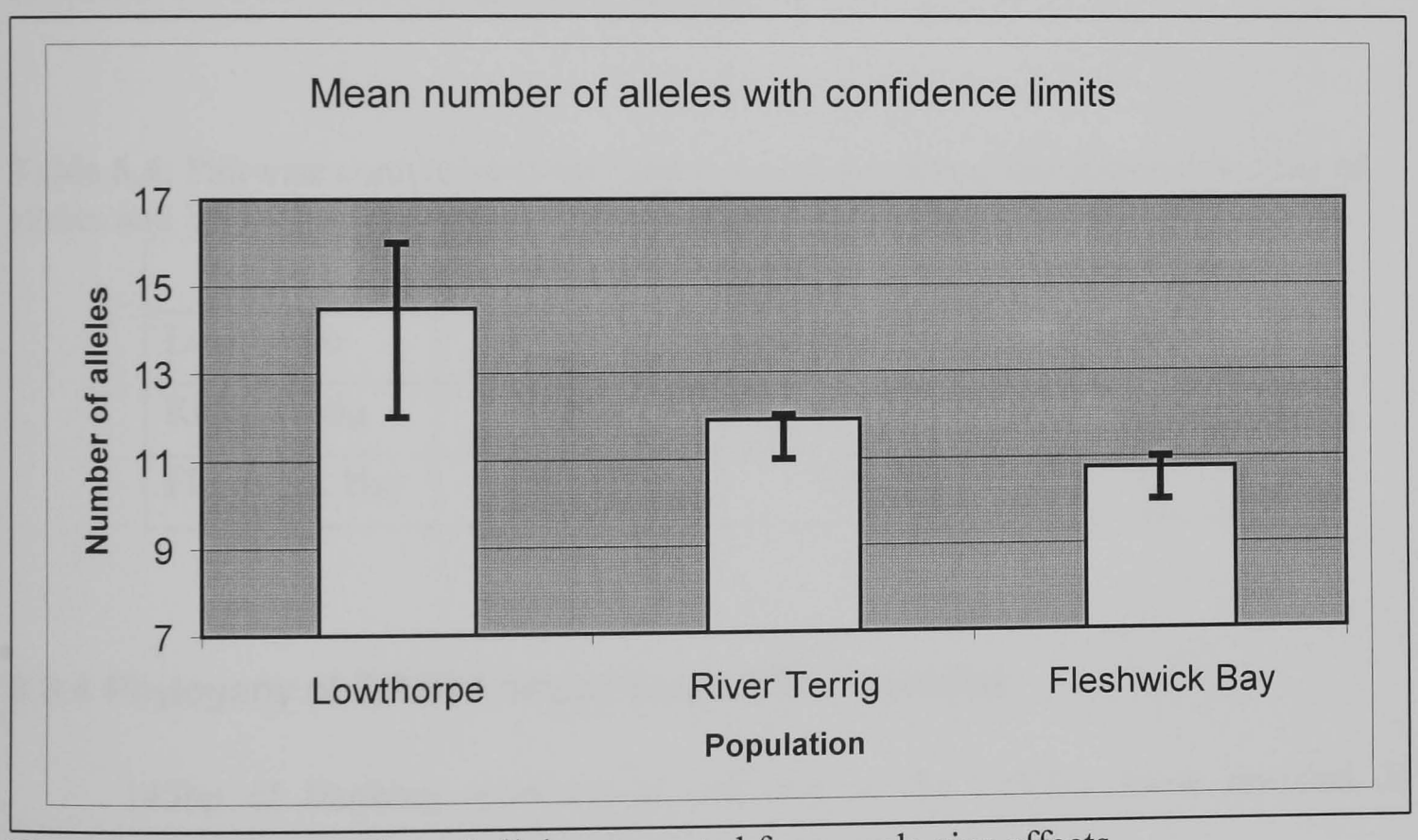

Figure 6.5: Mean number of alleles corrected for sample size effects.

Overall, the estimates of population differentiation were low (F_{st} = 0.016, NS; R_{st} 0.069, NS), and statistically significant differentiation between the three populations was only possible when taking into account allele size (using R_{st}, Table 6.3). The limited differentiation of the three populations was also evident in the pairwise comparisons (Table 6.3), with none of the F_{st} values being significant and the R_{st} value between River Terrig and Fleshwick Bay also not significant.

Table 6.3: Pairwise differences between populations, R_{ST} above the diagonal and F_{ST} below. (** P< 0.01)

	Lowthorpe	River Terrig	Fleshwick Bay
Lowthorpe		0.117**	0.069**
River Terrig	0.015		0.009
Fleshwick Bay	0.021	0.015	

Differentiation of the populations based on number of alleles was significant between Fleshwick Bay and the other populations (Table 6.4), but not significant between Lowthorpe and River Terrig. The only significant pairwise comparison based on heterozygosity was between Fleshwick Bay and River Terrig. (In all cases, significant values were calculated when there was no overlap in the confidence limits about the estimated mean (Figures 6.4 & 6.5))

Table 6.4: Pairwise comparisons between populations, above the diagonal number of alleles and below heterozygosity. All values are levels of significance.

	Lowthorpe	River Terrig	Fleshwick Bay
Lowthorpe	-	0.095	**0.042**
River Terrig	1.000	-	**0.037**
Fleshwick Bay	0.247	**0.025**	-

6.3.4 Phylogeny of GPGT1 alleles isolated from colonies

143bp of flanking sequence at one end of the GPGT1 locus revealed 21 polymorphic sites (Appendix 6.8). Of the 21 polymorphic sites four were informative parsimoniously and two of these split the alleles into two main clades (blue and red (C), see Figure 6.6 and Appendix 6.8). The larger blue clade could then split into two smaller groups (A, top & B, centre) by another parsimonious site.

Both blue clades (A & B) contained the largest alleles (repeat number) isolated with the exception of colony number 87, which was the only pure allele sequenced

(Appendix 6.6). The different sized alleles were spread within the phylogeny significantly differently from a random distribution (Kruskal Wallis test, $P<0.01$). Segregation of large and small alleles (with the above mentioned exception) was observed in the three clusters.

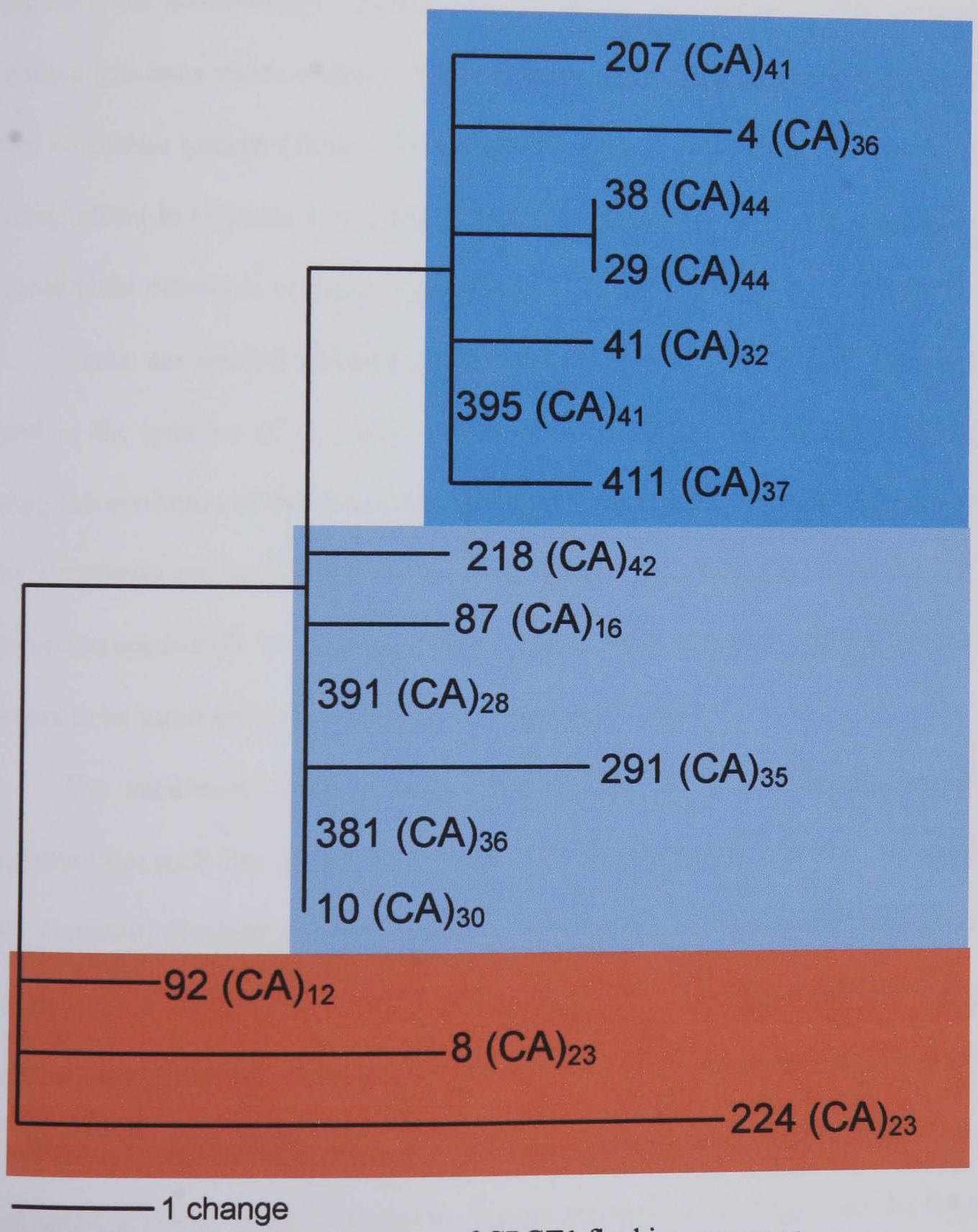

Figure 6.6: The most-parsimonious tree of GPGT1 flanking sequence. Numbers refer to colony number and the numbers of microsatellite repeats (not the structure of the alleles, Appendix 6.7). The three clusters are named from top to bottom, A, B and C (red) for later analysis.

6.4 Discussion

6.4.1 Microsatellite density

The lack of microsatellites isolated from *G. pulex* and the apparently low density of loci within the *G. pulex* genome estimated was surprising given that more than 50 $(AC)_n$ loci were identified for the guppy, *Poecilia reticulata* (Hadfield, *unpublished*) using the same methodology (Hammond *et al*, 1998). The density of microsatellite loci in animal genomes varies widely within and between different taxa and there appears to be no consistent pattern (Table 6.5), though even when considering this, the density of microsatellites in *G. pulex* is still low. Previously there have been very few estimates of microsatellite density in crustacean genomes (Table 6.5) e.g. Ball *et al.* (1998).

There are several possible explanations for the low density of microsatellites found in the genome of *G. pulex* in this study, including the loss of microsatellites during the evolution of the genome, as proposed in the Culicidae (Rai & Black, 1999). This hypothesis can be rejected, as more loci (at least three) have been isolated for other *Gammarus* species (*G. insensibilis*, Pearson *pers comm*), suggesting that the low density appears to be more species-specific, in this case to *G. pulex*.

The enrichment method used could be bias in its selection for the size of microsatellite, such that only large repeats are isolated from the library and small ones lost. Generally the size of repeats isolated from the library was greater than 20 repeats and reached a maximum of 65 in some guppy (*P. reticulata*) colonies sequenced (Hadfield *unpublished*). During the hybridisation of repeat probes and library DNA (see 6.2.1.2.1) the temperature was critical to optimise the hybridisation of probe to microsatellite DNA. If the hybridisation temperatures were too high, only the fragments containing the longest (and strongest) stretches of DNA that annealed to the probe would be retained. Such long stretches of DNA would contain large microsatellite

repeats and could explain the size bias observed in the data and the overall low density of microsatellite loci identified in this gammarid species.

Table 6.5: Descriptive data of $(AC)_n$ microsatellite loci from invertebrates and vertebrates

	Number of loci	Mean number of repeats	Average distance between repeats
Vespula rufa Yellow jacket wasp [1]	11	n/a	8kb
Apis mellifera honey bee [2]	23	12.5	34kb
Drosophilia melanogaster fruit fly [3]	41	10.1	60kb
Penaeus setiferus white shrimp [4]	39	n/a	51kb
Illex argentinus squid [5]	150	n/a	11kb
Dicentrarchus labrax sea bass[6]	11	26.1	17kb
Salmo salar Atlantic salmon [7]	45	11.6	63kb
Bos taurus cow [8]	11	16.1	180kb
Homo sapiens human [9]	36	13	28kb
Gammarus pulex freshwater shrimp[10]	0	n/a	>1.6Mbp

[1] Thoren *et* al (1995) [2] Estoup *et al* (1993) [3] Schug *et al* (1998) [4] Ball *et al* (1998) [5] Adcock *et al* (1999) [6] Garcia de Leon *et al* (1995) [7] Slettan *et al* (1993) [8] Toldo *et al* (1993) [9] Stallings *et al* (1991) [10] This study, using the 'shot-gun' methodology.

6.4.2 Size homoplasy and flanking site polymorphism at the GPGT1 locus

The data collected during the microsatellite isolation revealed multiple copies of the GPGT1 locus that not only differed in repeat number but also in structure and polymorphism in the flanking regions (Appendix 6.7). Unlike most previous studies,

where alleles, within a single species, from the same locus have been sequenced, (Angers & Bercnatchez, 1997; Angers *et al*, 2000; Fagergerg *et al*, 2001; Orti *et al*, 1997; Viard *et al*, 1998), size homoplasy due to both flanking site mutations and interruptions were observed here.

The high level of homoplasy observed at the GPGT1 locus suggests that the mutation rate of GPGA1 locus, as revealed by size alone, could be a very biased estimate. The flanking site phylogeny revealed three main clades, which was also the number of populations pooled for the construction of the genomic library. However it is impossible to assign an identity to any of the sequenced alleles to a specific population, due to the probing procedure and the phylogeny could be by chance alone.

6.4.3 Genetic diversity

Measures of diversity and variation within the three populations screened may not truly reflect the overall genetic pattern as only a single microsatellite locus was used for the screening. Nevertheless, the limited microsatellite data indicates high allele diversity and heterozygosity in the Lowthorpe population, supporting the previous allozyme results (Chapter 3) and give further support to the prediction that this population is large and seasonally stable. The concordance between the microsatellites and the allozymes was not supported in the other two populations. The high level of microsatellite heterozygosity in River Terrig was unexpected, when compared to the allozyme data, as the population appears to show strong seasonal fluctuation in structure and has a smaller size, when compared to Lowthorpe. One possible explanation could be that the level of heterozygosity in the River Terrig population was a result of genetic drift of allele frequencies, which would not affect the number of alleles (Leberg, 1992).

6.4.4 The effect of an introduction

The effect of artificial introductions has been referred to a "one generation bottleneck" (Carvalho and Hauser, 1995). It has also been shown that there is a loss of genetic diversity associated with populations that have undergone a bottleneck (Nei *et al*, 1975). The duration and scale (reduction in population size) of a bottleneck (or founder event) will determine the potential loss of genetic diversity in a population (Leberg, 1992). There are two indicative statistics of populations that can be used to suggest a bottleneck or a founder event: the number of alleles; and the heterozygosity. The number of alleles (especially rare ones) in a population would be expected to be reduced after a bottleneck or a founder event by an initial reduction in the number of animals, and then by the process of genetic drift (Shaw *et al*, 1992). Alleles at higher frequencies in a population would be maintained, as they are more numerous in the population as a whole and are therefore less likely to be lost. The loss of alleles (especially rare ones) has been predicted to be a more sensitive indicator of a reduced population size than levels of heterozygosity (Nei *et al*, 1975).

Lower levels of heterozygosity in a population after a bottleneck do occur, but only where the population (effective population) size has been severely reduced (~10-20) as in the case of the elephant seal (Hoelzel, 1999) and lions (Packer *et al*, 1991). From examples of introductions in the literature the reports of reduced heterozygosity are rare compared to the frequent loss of alleles (Cabe, 1998; Huettel *et al*, 1980; Parkin & Cole, 1985). However some of these studies investigated introductions of at least 100 animals. A reduction in the heterozygosity alone has also been considered a poor indicator of past population bottlenecks as an increase in variance can still occur as a result of a drift in allele frequencies (Leberg, 1992). Carvalho *et al* (1996) support the cautious use of heterozygosity as an indicator of past population bottlenecks and suggest the use of allelic diversity instead.

This study is the first to examine the effect of a well-documented introduction of *Gammarus pulex*. The lack of any apparent loss of genetic diversity in allozymes (Chapter 3), mtDNA (Chapter 5) and limited loss at a single microsatellite locus between source and introduced populations of *G. pulex* suggests that the introduction was successful. There are several possible hypotheses to support the maintenance of genetic diversity observed, and they all rely on the assumption that most, if not all, the founder animals were all capable of reproduction and that they would be approximately equal to the effective number of founders. This assumption is very likely to be supported as no mention has been given of the introduction of juveniles in this case (Hynes *pers comm*).

The first hypothesis is that the founders of the introduced population were sufficient to contain all the detectable genetic diversity of the source population. This hypothesis would assume that there was little initial mortality in the founder animals or that the number of founders needed to maintain genetic diversity was actually lower than 75. Another assumption is that the effective population size of the River Terrig (source) population is much smaller than the abundance estimate suggests (Chapter 2). There have been no estimates of either census (N) or effective population size (N_e) at the River Terrig, but an effective population size of greater than 75 was suggested by abundance estimates and the number of animals sampled (Chapter 2) (assuming a order of magnitude difference between N_e and N, Nelson & Soule, 1987).

A second hypothesis would be that the effect of genetic drift has been limited as randomly mating founder animals rapidly populate their new location. A rapid expansion into an empty habitat could reduce the time the population remains small. Although population sizes of *G. pulex* can rapidly increase during favourable periods (Adams *et al*, 1987; Iversen *et al*, 1977; Welton, 1979), the time to reach a equilibrium population size would take many generations. During the period where the population

was expanding the effect of genetic drift would be significant as the population would still be small. Another compounding factor is the strong influence of assortative mating in populations of *G. pulex* (Ward, 1984), which would have a similar effect to genetic drift, reducing rare alleles and fixation of some loci.

In conclusion, the maintenance of genetic diversity in an introduced population was unexpected considering the small number of founders. However the effective population size of the source population coupled with a rapid expansion into an empty habitat would be unlikely to have limited the time that the population was bottlenecked. This finding while significant in the context of other introductions of freshwater animals should be treated with some caution due to the limited molecular marker loci utilised (three polymorphic allozymes and one microsatellite loci).

6.5 Summary

Using two different methodologies for the isolation of microsatellites from the genome of *G. pulex* three loci were identified, only one of these loci proved reliable for routine scoring (GPGA1). The single locus was used to screen samples from three populations, with particular reference on the source and introduced populations used in an introduction experiment in 1950 by Hynes. Data from the source and introduced populations indicated there was a significant loss of heterozygosity in the introduced population, though caution was placed on further analysis as only single locus was used.

Flanking sequence of the GPGT1 locus (not screened) from 16 colonies (Figure 6.6) showed a significant clustering of allele sizes based on flanking site polymorphisms, with three distinct groups. The original library was constructed using three populations and allele sizes and sequence polymorphism may have correlated to specific populations.

Chapter 7

General discussion

7.1 Summary of main findings

Genetic diversity within the UK, as revealed with allozymes and mtDNA sequencing, was very low. Isolated populations, such as those located in the Malham Tarn area, showed some structuring, thought to be a consequence of geographical barriers restricting gene flow. The effect of population isolation and restricted gene flow was most apparent at the Horrabridge site, which is an isolated river draining off Dartmoor, where all sampled animals were monomorphic at 12 allozyme loci and at the mtDNA gene sequenced. In contrast, populations sampled within mainland Europe revealed high levels of divergence within *G. pulex*, which was previously thought as an homogenous distribution of a single species (Scheepmaker & van Dalfsen, 1989). Sequence data identified four genetically distinct clades of *G. pulex* in Europe, separated by at least 5% sequence divergence, and were proposed to represent separate 'waves' of colonisation during the climatically violent tertiary and quaternary periods.

Data collected from the known source (River Terrig) and introduced (Fleshwick Bay) populations of one of Hynes' transplantation experiments on the Isle of Man in the 1950's (see review by Dick *et al.*, 1997) presented a complex picture of *G. pulex*'s response to a potential bottleneck event as a consequence of the introduction. Molecular data were inconclusive as to whether there was a significant loss of molecular variation in the introduced population (when compared to the source). The allozyme loci and mtDNA sequencing (COI gene) showed no significant loss, though there was a significant reduction in heterozygosity at the single polymorphic microsatellite locus screened. Data from the morphological analysis of the populations

showed that there was an increase in morphological (phenotypic) variation in the introduced population using all of the trait data pooled, though individually this was not always the case.

Morphological variation within the three populations examined (Lowthorpe, River Terrig and Fleshwick Bay) was similar, when compared across all measured traits. Variation within the samples from the Fleshwick Bay population showed the highest levels of variation, with variation at 12 of the 14 traits greater than the mean across all populations (Table 4.4, Chapter 4). Analysis of variation in the three populations and from samples reared in the laboratory revealed that two sets of traits (pleopods 1 and gnathopod 1) were significantly different between environments (laboratory and wild), and a further two (eye traits and antenna 1) were significantly different between populations. These data indicate that the former traits are likely to be phenotypically plastic, and the latter have a genetic basis that showed little response to different environments. The remaining traits showed significant differences between both populations and environments indicating a potentially more complex genotype by environment interaction response.

7.2 Population structure in Gammarus pulex

Data from populations sampled in the UK showed no genetic structuring across drainages with all of the marker systems used. Unlike *G. fossarum*, *G. pulex* shows no clear correlation between genetic structure and drainage hierarchy, and the associated low levels of gene flow (Müller, 1998). There are several explanations for the lack of differentiation and apparent limited genetic diversity observed: The first is that barriers to migration are not sufficient to prevent the movement of *G. pulex* between populations and hence gene flow; the second is the hypothesis that the diversity of the colonists of

the UK was limited, or that the time since colonisation has been insufficient for a population to reach equilibrium.

The former hypothesis is unlikely when considering the life history of *G. pulex*, its actual (rather than its potential) dispersal ability between drainages and the many natural barriers to migration within lotic systems. Although *G. pulex* can migrate long distance within drainages (Litterick, 1973) there is little evidence to suggest that migration between distant drainages is possible. However, at the end of the last glacial period, when the ice sheets began to melt and most of the major drainages in Europe flooded (Gibbard, 1988), opportunities for cross-drainage migration by the freshwater gammarids would have been enhanced. Since this time, there have been no floods on this scale, leaving considerably less opportunity for *G. pulex* to move between drainages within the UK.

The latter hypothesis gains support from work conducted on a European caddisfly (Wilcock *et al.*, 2001), where limited population structuring in the UK was though to be a consequence of insufficient time for significant population differentiation to occur since colonisation.

7.3 Hynes' transplantation of G. pulex *to Fleshwick Bay*

When comparing the variation in markers, caution is needed as only one marker shows a reduction in variation (microsatellites) and this was based on only a single locus and may not be representative of the genome as a whole. Indeed the remaining markers, while showing weaker polymorphism and hence less powerful, do not show a significant reduction in diversity between the source and introduced populations.

The work of Hynes (1954; 1955) on the ecology of gammarids and his transplantation experiments (Dick *et al.*, 1997) have left present day ecologists with an ideal opportunity to study the effect of an introduction on genetic diversity. An

introduction provides a useful tool to study the effect of contemporary forces shaping population structuring (Leberg, 1992), without the comfounding effects of historical forces. As part of this thesis I investigated the genetic and morphological diversity of one introduced population (Fleshwick Bay) and compared the data to the known source (Hynes *pers comm.*) of the introduction (River Terrig). Assuming that the average number of generations per year is 1.5 (Hynes, 1955; Sutcliffe, 1992) and that the population has been established for 50 years, the total number of generations since introduction would be approximately 75.

The introduction of 75 *G. pulex* from River Terrig would not be classified as a severe bottleneck event, and any reduction in heterozygosity would be expected to be limited. A bottleneck down to two individuals for a single generation would still retain 75% of the pre-bottleneck heterozygosity (Hoelzel, 1999). However, it is the time in which a population spends bottlenecked that is crucial to the recovery of variation in the population (Amos & Harwood, 1998). The introduction in Fleshwick Bay would have created a bottleneck of 75 animals for at least one generation. The lack of animals sampled within the stream during a survey in 1952 (Dick *et al.*, 1997) does suggest that the number of animals remained at low abundances for more than one generation, making it unlikely that a rapid population explosion occurred after introductions. The survey data would suggest that the bottleneck may have persisted longer than previously thought, though not sufficiently so to erode genetic variability.

Collectively, therefore data from all the molecular and morphological marker systems employed here indicate no loss of diversity as a consequence of the introduction on the Isle of Man to the Fleshwick Bay site. Table 7.1 shows the variation in markers systems for the two populations. Molecular variation is overall higher in the samples from River Terrig; thought the variation in morphological characters was lower.

Table 7.1: Marker system variability of River Terrig (sourced) and Fleshwick Bay (introduced) populations.

Marker system and measure of variation	River Terrig	Fleshwick Bay
Allozymes Heterozygosity (observed, Chapter 3)	2.3%	2.1%
Cytochrome oxidase I Sequence haplotypes (Chapter 5)	1 (common type)	1 (common type)
Microsatellite data Heterozygosity (observed, Chapter 6)	89.8%	86.1%
Number of alleles	12	11
Morphology Coefficient of variation (Chapter 4)	14.00%	17.89%

7.4 Comparative Phylogeography

The distribution of *Gammarus pulex pulex* was thought to be almost continuous across the range (Sweden to Northern Africa and Britain to Afghanistan) and consist of a single species, with several other geographically isolated sub-species (Karaman & Pinkster, 1977a). The assumption of a single species range was based on reputably 'stable' morphological traits and has in general been supported by later work (Scheepmaker & Van Dalfsen, 1989; Scheepmaker, 1990), though variation within the species has also been documented (Pinkster, 1983). Following further studies, especially with molecular markers, the distribution of the freshwater gammarids are now questioned, and the hitherto 'homogenous' distribution of *G. pulex* appears to be far more complex than previously described. Within the phylogeographic study

undertaken (Chapter 5) there were two main areas of interest, the colonisation (timing and route) of the UK and the wider scale pattern of *G. pulex* within European drainages.

7.4.1 Colonisation and diversity in the UK

The route and timing of colonisation of the UK by *G. pulex* provided a challenge to new methods developed in the field of molecular ecology (namely the nested clade analysis). From the data collected in this study the limited divergence within the UK clade supports the geological history and existence of a land-bridge between the UK and mainland Europe which submerged ~ 8000 years ago (Wilcock *et al.*, 2001 and references within). The most likely route of colonisation was across the land-bridge from populations on the Elbe, Mosel and Rhine drainages that were nested within the same clade as samples from the UK (Figure 7.1). To date there has been little work carried out on the colonisation of the UK after the last glacial period, though work on the bullhead (Hänfling *et al.*, 2002) and caddisly (Wilcock *et al.*, 2001) have presented data on the likely colonisation routes and locations of putative refugia.

7.4.2 How many species? Diversity in Europe

Within Europe the genetic diversity of *G. pulex* revealed by mtDNA sequencing was greater than that of the whole UK, suggesting that Europe, unlike the UK, was colonised either (a) many times or (b) from several different locations. The data presented here suggests that *G. pulex* colonised from at least two distinct locations (the Balkans and the Pyrenees), and in two separate waves, one during the Pliocene and the other in the early Pleistocene (Chapter 5). The pattern of colonisation of *G. pulex* across Europe is consistent with the 'hedgehog' pattern identified as a common route for colonsation by Hewitt (1996).

This study presents data that in conjunction with other studies on freshwater animals in Europe suggest that there were northern refugia in Belgium during the last

glacial cycle (Stewart & Lister, 2001). The current data also supports many other trends and patterns recorded in other European species: the existence of a hybrid zone in Germany (Müller, 1998; Santucci *et al.*, 1998); colonisation in an east to west direction along the axis of the river Danube along with a more recent expansion on a north south axis (Bernatchez & Wilson, 1998; Volkaert *et al.*, 2002).

Within Europe, two subspecies of *G. fossarum* account for the genetic diversity recorded (Müller, 1998), but this was not thought to be the case in *G. pulex*, where subspecies were thought to be geographically isolated (Karaman & Pinkster, 1977a). Sequence data present here provides evidence for the existence of at least four subspecies, which have a wide geographical range (Figure 5.6, Chapter 5).

The formation of drainage basins during the Miocene epoch (Gibbard, 1988) appears to be one of the most significant factors in determining the present-day distribution of freshwater gammarids in Europe. However such historical processes can be superseded by anthropogenic influences. For example, the alteration of the direction of rivers, through damming, navigation channels and flooding areas for reservoirs. More recent anthropogenic activities can hide the older natural processes which are often more significant in large scale population structuring. This hypothesis can be supported by one known case study: The opening of the Main-Danube canal in 1992, which provided a perfect opportunity for many Ponto-Caspian species to invade 'new' habitats (Müller *et al.*, 2002; Wittmann & Ariani, 2000). One gammarid species in particular, *Dikerogammarus villosus*, was able to quickly colonise rivers in Germany and later rivers in France (Devin *et al.*, 2001) and the Netherlands (Dick & Platvoet, 2000), within the space of ten years. The invasion by this exotic species caused the eradication of native gammarid fauna (*G. pulex*, *G. fossarum* & *G. tigrinus*) leaving a 'break' in the distribution of the native species. This 'break' obscures the underlying historical processes that shaped population structuring in the native gammarids.

7.5 Morphological variation

As many authors have noted there is considerable morphological variability within the gammarid species (Karaman & Pinkster, 1977a; Scheepmaker, 1990), though molecular variation is generally lower. The evolution and response of marker systems within a population will be very different; morphological traits are though to be encoded for by multiple genes and will respond more slowly to changes in population size. Life history traits (not studied here) are expected, and have been shown, to have lower levels of variability than other morphological traits due to fitness constraints. Neutral molecular markers are thought to respond to changes in populations more rapidly and will contain a genetic signature of past population history (Avise, 1994).

The differential response of morphological and molecular markersto past changes can be seen in the process of speciation. In *G. fossarum* speciation, as defined by molecular analysis, has taken place even when there has been morphological stasis (Müller *et al.*, 2000). Other freshwater invertebrates (Wellborn, 2000) have also indicated that even during speciation, morphological differentiation is limited (between cryptic species forms) and is unlikely to be a good indicator for speciation. The data from the two examples above support the data presented here, with molecular differentiation of UK and Belgian samples, which displayed considerable morphological overlap.

7.6 Future work

This study presents molecular data on a ubiquitous freshwater invertebrate, that has until very recently been thought of as homogenous and limited in structure and diversity within its range. Although the UK gammarids appear limited in diversity there is now strong evidence to support the pattern of colonisation of the UK, and an insight into the reasons for the limited diversity recorded. Even though the present study does

shed light on to the colonisation of the UK by *G. pulex* there are similar species that have colonised the UK but as yet have not been examined. Data from bullhead (Haenfling *et al.*, 2002) does show support for a southern English refugium, though without more evidence (currently unavailable), the refugia could be unique to the bullhead. Data from a greater range of freshwater species would resolve any potential congruence's between the colonisation of the UK and their timing.

On a wider theme, the classification of the freshwater gammarids, by morphology, has shown even more limitations for identification of population samples within Europe (chapter 5). Work by Müller (1998) and his colleagues (Müller *et al.*, 2000) and myself have provided a wider insight into the complex processes of speciation and reconstructing historical separation and structuring of populations. Further work still needs to be undertaken on the same geographical scale as covered by Karaman & Pinkster (1977a, 1977b & 1987), but with the use of molecular markers to truly resolve the complex taxonomy and species complexes that appear to exist in most if not all of the freshwater gammarids. The nature and complexity of population structuring has in general become even more difficult to predict as a consequence of many invasions and the artificial introductions of exotic species. The effect of introduction has begun to be understood as a result of the work by Dick *et al.* (1993; 1997) though much is still unclear, especially with respect to the maintenance of genetic diversity within a species.

The introduction experiment in Fleshwick Bay would benefit from a more detailed analysis to answer the question "is there really a loss in genetic diversity in the Fleshwick Bay population due to the introduction carried out by Hynes?" One way to answer this question would be to isolate more microsatellite loci or additional polymorphic markers. A more detailed molecular analysis of the introduction

experiment would provide an insight into the contemporary forces that shape population structuring in a 'new' habitat.

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APPENDIX

Appendix 3.1

Buffer systems used for screening resolvable allozymes in *Gammarus pulex*. For routine screening only CM 6.2, TBE 8.3, TC 8.0 and TG 8.5 were used.

Buffer	Electrode (g/l)	Gel (g/l)	Current & duration of run
Citrate morpholine (pH 6.2) CM 6.2	8.4g citric acid adjust to pH 6.2 with N-(3-aminopropyl)-morpholine	Dilute 1:10	25mA 15h
Tris-borate EDTA (pH 8.5) TBE 8.5	1.08g boric acid 21.12g Tris 0.74g EDTA	Dilute 1:2	25mA 15h
Tris-borate EDTA (pH 8.3) TBE 8.3	60.6g Tris 40.0g boric acid 6.0g EDTA	Dilute 1:9	25mA 15h
Tris citrate (pH 8.0) TC 8.0	30.3g Tris 11.98g citric acid	Dilute 1:25	35mA 6h
Tris-maleic (pH 7.4) TM 7.4	12.1g Tris 11.6g maleic acid 3.7g EDTA 4.05g $MgCl_2$	Dilute 1:10	25mA 15h
Mod. Ridgeway's (pH 6.8) TCB 6.8	18.6g boric acid 4.2g lithium hydroxide	9.2g Tris 1.05g citric acid 53 ml electrode buffer	25mA 15h
Tris-glycine (pH 8.5) TG 8.5	30.0g Tris 144.0g Glycine Dilute 1:9	Cellulose acetate gel soaked in electrode buffer	90mA 14 mins

Appendix 3.2

Stain recipes used for the routine screening of *Gammarus pulex* samples. Recipes are modified from Harris & Hopkinson (1976), Ferguson (1985), Murphy *et al.* (1996) and Piertney (1994). With the exception of AAT, EST and PGM, all stains also contained 1ml 10mg/ml MTT (tetrazolium salt), 1ml 10mg/ml PMS (phenazine methosulfate) and 20ml of 2% agar solution.

Enzyme	EC no.	Running buffer	Ingredients	Stain buffer	Linking enzymes
AAT	2.6.1.1	TBE 8.3	75mg Ketoglutaric acid 250mg L aspartic acid 10 mg Pyridoxal-5-phosphate Adjust to pH 8 (3 drops 10M NaOH) 100mg Fast Blue BB in 5ml water 20ml Agar	15 ml 0.1M Tris-HCL pH 8	
ADA	3.5.4.4	TC 8.0	40mg Adenosine 80mg Arsenic acid	15 ml 0.1M Tris-HCL pH 8	0.4u XO 1.8u NP
ARK	2.7.3.3	TBE 8.3	15mg NADP 10mg ADP 100mg Glucose 10mg Phospho-L-Arginine 1ml 1M $MgCl_2$	15 ml 0.1M Tris-HCL pH 8	20u G6PDH 10u Hex
EST	3.1.1.1	TBE 8.3	1mg 4-Methyl-umbelliferyl acetate *dissolve in 5ml acetone*	50ml 0.1M Na_2PO_4 pH 6.5	
GAPDH	1.2.1.12	CM 6.2	30mg NAD 50mg Arsenic acid 30mg Fructose 1-6 Diphosphate	15 ml 0.1M Tris-HCL pH 8	20u Aldolase
GPI	5.3.1.9	TBE 8.3	40mg Fructose-6-phosphate 8mg NADP 1ml 1M $MgCl_2$	15 ml 0.1M Tris-HCL pH 8	20u G6PDH
IDH	1.1.1.14	CM 6.2	150mg Isocitric acid 8mg NADP 1ml 1M $MgCl_2$	15 ml 0.1M Tris-HCL pH 8	
MDH	1.1.1.37	TC 8.0	250mg Malic acid 20mg NAD	15 ml 0.1M Tris-HCL pH 8	
PEP	3.4.11.-	TBE 8.3	60mg Leucyl-Glycyl-Glycine 10mg O-dianisine 5mg L-amino acid oxidase 10mg Peroxidase 1ml 1M $MgCl_2$	15 ml 0.1M Tris-HCL pH 8	
PGDH	1.1.1.44	TBE 8.3	40mg Phosphogluconic acid 20mg NADP 1ml 1M $MgCl_2$	15 ml 0.1M Tris-HCL pH 8	
PGM	5.4.2.2	TG 8.5	1.5ml NAD (2mg/ml) 8 drops $MgCl_2$ (200mg/ml) 8 drops Glucose-1-phosphate (10mg/ml) 2 drops MTT (10mg/ml) 2 drops PMS (10mg/ml) 2ml Agar (2%)	1ml 1M Tris-HCL pH 8	25µl G6PDH (200u/ml)

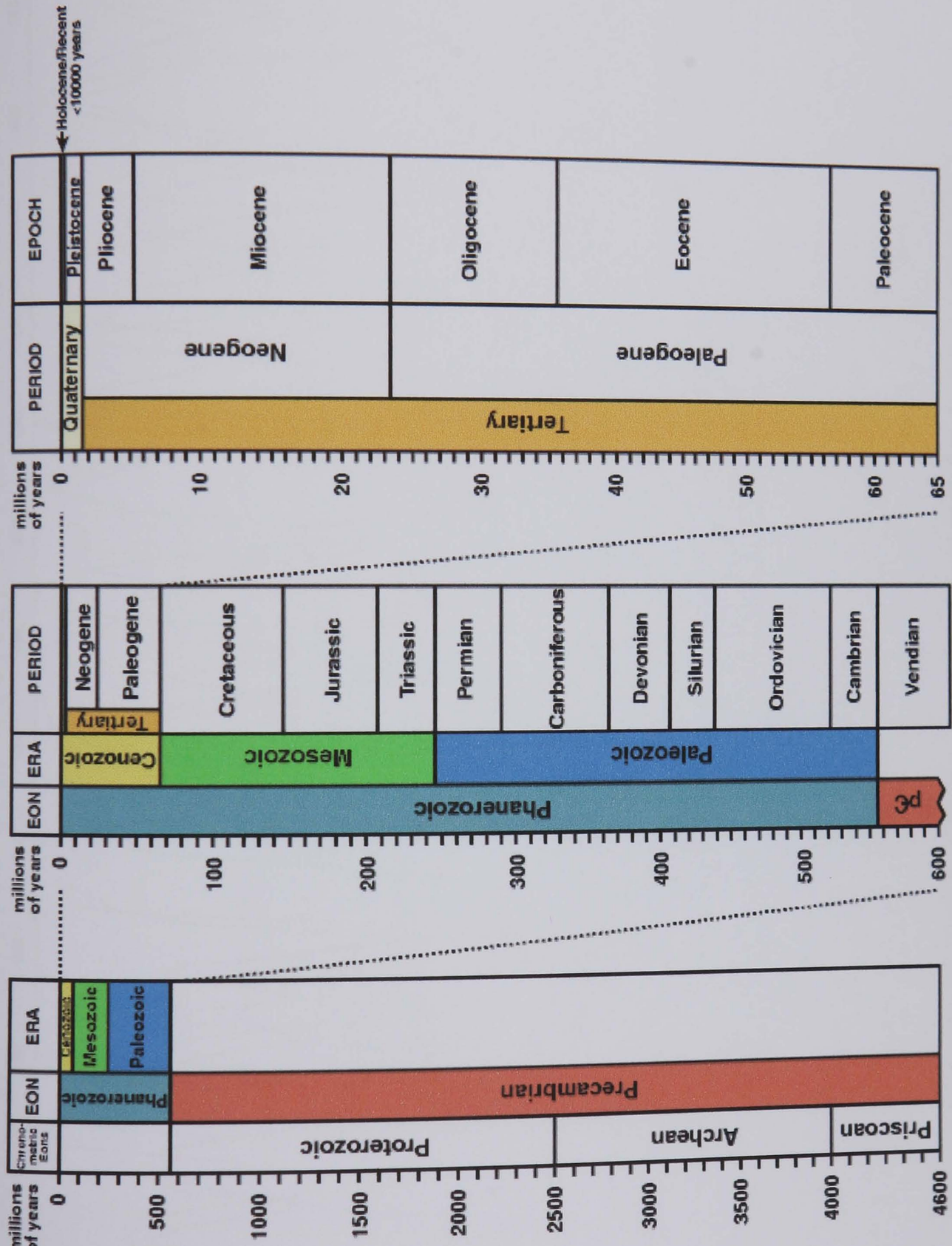
Appendix 3.3

Enzyme systems used initially for screening. Legend: A: activity; R: resolution; 1 excellent, 2 good, 3 moderate, 4 poor, 5 unscorable

Enzyme	Abbrev	EC number	A	R
Aspartate aminotransferase	AAT	2.6.1.1	1	2
Adenosine deaminase	ADA	3.5.4.4	2	2
Alcohol dehydrogenase	ADH	1.1.1.1	5	5
Adenylate kinase	AK	2.7.4.3	4	4
Arginine kinase	ARK	2.7.3.3	2	2
Carbonate hydratase	CAR	4.2.1.1	5	5
Creatine kinase	CK	2.7.3.2	5	5
Esterase-D	EST-D	3.1.1.1	1	1
Formaldehyde dehydrogenase	FDH	1.2.1.1	5	5
Fructose 1,6 diphosphataes	FDP	3.1.3.11	4	4
Fumurate hydratase	FH	4.2.1.2	5	5
Glyceraldehyde-3 phosphate dehydrogenase	GAPDH	1.2.1.12	2	2
Guanine deaminase	GDA	3.5.4.3	5	5
Glucose dehydrogenase	GDH	1.1.1.47	5	5
Glutamate dehydrogenase	GTDH	1.4.1.2	3	3
Glycerol-3-phosphate dehydrogenase	G3PDH	1.1.1.8	3	4
Hexokinase	HK	2.7.1.1	2	4
Isocitric dehydrogenase	IDH	1.1.1.42	2	2
Iditol dehydrogenase	IDDH	1.1.1.14	4	4
Lactate dehydrogenase	LDH	1.1.1.27	3	3
Malate dehydrogenase	MDH	1.1.1.37	1	1
Malic enzyme	MEP	1.1.1.40	3	3
Mannose phosphate isomerase	MPI	5.3.1.8	5	5
Peptidase	PEP	3.4.11.~	1	2
Phosphogluconate dehydrogenase	PGDH	1.1.1.44	1	1
Phosphoglucose isomerase	PGI	5.3.1.9	1	1
Phosphoglycerate kinase	PGK	2.7.2.3	5	5
Phosphogucose mutase	PGM	5.4.2.2	2	2
Pyruvate kinase	PK	2.7.1.4	3	4
Superoxidase dismutase	SOD	1.15.1.1	3	4
Xanthine dehydrogenase	XDH	1.2.1.37	5	5

Appendix 4.1: Mean morphometric data of the seven groups of animals analysed, with standard error in parenthesis. Sample size and environment (Laboratory reared or wild) in first row. Description of morphological traits corresponding to abbreviations in Table 4.2 (Chapter 4)

	Lowthorpe Wild *N=50*	Lowthorpe Laboratory *N=50*	River Terrig Wild *N=50*	River Terrig Laboratory *N=50*	Fleshwick Bay Wild *N=50*	Fleshwick Bay Laboratory *N=50*	Schendelbeke Wild *N=13*
TL	14.68 (2.29)	10.93 (2.15)	13.86 (1.88)	11.75 (1.70)	12.68 (2.57)	11.57 (2.88)	14.59 (2.39)
EYEL	0.42 (0.04)	0.37 (0.07)	0.44 (0.04)	0.41 (0.04)	0.38 (0.07)	0.40 (0.08)	0.43 (0.06)
EYEW	0.23 (0.03)	0.22 (0.03)	0.23 (0.03)	0.22 (0.02)	0.24 (0.04)	0.22 (0.05)	0.26 (0.04)
EYEO	52.60 (5.15)	45.48 (9.74)	56.28 (7.68)	56.58 (9.04)	54.94 (9.58)	49.22 (11.30)	55.46 (11.30)
A2H	1.66 (0.99)	2.06 (0.74)	2.82 (0.63)	2.80 (0.40)	2.78 (0.42)	2.24 (0.53)	2.69 (0.85)
A1S	31.36 (3.96)	27.12 (2.93)	28.80 (3.89)	28.76 (4.02)	29.40 (6.26)	28.10 (6.80)	27.38 (4.05)
GL	1.24 (0.21)	1.04 (0.19)	1.03 (0.20)	0.83 (0.17)	1.01 (0.20)	0.92 (0.22)	1.14 (0.14)
GW	0.68 (0.11)	0.58 (0.09)	0.60 (0.11)	0.49 (0.11)	0.57 (0.11)	0.52 (0.13)	0.65 (0.08)
P5L	1.16 (0.17)	0.80 (0.11)	0.98 (0.13)	0.84 (0.14)	0.85 (0.16)	0.80 (0.19)	1.04 (0.17)
P5W	0.44 (0.06)	0.34 (0.06)	0.40 (0.05)	0.35 (0.06)	0.35 (0.06)	0.34 (0.07)	0.43 (0.06)
P5BS	12.90 (1.60)	11.36 (1.24)	12.72 (1.31)	12.96 (1.95)	12.44 (1.55)	12.32 (2.22)	14.00 (1.73)
P7BS	14.18 (1.51)	12.44 (1.97)	14.72 (1.80)	13.98 (2.09)	14.68 (2.02)	14.02 (2.66)	14.69 (1.75)
P0	2.07 (0.28)	1.46 (0.22)	1.78 (0.23)	1.73 (0.22)	1.67 (0.34)	1.55 (0.38)	1.93 (0.34)
P1	2.54 (0.32)	1.85 (0.27)	2.27 (0.28)	2.16 (0.29)	2.09 (0.37)	1.99 (0.43)	2.40 (0.37)
Area	0.31 (0.06)	0.25 (0.07)	0.33 (0.06)	0.30 (0.06)	0.29 (0.10)	0.28 (0.10)	0.36 (0.09)
Density	176.87 (42.53)	188.60 (41.77)	176.54 (32.78)	194.86 (28.74)	206.27 (61.94)	182.95 (65.49)	158.78 (29.90)


millions of years
Chrono-metric Eons
EON
ERA
PERIOD
EPOCH
Holocene/Recent <10000 years
Pleistocene
Pliocene
Miocene
Oligocene
Eocene
Paleocene
Quaternary
Neogene
Paleogene
Tertiary
Cretaceous
Jurassic
Triassic
Permian
Carboniferous
Devonian
Silurian
Ordovician
Cambrian
Vendian
Cenozoic
Mesozoic
Paleozoic
Phanerozoic
Proterozoic
Archean
Priscoan
Precambrian
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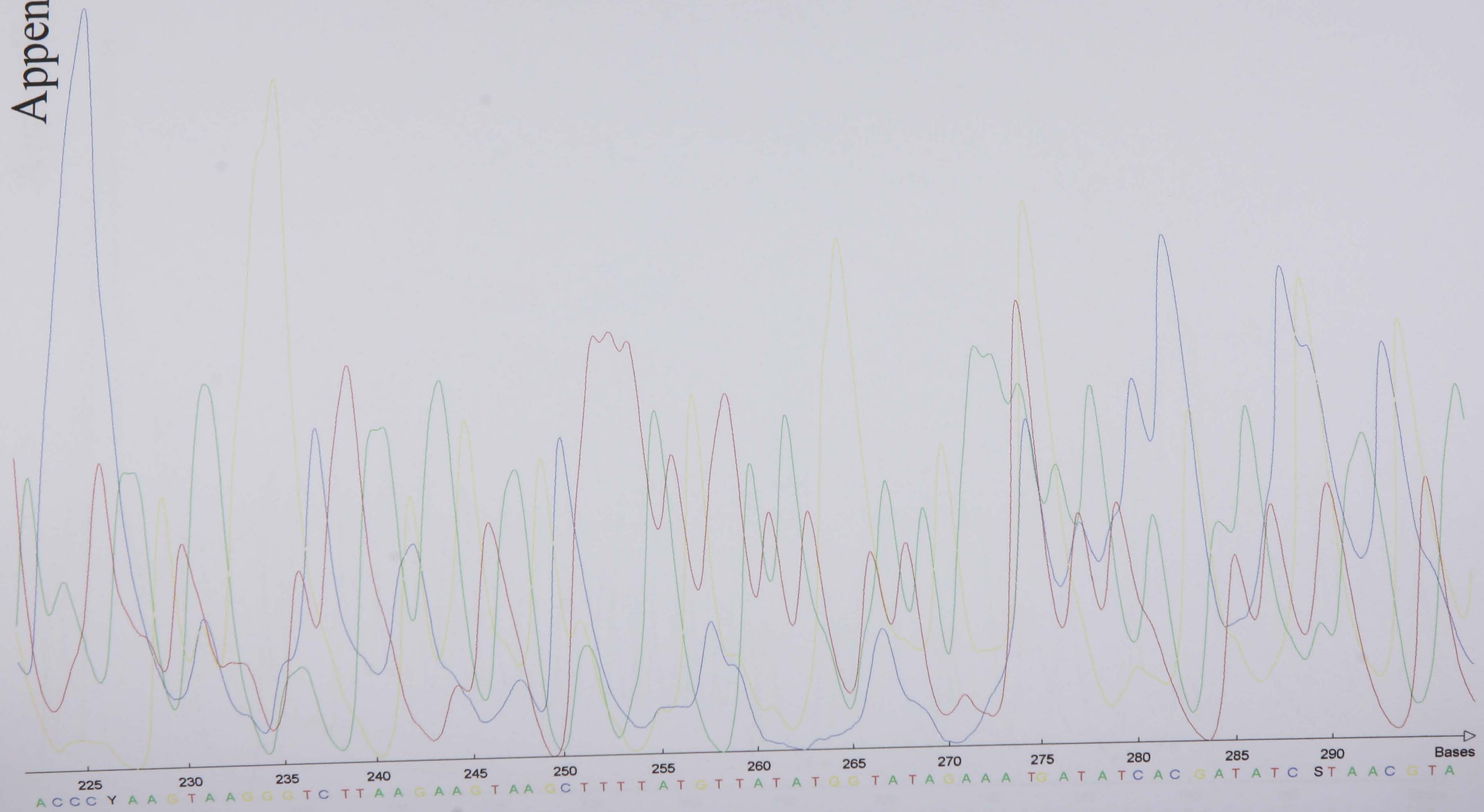
4600

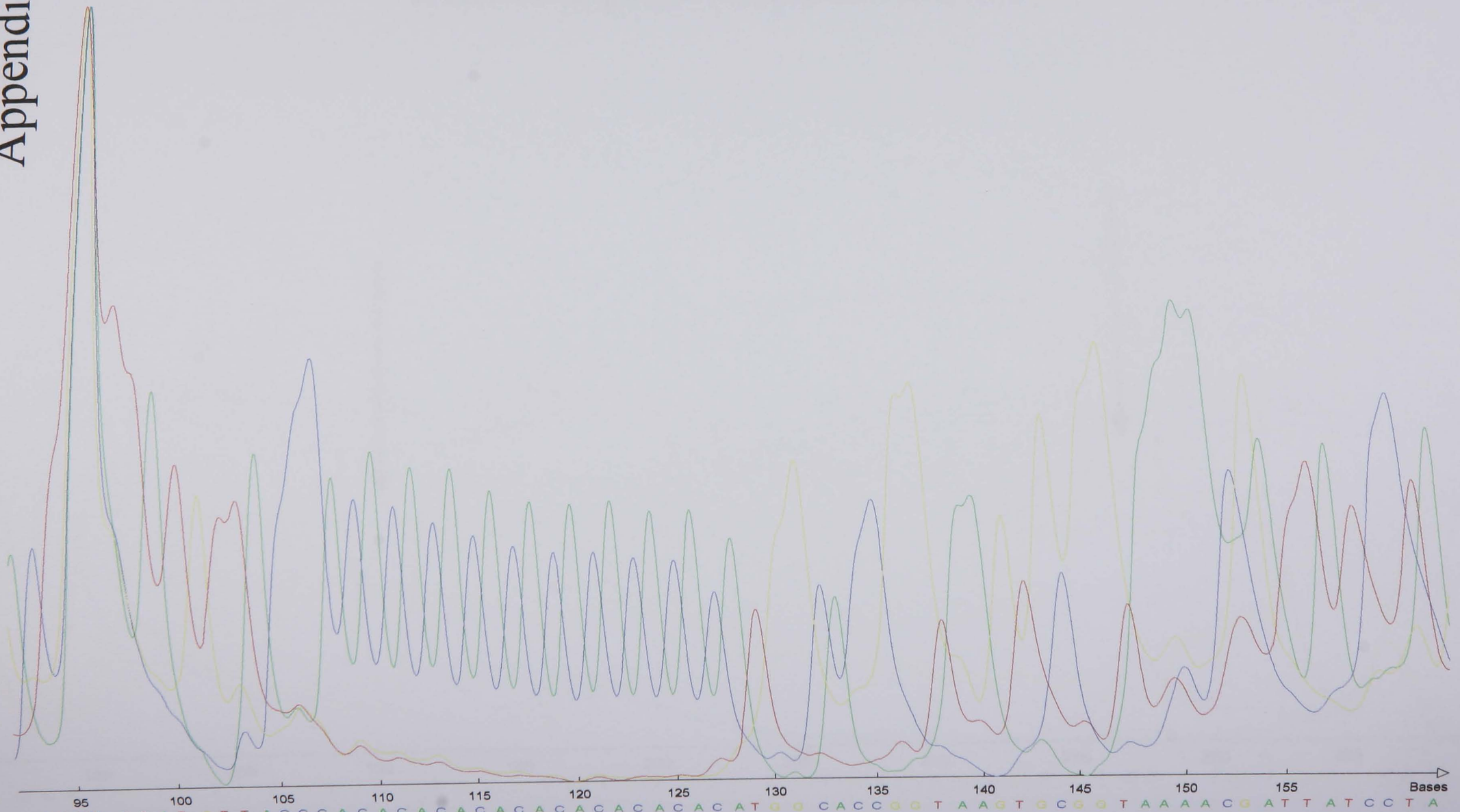
Appendix 6.1

Weak AT repeat motif in false positive colony



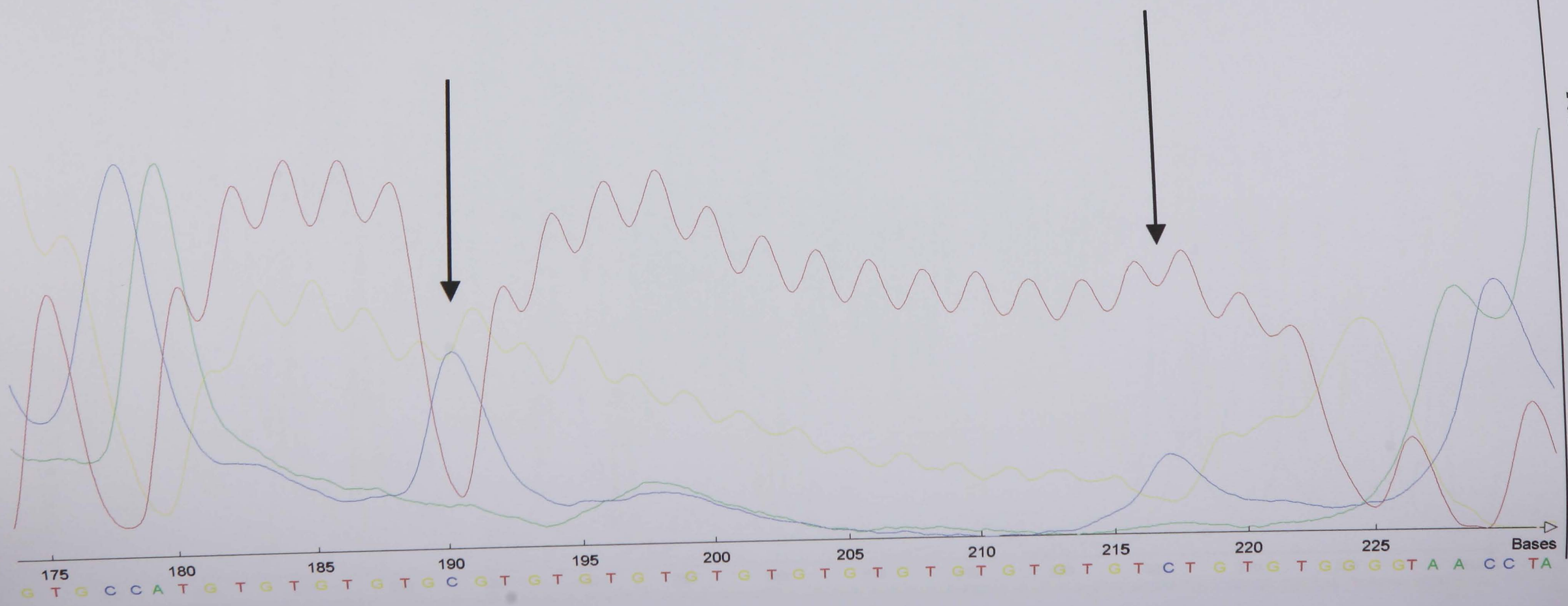
Appendix 6.2

Perfect copy of GT1 microsatellite locus



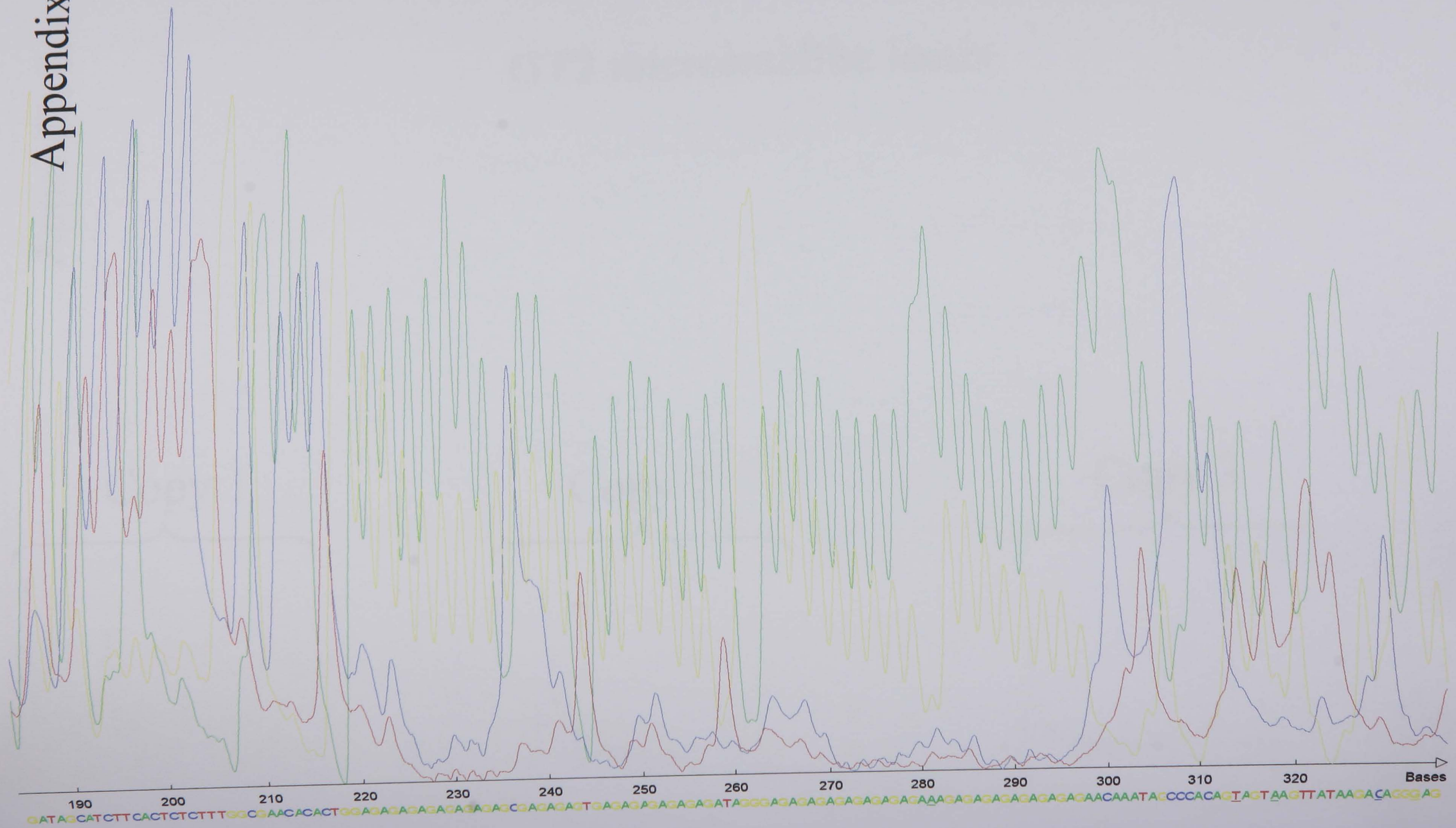
Appendix 6.3

Interrupted copy of GT1 microsatellite locus, with two point mutations (→)



Appendix 6.4

GA1 microsatellite locus



Appendix 6.5

GT2 microsatellite locus

Appendix 6.6: Microsatellite structure from 22 of the sequenced colonies

Colony	Microsatellite repeat structure
1	TC CA CG $(CA)_6$ CG $(CA)_9$ TA $(CA)_6$ TC CA TA $(CA)_{10}$
2	$(CA)_{34}$ TA $(CA)_7$ CG $(CA)_6$
4	$(CA)_9$ TT $(CA)_9$ CT CA CT CG $(CA)_2$ TA $(CA)_2$ CT $(CA)_5$ TA CA
7	$(CA)_{17}$ TT $(CA)_7$ AT CC $(CA)_9$
8	CC $(CA)_3$ GA $(CA)_{12}$ CG $(CA)_5$
10	CC $(CA)_{17}$ CT $(CA)_{11}$
29	TC $(CA)_{33}$ CT $(CA)_7$ TA CA
38	TC $(CA)_{31}$ TA $(CA)_9$ TA TA
41	CC $(CA)_9$ TG $(CA)_2$ CC $(CA)_3$ TC CC TA $(CA)_{12}$
59	TC CA CG $(CA)_{12}$ CC CA TT CA CC $(CA)_5$ TA CA
87	$(CA)_{16}$
92	CC $(CA)_{11}$
207	TC CA CG $(CA)_6$ TT $(CA)_{14}$ CG $(CA)_3$ TC CA TA $(CA)_2$ CT $(CA)_5$ TA CA
218	CC $(CA)_{25}$ TC $(CA)_{15}$
224	TC $(CA)_{24}$
291	$(CA)_{25}$ TA $(CA)_9$
298	TC CA CG $(CA)_{24}$ CC $(CA)_7$ TA CA TA $(CA)_6$ TA
379	TC $(CA)_{24}$
381	$(CA)_{14}$ TA $(CA)_6$ CG TC $(CA)_{13}$
391	$(CA)_9$ CC $(CA)_2$ CC $(CA)_{15}$
395	TC CA CG $(CA)_7$ TT $(CA)_{14}$ CG $(CA)_3$ TC CA TA $(CA)_2$ CT $(CA)_5$ TA CA
411	TC CA CG $(CA)_{21}$ TC CA TA $(CA)_8$ TA CA

Appendix 6.7: Isolation protocols and recipes

6.7(i): Restriction digest reaction mix (10µl) (37°C overnight)

10X reaction buffer	1µl	
Extracted DNA	3µl	
Sau3A enzyme	0.5µl	(Boehringer)
ddH_2O	5.5µl	
	10µl	

6.7(ii): pUC18 ligation reaction mix (10µl) (13°C overnight)

pUC 18 plasmid vector	2µl (50ng/µl)	
Size selected DNA	4µl (12ng/µl)	
T4 ligase buffer (10X)	1µl	
T4 ligase enzyme	1µl	(Amersham)
ddH_2O	2µl	
	10µl	

6.7(iii): Linker ligation reaction mix (35µl) (13°C overnight)

Size selected DNA (36ng/µl)	5.6µl	
SAULA SAULB linkers	20.0µl	
T4 ligase buffer (10X)	3.5µl	
T4 ligase enzyme	1.0µl	(Amersham)
ddH_2O	4.9µl	
	35µl	

The best ratio of linkers to genomic DNA was a 250 fold excess of linkers.