GENETIC VARIABILITY AND PHYLOGEOGRAPHY OF THE INVASIVE ZEBRA MUSSEL, *DREISSENA POLYMORPHA* (PALLAS)

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of the requirements for the degree of Doctor of Philosophy

Galway-Mayo Institute of Technology

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Submitted to the Higher Education and Training Awards Council
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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Julien Astorci Candidate

ID No.: ____________

Date: 9th Sept 2005

In memory of my grand father…

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CHAPTER I

INTRODUCTION

I.1. SYSTEMATIC POSITION

Kingdom:	**Animalia**
Phylum:	**Mollusca**
Class:	**Bivalvia (Pelecypoda, Lamellibranchia, Linnaeus, 1758)**
Subclass:	**Heterodonta (Neumayr, 1884)**
Order:	**Veneroida (Adams, 1856)**
Suborder:	**Dreissenacea (Gray, 1840)**
Superfamily:	**Dreissenoidea (Gray, 1840)**
Family:	**Dreissenidae (Gray, 1840)**
Genus:	***Dreissena* (van Beneden, 1835)**
Subgenus:	***Dreissena* (van Beneden, 1835)**
Species:	***Dreissena (D.) polymorpha* (Pallas, 1771)**

Representatives of the small Dreissenidae family have never been diverse and abundant despite their relatively long evolutionary history (ca 50 million years, Andrusov 1897; Starobogatov 1994). About 10-11 million years ago numerous genera, subgenera, species, and subspecies evolved in large, brackish-water lakes of central and eastern Europe (Paratethys), colonizing all accessible aquatic habitats and leaving large depositions of shells. The majority of these forms disappeared as a result of periodical regressions and transgressions of Paratethys's Seas-Lakes (Starobogatov 1994; Starobogatov & Andreeva 1994). At present, the Dreissenidae family is represented by only two genera – *Congeria* and *Dreissena* (classification of Starobogatov, 1994). *Dreissena* species and subspecies can be subdivided into two groups - brackishwater (mezohaline) and freshwater. The zebra mussel (*Dreissena polymorpha*) belongs to the freshwater, euryhaline group. The first

records of *D. polymorpha* date from 10-11 million years ago in estuaries of the central Paratethys (Starobogatov 1994).

The famous Russian scientist and explorer Pyotr Simon Pallas originally described the species from an Ural River population in the Caspian Sea Basin (Pallas 1771). The common name of the species derives from the zebra-like stripes on the shell, while the name "*polymorpha*" refers to the stripe pattern on the shells, which can be very variable (Fig. I.1).

Fig. I.1: Zebra mussels showing a variety of shell markings.

I.2. DISTRIBUTION

Prior to the 19th century, zebra mussels were endemic to the Black, Caspian, and Azov seas (Stanczykowska 1977). Their expansion into other areas was as a result of unintentional introductions. Between 1800 and 1900, the zebra mussel more than doubled its range in Europe (Schloesser 1995), spreading to almost all major drainages. The spread

was facilitated by the development of extensive canal networks and by increased shipping trade following the 19th century industrial revolution (McCarthy & Fitzgerald 1997; van der Velde 2001; Minchin & Moriarty 2002; Minchin *et al.* 2003; Stokes *et al.* 2004).

Zebra mussels first appeared in Great Britain in 1824, following imports of damp timber from the Baltic region (Minchin *et al.* 2002). The timber was exported to several other European ports, and the Netherlands was invaded soon after (1826). Since then, zebra mussels have expanded their range into Sweden, Finland, Switzerland, Northern Italy, France and Ireland (Fig. I.2).

Zebra mussels were first discovered in North America in 1988 in the Great Lakes. The first account of an established population came from Canadian waters of Lake St. Clair, a water body connecting Lake Huron and Lake Erie (Hebert *et al.* 1989). In a few years zebra mussels spread from the Great Lakes basin into the Illinois and Hudson rivers. The Illinois River was the key to their introduction into the Mississippi River drainage, which covers over 1.2 million square miles. In the space of seven years, zebra mussels had spread to eighteen American states and two Canadian provinces (Johnson & Padilla 1996). By 2000 the zebra mussel had colonized Quebec and Ontario provinces in Canada, and 21 states in the USA. They had also invaded isolated water bodies due to the overland transportation of boats (Fig. I.3).

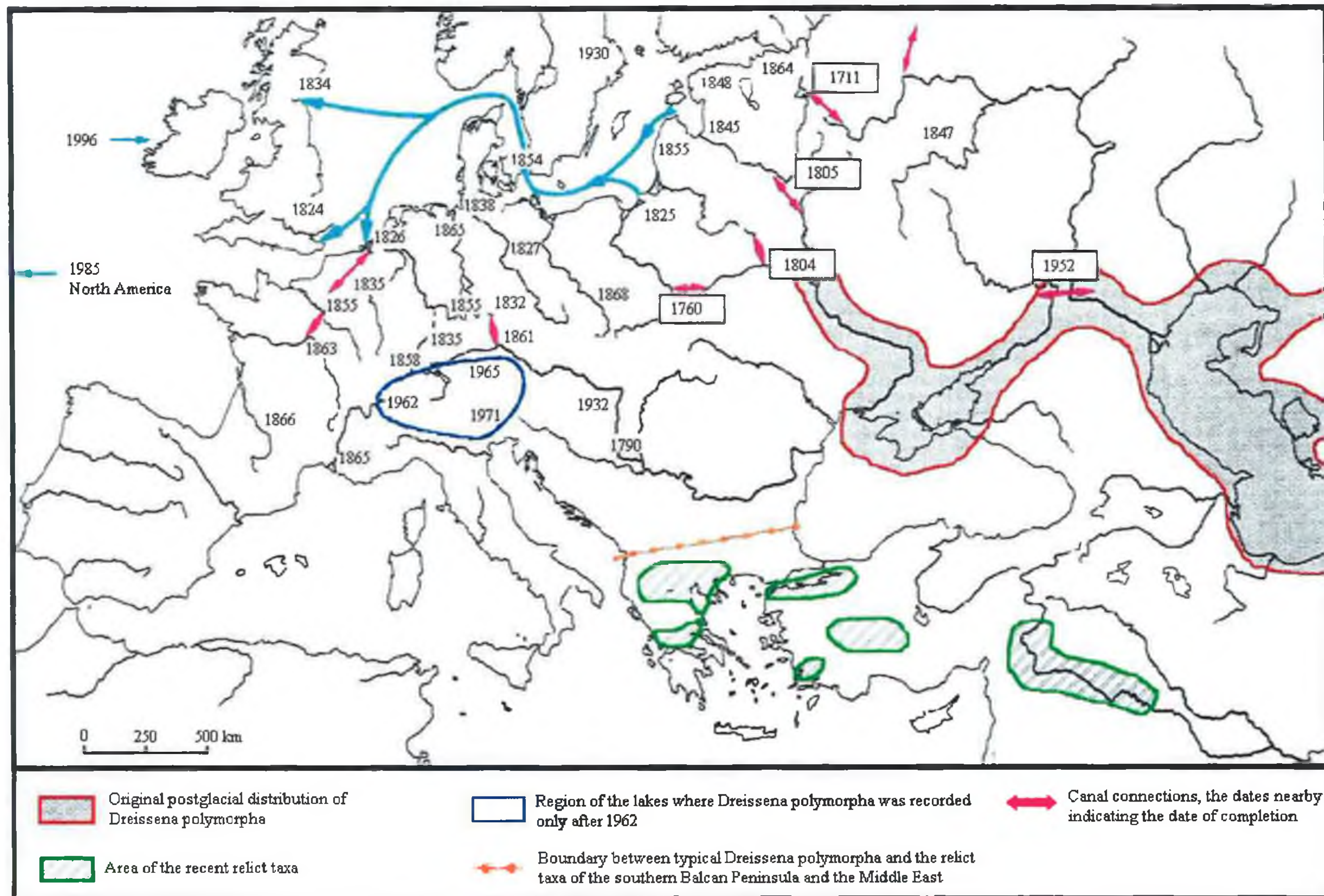

Fig. I.2: The spread of zebra mussels in Europe (http://www.nuigalway.ie/freshwater/zebra/Europe%20c.jpg)

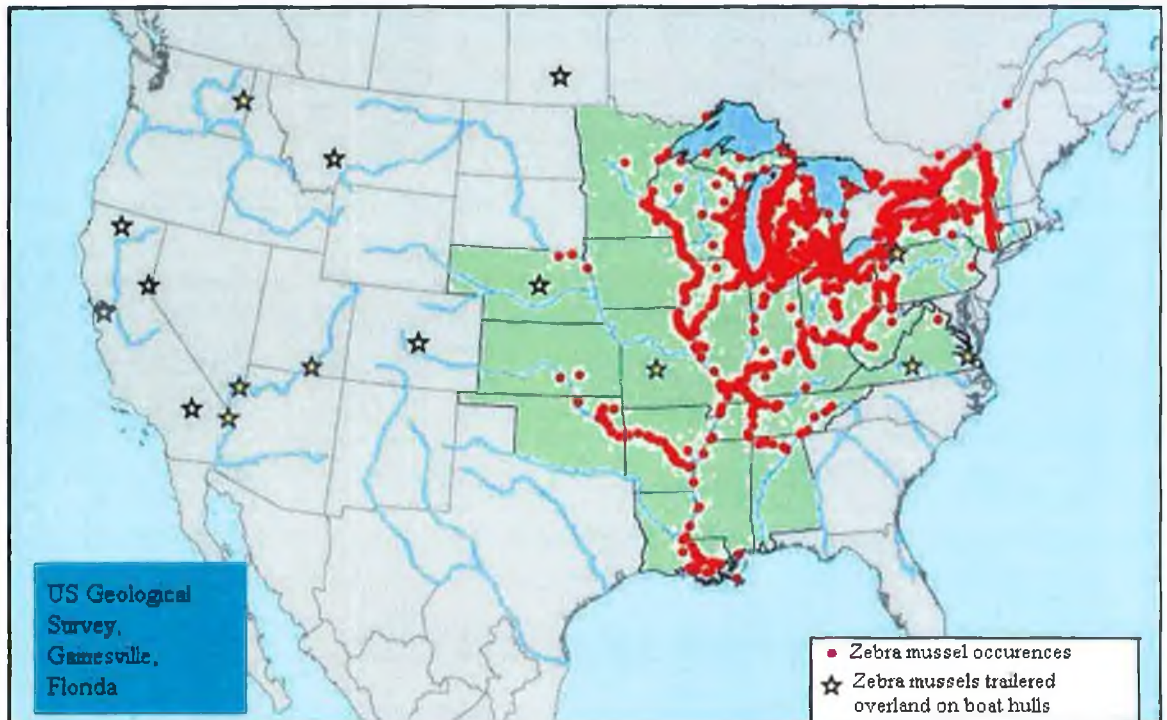

Fig. I.3: Zebra mussel distribution in North America (April 2005) (http://nas.er.usgs.gov/taxgroup/mollusks/zebramussel/maps/zmvr2004.gif)

The mussel was first reported in Ireland in the major river system, the lower Shannon (Fig. I.4), in 1997 (McCarthy & Fitzgerald 1997), but was probably introduced in 1994 or even earlier (Minchin & Moriarty 1998). By 1999 the species was well established throughout Lough Derg on the lower Shannon, and a rapid expansion up the Shannon waterway took place subsequently, reaching densities, in some places as high as 148 000 m^{-2} (Lucy & Sullivan 2001). From the Shannon the species spread into connecting canals, and is now in a second river system, the Erne, probably introduced via boats moving through the recently restored Shannon-Erne canal (Rosell *et al.* 1999). Surveys (Lucy & Sullivan 2001; Minchin *et al.* 2002) show general increases in abundance in the Shannon and associated lakes as well as new

colonizations e.g., at Dublin on the east coast of Ireland, and in the Grand Canal linking Dublin with the Shannon river system.

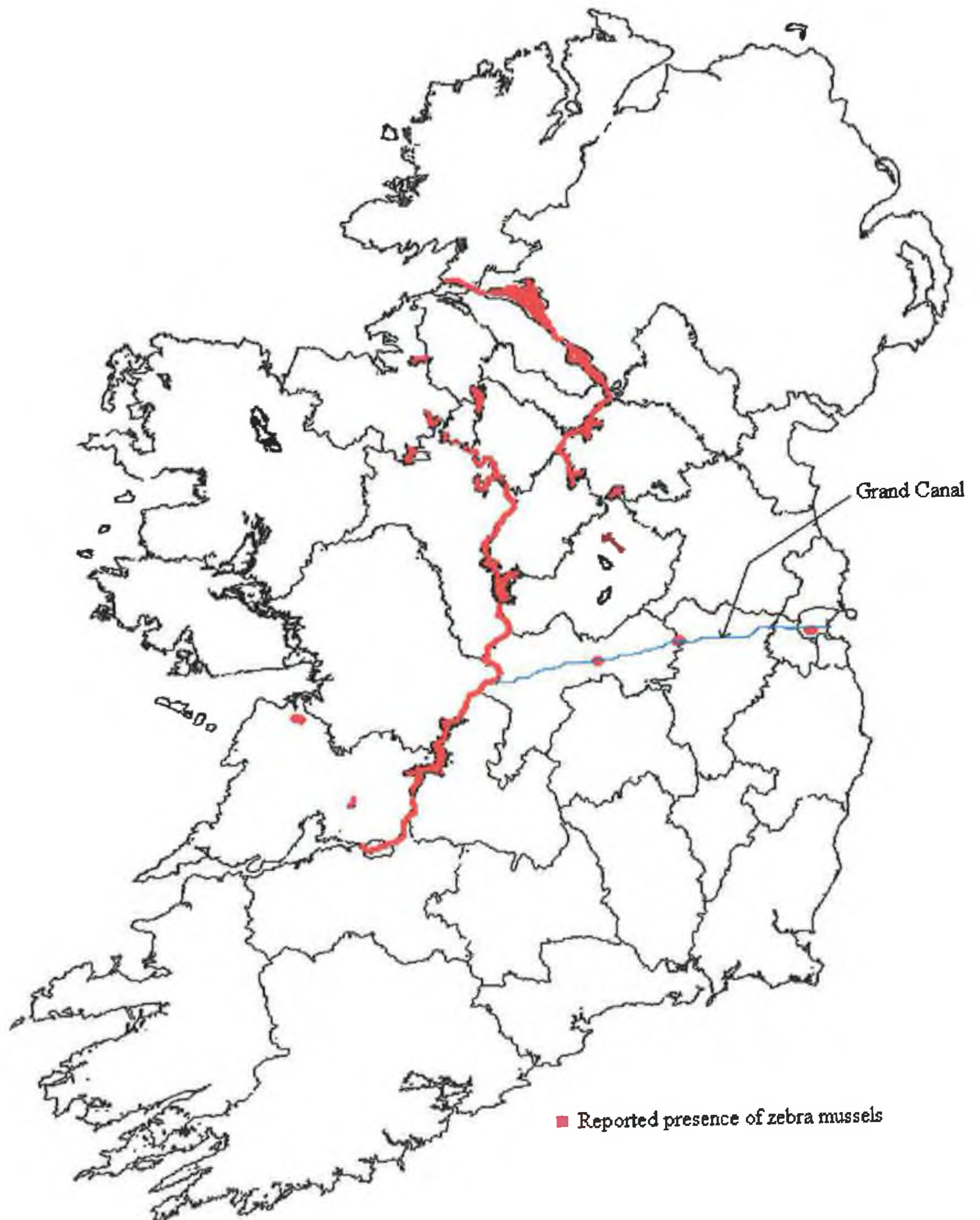

Fig. I.4: Zebra mussel distribution in Ireland (Sullivan 2004)

Dispersal of zebra mussel is mediated by natural (e.g. streams, birds and other animals) and human-mediated (e.g. navigation, fisheries) mechanisms. Several methods of introduction have been suggested (Minchin & Moriarty 1998): the release of ballast water containing mussel larvae, detachment of adults from boats, or transport of aquatic plants or other invertebrates. A recent paper (Pollux *et al.* 2003) has suggested that zebra mussels in Irish waters have a British origin, and that the mussels arrived attached to boat hulls. The pattern of spread of zebra mussels within Ireland has indeed indicated that recreational boating is an important or even the main dispersal vector. This is chiefly because zebra mussel spawning in Ireland occurs between June and September when a large number of vessels come together for festivals (Minchin *et al.* 2003). The presence of zebra mussels in the Shannon and Lough Erne systems therefore increases the possibility of colonization of angling lakes, many of which are still free of zebra mussels (Minchin *et al.* 2002).

The rapid spread of the zebra mussel is aided by several notable life history characteristics. First, the species has a high fecundity (up to one million eggs per female/season) which means that only a few mature individuals are required to supply sufficient recruits and thus, once established, even remote populations have the potential to be not just self-sustaining but rapidly increasing. Second, zebra mussels have a planktonic veliger stage, so that free-living larvae remain suspended in the water column for up to several weeks (Carlton 1993), and may travel downstream more than 300 km before settling and founding new populations (Stoeckel *et al.* 1997). Third, the mussel can attach to a wide variety of hard substrates by byssal threads and densities of 150 000 m^{-2} with biomasses exceeding 10 kg m^{-2} are not uncommon (Stewart & Haynes 1994). The species also exhibits great variability in its morphology and physiology and has the potential to adapt to a wide variety of ecological conditions

and to differentiate through founder effects into ecologically distinct populations (Hebert *et al.* 1989).

I.3. BIOLOGY

Adult zebra mussels are epifaunal (like most marine bivalves) and attach to solid substrates by byssal threads with adhesive pads. The byssal gland, situated in the foot on the ventral side of the mussel, secretes byssal threads, which may number in hundreds. The shell is of heteromyarian type, in which the anterior end is greatly reduced while the posterior end is inflated. The biology, ecology and physiology of the zebra mussel have been very well documented: Morton (1969 *a*, b), Mackie (1991), Mackie & Schloesser (1996), Morton (1993), Claudi & Mackie (1994), Ekroat *et al.* (1993), Ackerman *et al.* (1994), Marsden *et al.* (1996), Mc Mahon (1996), Mills *et al.* (1996), Nichols (1996).

The success of the zebra mussel as a colonist is largely determined by its ability to tolerate and adapt to a wide range of environmental conditions. While tolerating salinity of up to 18.4 ‰ (Lyakhnovich *et al.* 1994), the peak of larval abundance is observed in areas with a salinity of 0.3-0.7 ‰ (Wictor 1969). Temperature tolerance limits vary between 0 and 32°C (Luferov 1965; Shkorbatov *et al.* 1994). Shkorbatov *et al.* (1994) have shown that the oxygenation critical threshold for *Dreissena polymorpha* is 25%, although zebra mussels can survive for several days in anoxic conditions, depending on temperature (Karatayev *et al.* 1998).

Zebra mussels inhabit depths from 0.1 m (Kachanova 1963) to 50-60 m (Dario 1978), depending on the food availability and the presence of accessible substrata. Although the most suitable substrates in most lakes are hard surfaces (rocks, concrete, stones), high densities of zebra mussels are also found on submerged macrophytes and other invertebrates (Karatayev *et al.* 1998, Minchin *et al.* 2002). High abundances

of *Dreissena polymorpha* have been found on artificial substrata, densities reaching 500 000 individuals/m^2 on water pipes of power plants. The poorest substrate for settlement is reported to be silt (Karatayev *et al.* 1998).

I.4. FEEDING

Zebra mussels, like most bivalves, are suspension feeders that can pump considerable volumes of water through their gills and filter out small particulate food items from the water column. Filtration normally occurs at a temperature of 5-30 °C (Kondratiev 1962), and at a pH of 8-9 (Morton 1971). They feed on suspended food particles (bacteria, unicellular algae and fine particulate organic detritus), which they take in via an inhalant siphon and subsequently filter on specialised cilia from water currents maintained over their gills. They are extremely efficient filter feeders, removing particles from 1 μm to > 50 μm diameter (Horgan & Mills 1997). Selection of filtered particles occurs on the epithelium of the gills and labial palps. Besides rejecting non-nutritious, inorganic particles, mussels usually reject mats of diatoms, big-sized colonies of green and blue-green alga and emulsions of organic liquids (Mikheev 1994). All rejected materials are collected in the mantle cavity and then expelled via the inhalant siphon.

By removing large amounts of suspended matter, populations of zebra mussels have the ability to alter transparency and plankton abundance, thereby performing an important role in the transformation and circulation of material and energy in lake ecosystems (Karatayev & Burlakova 1993; Karatayev *et al.* 1994).

I.5. REPRODUCTION

In *Dreissena* the sexes are separate and mussels usually spawn in the summer season, starting when they are about one year old (~ 6 mm shell length). Hermaphrodites have, however, been found in North American populations (Nichols 1996). Adults release gametes over a period of six to eight weeks and fertilization occurs externally. The life cycle (Fig. I.5) consists of a planktonic free-living larval stage and a relatively sessile adult phase. Such a life cycle, involving high reproductive output and the presence of an extended planktonic stage, is very unusual among freshwater bivalves, but is typical of many species of marine invertebrates.

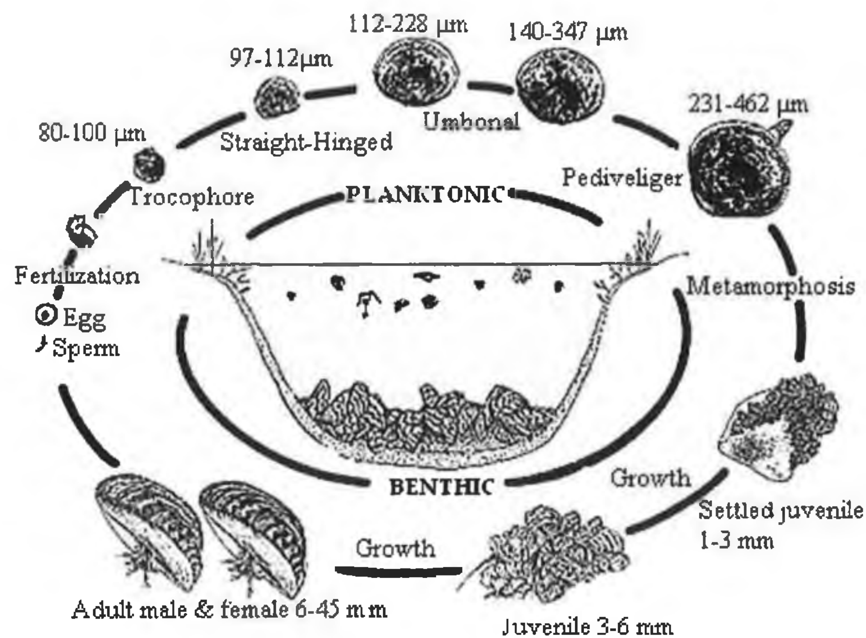

Fig. I.5: The life cycle of *Dreissena polymorpha*

(http://el.erdc.usace.army.mil/zebra/zmis/)

Spawning takes place at temperatures above 12°C, but is also influenced by other factors, such as food availability and current patterns (Haag & Garton 1992). The time required for a fertilised egg to develop into a juvenile varies inversely with water temperature, from 8 to 24 days (Nichols 1996). For example, in North American

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waters larval development takes 8 to15 days (Marsden 1992), while development time in Irish waters is two to three weeks (Lucy & Sullivan 2001). Larval densities as high as 400 000 m^{-3} have been reported (Marsden 1992). High mortality rates (20-100%) have been reported in larvae and newly settled individuals (Nichols 1996). Zebra mussels usually live three to five years, although they may survive as long as nine years (Marsden 1992). The life span in Irish waters is approximately three years (Lucy & Sullivan 2001).

I.6. IMPACTS

The zebra mussel has become the most serious non-indigenous biofouling pest in freshwater systems (O'Neill 1997) and has been nominated among the 100 worst invasive alien species of the world (ISSG, 2001;http://www.issg.org/database/species). The negative economic impact of zebra mussels in the Great Lakes only, has been estimated at 120 million US dollars over five years (van der Velde 2001).

Using byssal threads zebra mussels attach to solid substrates such as boat hulls, stones and bedrock, other molluscs, aquatic plants (including mosses), plastics, metals and sticks (Minchin & Moriarty 2002). This has led to problems such as the blocking of pipes in urban water supply systems, at power plants, lockgates, boats, moorings etc. (Minchin & Moriarty 2002; Minchin *et al.* 2003).

The ecological consequences of this invader are severe. Dense colonizations of this species alter macro-invertebrate communities on hard substrata (Strayer *et al.* 1996), and have been responsible for the decline and even the disappearance of native unionid mussels (Schloesser *et al.* 1996; Burlakova *et al.* 2000). In the Hudson River, USA, there has been an 80-90% reduction in phytoplankton biomass and more than a 70% reduction in zooplankton biomass since the introduction of *Dreissena polymorpha* in 1992 (Pace *et al.* 1998). Zebra mussels may alter nutrient cycling in a

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water body by inducing an increase of total phosphorus, ammonia, nitrate and nitrite (van der Velde 2001; Minchin *et al.* 2003). They also have the potential to reduce the overall native biodiversity of the ecosystem and change the soft bottom of the water body into a hard substrate that may be unsuitable for other organisms (van der Velde 2001).

In Ireland the environmental impacts of the species have been equally severe (Minchin *et al.* 2002). In the Shannon and Erne system, zebra mussels are a major threat to the plankton-feeding, endangered fish species *Coregonus autumnalis pollan* (McCarthy & Fitzgerald 1997), found nowhere else in western Europe, except Ireland. In addition, once zebra mussels become abundant in a water body this is followed shortly afterwards by the extirpation of whole beds of the native clam species, *Anodonta anatina* and *Anodonta cygnea* (Minchin *et al.* 2002). Another clam, *Margaritifera margaritifera* is likewise under threat (Lucey 1993; Beasley & Roberts 1999; Moorkens 1999), despite being included in the EC Habitats and Species Directive (92/43/EC) and listed in the Annex to the Bern (1979) Convention, and under the protection in Ireland of the Wildlife Act (1976). Two other Irish freshwater bivalves, *Pisidium pseudosphaerium* and *Pisidium moitessierianum* are regarded as having Red Data Book status (Killeen *et al.* 2004).

Aside from negative impacts, zebra mussel invasions can have several, seemingly positive, impacts. Water clarity can be increased by 20-100% due to filtering activities of zebra mussels, which result in reduced concentrations of suspended solids and phytoplankton within water bodies (van der Velde 2001). However, clear water may facilitate the expansion of macrophytes due to higher levels of light in the water column (Minchin & Moriarty 2002; Minchin *et al.* 2003). On the other hand, zebra mussels may be an important food resource for other animals, such as the roach (*Rutilus rutilus*), the carp (*Carpinus carpio*), the tufted duck (*Aythya fuligula*)

(Minchin & Moriarty 2002). The downside however, is that zebra mussels being filter feeders may accumulate, concentrate and transfer contaminants (bioaccumulation), such as cadmium, to these predators (van der Velde 2001).

I.7. GENETIC VARIATION

In spite of the vast literature documenting the ecological and economic impacts of *Dreissena polymorpha*, there are few publications on the population genetics of this invasive species. High levels of genetic variability have been reported for European populations with heterozygosity values at allozyme (protein-coding) loci between 0.27 and 0.49 for 3-15 polymorphic allozyme loci (Boileau & Hebert 1993; Marsden *et al.* 1995; Zielinski *et al.* 1996; Soroka *et al.* 1997; Müller *et al.* 2001). Similar high levels have also been reported for North American populations: 0.30-0.50 for 6-17 polymorphic loci (Hebert *et al.* 1989; May & Marsden 1992; Boileau & Hebert 1993; Marsden *et al.* 1995; Lewis *et al.* 2000; Elderkin *et al.* 2001).

It is obvious that there has been no detected loss of genetic variability in North American populations following the Great Lakes invasion, consistent with the hypotheses of large numbers of founding individuals and/or multiple colonisation events. While some genetic surveys revealed significant differentiation between North American populations (Boileau & Hebert 1993; Elderkin *et al.* 2001), some others failed to find significant population differentiation on a regional scale (Marsden *et al.* 1995; Lewis *et al.* 2000) although significant genetic heterogeneity was observed on a local scale within lakes (Lewis *et al.* 2000).

In Europe, two major clusters corresponding to the Danube and Northern Europe (from the Netherlands to the Volga River) have been revealed using allozymes (Marsden *et al.* 1995; Müller *et al.* 2001). Construction of the Main-Danube canal in 1992, linking the Rhine with the Danube, provided the opportunity for the two major

phylogroups to mix extensively. Müller *et al.* (2001, 2002) recently provided supporting evidence from allozyme and microsatellite markers that such exchange is indeed occurring.

Recent results from AFLP (amplified fragment length polymorphisms) analysis on Irish and British samples suggest that zebra mussels may have been introduced into Ireland from Britain (Pollux *et al.* 2003). This was later corroborated by results from the present work (see Astanei *et al.* 2005).

Using randomly amplified polymorphic DNA (RAPD) loci, Stepien *et al.* (2002) showed that the North American populations were probably founded by multiple source populations from north-western and northcentral Europe. The authors also report significant genetic differentiation between populations.

I.8. AIMS

The arrival and the explosive spread of zebra mussels in Ireland provided a unique opportunity to study the genetics of this invasive species. This is the first genetic study of zebra mussels in Ireland and the first major geographic survey of *Dreissena polymorpha* populations using microsatellite loci.

The main objectives of this study were to:

- Analyze genetic variability in five recently established Irish populations, using allozyme and microsatellite loci;
- Compare genetic variation in Irish populations with samples collected from European and North American locations;
- Attempt to identify the source population(s) of Irish zebra mussels, the probable number of introductions involved, and the size of the founder population(s); and also to identify the European source population(s) that have contributed to North American invasion;

- Analyse spatial, temporal and ontogenetic variation in the genetics of larvae and adults at a single Irish site, using microsatellite loci;
- Evaluate the importance of larval dispersal and/or human-mediated factors in the spread of zebra mussels to new sites in Ireland;
- Investigate the relationship between allozyme heterozygosity and the fitness traits growth and scope for growth in an Irish population.

CHAPTER II

MICROSATELLITE VARIATION

II.1. MACROGEOGRAPHICAL VARIATION

II.1.1. INTRODUCTION

Microsatellites markers are tandemly repeated motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes. They are present in both coding and noncoding regions and are usually characterised by a high degree of length polymorphism. When flanking regions have been determined, microsatellite loci can be amplified by polymerase chain reaction (PCR) and run on high resolution polyacrylamide gels to reveal copy number variation.

Microsatellites have many advantages over other markers in that they exhibit high polymorphism, are found in large numbers, are inherited in a simple Mendelian fashion, and, because they are non-coding, they are presumed to act as neutral markers. Microsatellites have been found to be variable even in populations that have low levels of allozyme and mitochondrial variation (Estoup *et al.* 1995). Soon after their first description (Weber & May 1989; Litt & Luty 1989), microsatellites were widely employed in many fields, such as genetic mapping (Weissenbach *et al.* 1992), analysis of paternity and kinship (Queller *et al.* 1993), estimating degrees of hybridisation between closely related species (Gotelli *et al.* 1994), comparing levels of variation between species and populations (Gotelli *et al.* 1994; Taylor *et al.* 1994) estimating effective population size (Allen *et al.* 1995), estimating the degree of population structure, including the amount of migration between subpopulations (Gotelli *et al.* 1994; Allen *et al.* 1995) and genetic relationships among various subpopulations (Bowcock *et al.* 1994; Forbes *et al.* 1995; Estoup *et al.* 1996), and in linkage analyses in association with disease susceptibility genes (Holden *et al.* 1996; Robinson *et al.* 2001).

Microsatellites mutate at rates between 5 x 10^{-3} and 5 x 10^{-5} (Estoup & Angers 1998). The higher mutation rate of microsatellite loci is reflected in their higher

polymorphism and their greater potential to detect population differentiation than less variable markers (Estoup *et al.* 1998; Ruzzante *et al.* 1999; Shaw *et al.* 1999; Wirth & Bernatchez 2001). However, the mechanisms by which microsatellites mutate are poorly understood. Understanding the mutational process is essential before genetic variation or population structure can be inferred. In allozymes, it has been assumed that most new mutations gave rise to new distinguishable alleles. In addition, the electrophoretic mobility, upon which alleles were assigned, could not be used to assess the mutational relationship amongst the alleles. An Infinite Allele Model (IAM) modelled this process, where every new mutation was assumed to give rise to a new electrophoretically distinguishable allele (Nei 1987). The situation is very different for microsatellite markers. First, since most of the mutations seem to involve the gain or loss of a single repeat unit (Weber & Wong 1993), it is clear that there exists a high amount of homoplasy (alleles of the same size, but not identical by descent). This is important as homoplasy leads to the underestimation of the total amount of variation and genetic distance, and to the overestimation of similarities among populations. The Stepwise Mutation Model (SMM) has been used to simulate this situation (Shriver *et al.* 1993). In this model alleles can only mutate by the gain and loss of one repeat unit. Di Rienzo *et al.* (1994) have developed the Two Phase Model (TPM), which incorporates the mutational process of the SMM, but allows for mutations of a larger magnitude to occur.

Despite rapidly rising interest and an increasing number of studies using microsatellites, the data are scarce and incomplete, and for many taxa still totally absent. In general, the abundance of microsatellite loci in the genomes of invertebrates is lower than in vertebrates (Amos 1999).

Up to relatively recently, information on the genetic structure of European and North American zebra mussel populations, has been based on polymorphic allozyme

markers (see Chapter I.7). However, allozymes are much less polymorphic than microsatellites, and there is extensive evidence that they do not always act as neutral markers (Karl & Avise 1992; Pogson *et al.* 1995; Barker *et al.* 1997; Riginos *et al.* 2002). Other markers, such as randomly amplified polymorphic DNA (RAPD) (Stepien *et al.* 2002) and amplified fragment length polymorphisms (AFLP) (Pollux *et al.* 2003) have also been used to uncover genetic structure between zebra mussel populations (see Chapter I.7). In spite of the increasing importance of microsatellites in studies of population genetics, there are little data on *Dreissena polymorpha*. Five microsatellite primers have been developed for *Dreissena polymorpha* (Naish & Boulding 2001). Four of these loci have been used by Müller *et al.* (2002) in a study involving European populations. Results have revealed the existence of two major clusters corresponding to the Danube and Northern Europe (from the Netherlands to the Volga River) and provided evidence for extensive gene flow between the two phylogroups. The authors reported high polymorphism, with numbers of alleles per locus ranging from 30 to 35. By contrast, the highest number of alleles recorded for an allozyme locus was 9 (Marsden *et al.* 1995).

The present section investigates the genetic population structure of zebra mussels using five microsatellite loci. In addition to investigating genetic variability in five recently established Irish populations, a comparative study of Irish, European (UK, the Netherlands and Romania) and North American (Lake Ontario and Lake St. Clair) samples was also carried out in an attempt to establish the source population(s) of Irish zebra mussels.

II.1.2. MATERIAL AND METHODS

II.1.2.1. Sample collection

Mussels were collected from five locations in Ireland (Fig. II.1.1): Limerick Docks (LM, 52.65°N, 8.66°W), Dromineer, Lough Derg on the River Shannon (LD, 52.93°N, 8.26°W), Lough Key (LK, 53.98°N, 8.20°W), Assaroe, on Lower Lough Erne (AS, 54.49°N, 8.09°W) and Ringsend, Dublin (DB, 53.35°N, 6.19°W). European samples were obtained from Walthamstow on the River Lee (RL, 51.32°N, 0.06°W), which flows into the River Thames close to London, UK, the Prut River (PR, 47.16°N, 27.82°E), which flows from Romania into the Black Sea, and Blerick on the Maas River (MR, 51.22°N, 6.08°E) in the Netherlands. North American samples were collected from Oswego Harbor, Lake Ontario (LO, 43.40°N, 76.36°W), and Lake St. Clair (LC, 42.42 °N, 82.45°W) the site where zebra mussels were first recorded in the Great Lakes. Samples were placed in 95% ethanol after collection and stored at room temperature.

II.1.2.2. DNA extraction

The phenol/chloroform method (Sambrook *et al.* 1989) was used to extract genomic DNA from whole body tissue. Five g of dry tissue was placed into sterile 1.5 ml Eppendorf microcentrifuge tubes (Eppendorf AG, Hamburg, Germany). Three hundred µl hexadecyl-trimethyl-ammonium bromide (CTAB) extraction buffer (containing 100 mM Trizma base, 1.4 M NaCl, 20 mM EDTA and 2% CTAB) was added to each tube prior to adding 25 µl of 20 mg ml^{-1} proteinase K (Sigma Co., Dublin).

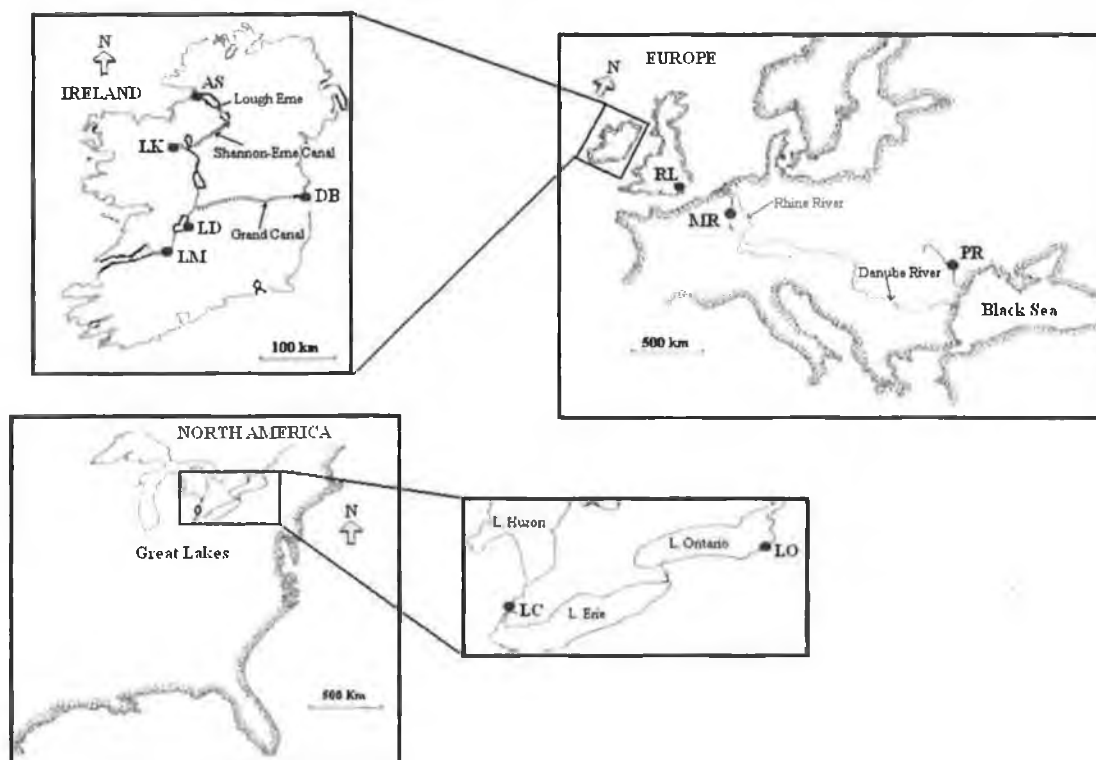

Fig. II.1.1: Location of the sampling sites. Ireland: LM: Limerick, LD: Lough Derg, LK: Lough Key, AS: Assaroe, DB: Dublin. Europe: RL: River Lee, UK; MR: Maas River, Netherlands; PR: Prut River, Romania. North America: LC: Lake St. Clair, LO: Lake Ontario.

The obtained mixture was vortexed briefly (20 s) and then incubated for 1 h at 56°C on a rotary shaker (Gio Gyrotary Shaker, New Brunswick Scientific Co. New Jersey, USA), allowing cell lysis and the release of nucleic acids. The mix was then allowed to cool down to 37°C and 25 µl of 20 mg ml^{-1} of ribonuclease A (Sigma Co., Dublin) was then added to each tube to digest the RNA. Following RNA digestion, the tubes were incubated at 37°C for 1 h. Subsequently, 350 µl phenol (Sigma Co., Dublin) pH8 was added to each tube. The role of the phenol is to digest the enzymes, proteins and any other hydrophilic compounds. The tubes were then incubated for 1 h at room temperature prior to adding 350 µl chlorophorm/isoamyl alcohol (24:1) solution to

each sample. The chlorophorm/isoamyl alcohol was used to neutralize the phenol. The mix was again incubated at room temperature for 1 h. The tubes were well shaken after adding each of the above solutions. Finally, the tubes were centrifuged at 8 000 rev min^{-1} for 15 min (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany). The upper phase was removed carefully into a sterile 1.5 ml Eppendorf microcentrifuge tube, avoiding touching the bottom layer (the supernatant liquid contains nucleic acids). A double volume of ice cold 100% ethanol was added to each tube and the obtained solution was well mixed. All the tubes were then incubated overnight in an ultrafreezer at -70°C allowing DNA precipitation. The precipitated DNA (as pellets) was collected the following day by centrifugation at 11 000 rev min^{-1} for 20 min. Following the removal of the supernatant the pellet was washed in 70% ethanol. The air-dried pellets were then resuspended overnight at 4°C in an appropriate volume (50–100μl) of TE solution (10mM Trizma base, 1 mM EDTA), depending on the size of the pellet.

II.1.2.2.1. DNA quantification

Once DNA was obtained, the DNA quality was checked out by electrophoresis through a 1% TAE (Tris Acetate EDTA) agarose (Sigma-Aldrich Co., Dublin) gel. An ethidium bromide solution (0.5 μg ml^{-1}) was used to stain the gel. The samples were loaded after prior mixing with a bromophenol blue dye to visualize the migration of samples on the gel. DNA concentrations were estimated against known concentrations of λ DNA/*Hind*III marker (New England, Bio Labs Ltd., MA, USA), which was also run on the gel as a ladder, to assist scoring.

Electrophoresis was performed in a mini-sub cell GT electrophoresis chamber (15 cm X 30 cm) using a Bio-Rad power supply (Bio-Rad Laboratories, California, USA) at a current of 120-130 mA. The ethidium bromide stained gels were viewed under UV light, using the Bio Profil Imaging system (Vilber Lourmat, Marne La

Valee, France), with Bio 1 DV 6.11 software (Vilber Lourmat) and a Mitsubishi video copy processor. The genomic DNA fragments were sized by comparing them with the standard λDNA/*Hind*III marker of known sequence.

The DNA quality (concentration and purity) was further analysed by spectrophotometric analysis at 260 and 280 nm (the DNA purity was assessed by calculating the 260/280 nm absorbance ratio). A Spectronic Genesys 2 PC spectrophotometer was used.

II.1.2.3. Microsatellite analysis

Polymerase chain reaction (PCR) amplification of five trinucleotide microsatellite loci (Naish & Boulding 2001) was carried out. The reaction mixture contained 10x reaction buffer (10 mM Tris-HCl, pH 8.3), 0.2 mM of each dNTP, 1 mM $MgCl_2$, 1 μM of forward and reverse primers, 0.5 units of Taq DNA polymerase (Sigma-Aldrich Co., Dublin) and 50 ng template DNA. The PCR amplification was carried out on a Perkin Elmer thermocycler (Perkin Elmer 9600, Perkin-Elmer Corp., Connecticut, USA). Lyophilised primers were resuspended in sterile water in a stock concentration of 100 μM and stored at -20°C. Ten μM primer aliquots were made from stock solution. The thermocycling regime consisted of an initial denaturation step at 96°C for 3 min, followed by 30 cycles of denaturing at 96°C for 1 min, annealing at 58, 61, 50, 57 and 53 °C (for *DpolA6*, *DpolB6*, *DpolB8*, *DpolB9* and *DpolC5*, respectively), and extension at 72°C for 45 s. Optimisations of PCR conditions were performed using an initial set of 10 cycles dropping from 5°C above the annealing temperature in 0.5°C increments.

Agarose gels were used to check the PCR amplification prior to performing radiolabeled microsatellite amplification. A volume of 5 μl of each PCR product was mixed with 1 μl bromophenol blue loading dye and 4 μl sterile water and the mix was

then run on a 2% agarose (Sigma-Aldrich Co., Dublin) gel (stained with 0.5 μg/ml ethidium bromide solution). A Tris Borate/EDTA (TBE) buffer was used. A 100 bp (base pairs) marker (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK) was also run on the same gel, allowing for the sizing of amplified DNA.

Primers for two loci (*DpolB8* and *DpolB9*) were modified using the program OLIGO v.4.1 (National Biosciences, USA). The sequences of the newly designed primers are given in Table II.1.1.

Primer	Sequence
*DpolB8*F	5'-TTTTTCTTCTGGTCTCGACG-3'
*DpolB8*R	5'-AATTTGAACATTAAACATTTGTC-3'
*DpolB9*F	5'-TGATCAGATATTTTCACAAACTGC-3'
*DpolB9*R	5'-GCGTGTGTTTTTGAAACGTG-3'

Table II.1.1: Primer sequences for *DpolB8* and *DpolB9* loci

II.1.2.3.1. Radiolabelled microsatellite amplification

The radiolabeled microsatellite amplification technique was identical to that described in the previous section, with the exception of adding 15 pmol of labelled forward primer with 0.5 μl of ($\gamma^{32}P$) ATP (Amersham Pharmacia Biotech. Ltd., Buckinghamshire, UK). Fifty ng of genomic DNA template was added to each well of a 96-well microtitre plate (Sigma-Aldrich Co., Dublin) prior to aliquoting 9.4 μl of the PCR reaction mix with a pippetor (Repeater pippetor, Eppendorf AG, Hamburg, Germany).

II.1.2.3.2. Preparation of M13 A/T DNA sequence ladder

An A/T DNA sequence ladder was prepared to allow for PCR product sizing. Components supplied with the T7 sequencing kit (Amersham Pharmacia Biotech. Ltd., Buckinghamshire, UK) were used for making up the ladder. A primer mix was prepared in a 1.5 ml microcentrifuge tube by adding 10 µl of M13 mp18 (+) strand DNA, 2 µl of annealing buffer (1 M Tris HCl pH 7.5, 100 mM $MgCl_2$, 160 mM DTT) and 2 µl of M13 universal primer (5'- d[GTA AAA CGA ACG GCC AGT] – 3' in aqueous solution). The primer mix was subsequently incubated at 65°C for 10 min followed by 10 min incubation at 25°C. A labelling mix was also prepared in another 1.5 ml microcentrifuge tube by adding 3 µl of dCTP labelling mix (1.375 µM each of dATP, dGTP and dTTP and 333.5 mM NaCl), 1µl $\alpha^{32}P$ dCTP (6 000 Ci/mmol, Amersham Pharmacia Biotech. Ltd., Buckinghamshire, UK), 2 µl of T7 DNA polymerase buffer (25 mM Tris HCl, pH 7.5, 5 mM DTT, 100 g BSA ml^{-1} and 5% glycerol) and 0.5 µl of T7 DNA polymerase. Six µl of the labelling mix was added to the primer mix, and the obtained mixture was incubated at 25°C for 5 minutes. Following incubation, 6.5 µl of solution was added to two separate 1.5 ml microcentrifuge tubes containing 5 µl of "A" long-mix (840 µM of each dGTP, dTTP and dCTP, 93.5 µM dATP, 2.1 mM ddATP, 40 mM Tris HCl, pH 7.5 and 50 mM NaCl) and respectively 5 µl of "T" long-mix (840 µM of each dCTP, dGTP and dATP, 93.5 µM dTTP, 2.8 mM dTTP, 40 mM Tris HCl, pH 7.5 and 50 mM NaCl). The tubes were then incubated for 5 min at 37°C. Thirty µl of kit stop solution (0.3% bromophenol blue, 0.3% xylene cyanol FF, 10 mM EDTA, pH 7.5 and 97.5% deionised formamide) were added after incubation. A 1:5 ladder dilution was finally made up. Ten µl aliquots of the M13 A/T DNA diluted sequencing ladder were subsequently made and stored at -20°C until use.

II.1.2.3.3. Preparation of denaturing polyacrylamide gel

The polyacrilamide gels were run using a Sequigen GT apparatus (Bio-Rad Laboratories, California, USA). For each gel, 100 ml gel solution (final concentration 5% polyacrylamide) was prepared from a 40% acrylamide stock solution. The 40% acrylamide solution was made as follows:

40% Acrylamide stock solution (100 ml)

Acrylamide (Sigma-Aldrich Co., Dublin)	38 g
Bis-acrylamide (Sigma-Aldrich Co., Dublin)	2 g
Amberlite MB-1 resin (Sigma Chemical Co., Missouri, USA)	5 g

This solution was incubated at room temperature for 1 h (with constant shaking) and then it was passed through a sinterred glass funnel (in order to remove the amberlite resin). The 5% polyacrylamide denaturing gel was prepared as follows:

5% Polyacrylamide denaturing gel (100 ml)

Urea (BDH Laboratory Supplies, Dublin)	42 g
40% Acrylamide stock solution	12.5 ml
10 X Tris-borate EDTA (TBE) solution	10 ml
Sterile water	to bring up to 100ml

The mix was heated until the urea was fully dissolved and was then allowed to cool. The 40% and 5% polyacrylamide solutions were stored at 4°C until use. A 10% ammonium persulphate (Sigma-Aldrich Co., Dublin) solution was freshly prepared before pouring the gel, and 500 µl of this was added to 100 ml of 5% polyacrylamide solution, together with 100 µl TEMED (Sigma-Aldrich Co., Dublin); the mixture was

then well mixed. Using a 100 ml syringe, the solution was quickly poured between two glass plates (38 cm x 50 cm) separated by plastic spacing strips (Bio-Rad Laboratories, California, USA). Plastic combs (Bio-Rad Laboratories, California, USA) were immediately placed at one end (with teeth facing upwards) to allow a uniform border to form. The gel was then allowed to set overnight. After setting the combs were removed carefully and reversed, so that the comb teeth could easily break the surface of the gel, creating wells in which the samples were loaded.

II.1.2.3.4. Electrophoretic separation of amplified microsatellites

A Tris Borate/EDTA (TBE) 1X solution was used as electrophoresis buffer. Immediately after PCR amplification, 10 μl of formamide stop solution were added to each well containing the amplified radiolabelled product. The formamide stop solution contained 40 ml of formamide solution (Sigma-Aldrich Co., Dublin), 0.1 g xylene cyanol FF (Sigma-Aldrich Co., Dublin), 0.1 g bromophenol blue (Sigma-Aldrich Co., Dublin) and 4 ml of 500 mM EDTA (BDH Laboratories Supplies, Dublin) pH 8.0. The solution was stored at room temperature covered in tin foil. The samples were then denatured at 96°C for 5 min, prior to loading on the denaturing gel. The gel was heated up to 50°C and the samples were then loaded using a multichannel Eppendorf pippette (Sigma-Aldrich Co., Dublin). Three to five M13 A/T DNA sequence ladders were also run on each gel to allow for alleles sizing; the ladder was also incubated for 5 min at 96°C prior to loading. To ensure reliability of allele scoring, two reference samples were rerun on each gel. The gels were run at a constant current of 50 mA. Following electrophoresis, gels were transferred onto 3 MM filter paper (Whatman International Ltd., Maidstone, UK), covered in cling film and dried for 3 h on a gel drier (Bio-Rad Laboratories, California, USA). The cling film was then removed and the gel was exposed to a Curix Blue HC-S Plus X-ray film (Agfa-Gevaert N.V., Mortsel,

Belgium). The X-ray film was placed over the gel in an autoradiography cassette, which was subsequently stored at -70°C for 2-3 days. The autoradiographs were then developed and fixed manually by immersing the film for 5 min in a tray containing developer (Champion Photochemistry International Ltd., Essex, UK), washing it with water and finally immersing it for 5 min in a tray containing fixer (Champion Photochemistry International Ltd., Essex, UK). The film was then thoroughly washed of fixing solution and air-dried. The microsatellite allele lengths were determined by comparing each allele with the M13 A/T DNA sequence ladder co-electrophoresed on the same gel.

II.1.2.4. Statistical analysis

II.1.2.4.1. Genetic diversity

Allelic distribution for each locus, expected (H_E) and observed (H_O) heterozygosity estimates for each population were obtained using the GENETIX v. 4.01 computer program (Belkhir *et al.* 2000). Conformance to Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP v 3.1 program (updated version of Raymond & Rousset 1995), with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. Tests for linkage disequilibrium between pairs of loci were performed using the same program.

II.1.2.4.2. Population differentiation

A number of methods were employed to assess population differentiation. First, heterogeneity in allele and genotype distributions between pairs of populations was tested using the exact probability test as implemented in GENEPOP. Second, the level

of genetic differentiation among populations was quantified using F_{ST} (Weir & Cockerham 1984), the significance of which was determined using permutation tests as implemented in the FSTAT program (Goudet 2001). Significance of multiple pairwise comparisons was determined using the sequential Bonferroni method (Rice 1989). The allele frequency-based F_{ST} statistic was used, rather than the allele size-based R_{ST} statistic of Slatkin (1995), which is derived specifically under the assumptions of the generalized stepwise mutation model. R_{ST} and F_{ST} values are not expected to differ greatly for short-term differentiation of populations within species (Slatkin 1995; Wenburg *et al.* 1998) but F_{ST}-based estimates of differentiation are considered more reliable when fewer than 20 loci are used (Gaggiotti *et al.* 1999). Third, dendrograms of relationships were constructed using the neighbour-joining method of clustering implemented in the PHYLIP program (Felsenstein 1989), based on both D_C and D_A distances among populations. Computer simulations have shown that for microsatellite data Cavalli-Sforza & Edwards (1967) chord distance (D_C) and Nei *et al.* (1983) D_A distance are most efficient in obtaining the correct tree topology (Takezaki & Nei 1996). Bootstrap values were computed by resampling loci over 1000 replications.

We explored how much differentiation between the five Irish population pairs might be explained by geographical distance. Pairwise F_{ST} values were used to calculate the relationship between genetic differentiation and geographical distance by regressing $F_{ST}/(1-F_{ST})$ values against the ln of geographical distance between sample pairs (Rousset 1997) using the Mantel test (Mantel 1967) with 1000 permutations, as implemented in GENEPOP.

Recent results have indicated that high locus polymorphism may underestimate the degree of genetic divergence between populations when using measures of differentiation such as F-statistics (Olsen *et al.* 2002, 2004; O'Reilly *et al.* 2004). In order to test this the relationship between F_{ST} and locus polymorphism was estimated

using two measures, allelic richness, an estimate of the number of alleles, which is independent of sample size and expected heterozygosity. Allelic richness estimates were obtained using the FSTAT program.

II.1.2.4.3. Bottlenecks

Three mutational models (the infinite allele model, IAM, the two-phased model, TPM and the stepwise mutation model, SMM) were considered in order to test for recent occurrences of population bottlenecks, using the BOTTLENECK program (Cornuet & Luikart 1996). Distributions of heterozygosity were compared under each mutational model with distributions predicted from the number of alleles detected in each sample. Populations that have recently undergone a bottleneck, i.e., in the past several dozen generations, will exhibit larger observed heterozygosities than expected from the models, because of more rapid reductions in allele numbers than in heterozygosity. Statistical significances of the results from each model were tested using the Wilcoxon test. The BOTTLENECK program was also used to examine allele frequency distributions in all populations. Non-bottlenecked populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles at low frequency (Luikart *et al.* 1998). However, in recently bottlenecked populations a mode-shift distortion in allele frequency distributions is expected such that alleles at low frequency (<0.01) become less abundant than alleles in one or more intermediate allele frequency classes (e.g., 0.1-0.2). This distortion is transient and will probably only be detected for a few dozen generations after the bottleneck has occurred (Luikart *et al.* 1998).

II.1.2.4.4. Null alleles

Where observed genotype frequencies deviated significantly from HWE expectations the method of Brookfield (1996) as implemented in the program MICRO-CHECKER

(Shipley 2003; Van Oosterhout *et al.* 2004) was used to determine the most likely cause. Null alleles are a common problem with microsatellite loci and can lead to large heterozygote deficiencies (Callen *et al.* 1993; O'Connell & Wright 1997; McCartney *et al.* 2004). They arise when alleles are not amplified because of base substitutions or deletions in PCR priming sites flanking microsatellite arrays. In the present study null alleles were inferred if homozygote excess was homogenously distributed across the homozygote-classes. MICRO-CHECKER estimates the null allele frequency and adjusts the observed allele and genotype frequencies appropriately. Brookfield's (1996) null allele estimator 2 was used in preference to Brookfield's estimator 1 (1996), Chakraborty *et al.* (1992) or van Oosterhout *et al.* (2004) null allele estimators. These estimators regard non-amplified samples to be a consequence of degraded DNA, human error, etc. in contrast to Brookfield's second estimator which assumes that a proportion of the samples are not amplified because they are in fact null allele homozygotes. Another source of heterozygote deficits results from preferential amplification of small alleles (i.e., large allele dropout or short allele dominance, Wattier *et al.* 1998) when the larger allele in the heterozygote fails to amplify. This source of heterozygote deficits was assumed if the excess of homozygotes was biased towards either extreme of the allele size-distribution. Heterozygotes deficits can also be caused by slippage during PCR amplification, thereby producing stutter products that differ from the original template by multiples of the repeat unit length (Shinde *et al.* 2003), making it difficult to distinguish between homozygotes and heterozygotes. PCR stutter was indicated if there were significant shortages of heterozygote genotypes with alleles differing in size by a single repeat.

II.1.3. RESULTS

II.1.3.1. Genetic diversity

All five loci were polymorphic in all populations, the number of alleles per locus varying from 15 (*DpolC5*) to 30 (*DpolB6*) across all populations, with the frequency of any one allele no higher than 0.51 for any individual locus. Allele size ranges were: 293-368 bp for *DpolA6*, 268-376 bp for *DpolB6*, 306-411 bp for *DpolB8* bp, 267-327 bp for *DpolB9* and 181-238 bp for *DpolC5*. Table II.1.2 shows that the highest numbers of alleles per locus were observed in the American samples (13-21), followed by the European and British samples (11-19) and the Irish samples (6-17). Mean number of alleles per locus for Irish samples was significantly lower than for the other samples (Mann-Whitney U test $P < 0.05$). Out of a total of 109 alleles, nine private alleles were recorded for the PR sample and eight for the American samples. Private alleles were also recorded for MR (1) and RL (1) samples. Surprisingly, one private allele at the *DpolB6* locus was observed in the Irish AS sample. The high polymorphism recorded is reflected in high expected heterozygosity (H_E) values, where between 79% and 89% heterozygous individuals were expected in each sample across all loci (Table II.1.2). The actual observed values (H_O) were lower than those expected for all populations. The RL population had the lowest observed heterozygosity (H_O = 0.504) followed by Irish populations (0.517-0.608) with the highest values in the LO (0.635) and PR (0.653) populations.

Locus/Sample	LM	LD	LK	AS	DB
DpolA6					
N	48	30	30	41	27
A	10	13	9	9	11
H_E	0.778	0.864	0.757	0.775	0.837
H_O	0.396	0.767	0.600	0.512	0.444
F_{IS}	**0.449**	0.129	**0.223**	0.350	**0.484**
DpolB6					
N	31	36	30	33	24
A	15	17	13	15	14
H_E	0.884	0.915	0.789	0.814	0.826
H_O	0.387	0.444	0.400	0.546	0.625
F_{IS}	**0.573**	**0.524**	**0.506**	**0.343**	**0.264**
DpolB8					
N	30	31	30	30	27

RL	MR	PR	LO	LC	Mean/locus
30	32	30	33	31	33.2
11	12	13	13	16	11.7
0.833	0.807	0.876	0.853	0.888	0.827
0.367	0.406	0.800	0.576	0.516	0.538
0.571	**0.508**	0.104	**0.339**	**0.432**	**0.369**
30	30	30	35	33	31.2
15	19	18	21	17	16.4
0.856	0.888	0.919	0.919	0.888	0.870
0.400	0.567	0.733	0.543	0.485	0.513
0.545	**0.377**	**0.214**	**0.422**	**0.466**	**0.429**
31	33	30	30	31	30.3

A	13	15	12	10	13
H_E	0.884	0.889	0.869	0.859	0.892
H_O	0.500	0.548	0.400	0.533	0.556
F_{IS}	**0.448**	**0.397**	**0.552**	**0.393**	**0.393**
DpolB9					
N	30	30	30	30	23
A	9	8	7	6	7
H_E	0.766	0.734	0.728	0.664	0.760
H_O	0.500	0.633	0.600	0.567	0.870
F_{IS}	**0.362**	0.154	0.192	0.164	-0.122
DpolC5					
N	30	31	30	33	22
A	10	10	10	10	9
H_E	0.851	0.852	0.849	0.842	0.825
H_O	0.800	0.645	0.867	0.788	0.546

17	14	17	13	15	13.9
0.907	0.905	0.913	0.886	0.905	0.891
0.452	0.606	0.533	0.567	0.516	0.521
0.514	**0.344**	**0.430**	**0.375**	**0.443**	**0.429**
31	31	30	32	28	29.5
12	12	15	14	14	10.4
0.786	0.809	0.903	0.891	0.903	0.794
0.581	0.774	0.633	0.750	0.714	0.662
0.277	0.059	**0.314**	**0.173**	**0.226**	**0.190**
32	32	30	31	31	30.2
11	10	13	14	13	11
0.864	0.686	0.876	0.842	0.854	0.834
0.719	0.531	0.567	0.742	0.452	0.666

F_{IS}	0.077	**0.258**	-0.004	0.079	**0.360**	**0.183**	**0.240**	**0.368**	**0.135**	**0.484**	**0.214**
All loci											
N	33.8	31.6	30	33.4	24.6	30.8	31.6	30	32.2	30.8	30.9
A	11.4	12.6	10.2	10	10.8	13.2	13.4	15.2	15	15	12.7
H_E	0.832	0.851	0.798	0.791	0.828	0.849	0.819	0.894	0.878	0.888	0.843
H_O	0.517	0.608	0.573	0.589	0.608	0.504	0.577	0.653	0.635	0.537	0.580
F_{IS}	**0.392**	**0.292**	**0.294**	**0.266**	**0.276**	**0.418**	**0.306**	**0.286**	**0.289**	**0.410**	**0.323**

Table II.1.2: Sample size (N), number of alleles (A), expected (H_E) and observed (H_O) heterozygosity, and deviations from HWE (F_{IS}) according to Weir & Cockerham (1984). Values in bold indicate samples which deviate significantly from HWE ($P<0.05$) after sequential Bonferroni corrections.

II.1.3.2. HWE and null alleles

Significant departures from HWE were observed in 39 of the 50 single locus exact tests after sequential Bonferroni correction (Table II.1.2). Although two negative F_{IS} values were found, permutation tests on F_{IS} values across loci revealed no evidence of heterozygote excess in any population.

Some specimens did not yield PCR products at some loci after two or three attempts; the same samples were, however, successfully amplified at the other loci, strongly suggesting the presence of null alleles. The second method of Brookfield (1996), as implemented in MICRO-CHECKER, was used to calculate the expected frequency (*r*) of null alleles per locus. Frequencies ranged from 0 to 0.46 (Table II.1.3), with mean frequencies per locus varying from 0.14 to 0.31. No evidence of upper allele dropout and/or stuttering during PCR amplification was found. Correcting the data for null alleles decreased the number of significant departures from HWE from 39 to 7.

Genotypic data were also tested for linkage disequilibrium but no significant disequilibrium was detected for any locus pair in any of the populations analysed ($P >$ 0.1 in every case).

II.1.3.3. Bottlenecks

There was no evidence for heterozygote higher than that expected at equilibrium in any of the populations tested under the three different mutation models either for uncorrected data or data corrected for null alleles. Allele frequencies followed an L-shaped distribution i.e., no skews of allele frequencies towards intermediate values were observed in any of the populations, as expected in a non-bottlenecked population at mutation-drift equilibrium.

Locus/Sample	LM	LD	LK	AS	DB	RL	MR	PR	LO	LC	Mean
DpolA6	0.37	0.07	0.15	0.23	0.37	0.46	0.35	0.11	0.28	0.27	0.27(0.040)
DpolB6	0.44	0.38	0.36	0.26	0.21	0.39	0.29	0.14	0.29	0.33	0.31(0.029)
DpolB9	0.26	0.09	0.16	0.11	0	0.22	0.05	0.19	0.12	0.15	0.14(0.025)
DpolC5	0.06	0.19	0.02	0.03	0.27	0.10	0.16	0.31	0.08	0.34	0.16(0.037)
DpolB8	0.31	0.28	0.41	0.27	0.28	0.38	0.26	0.29	0.24	0.28	0.30(0.017)
Mean	0.29 (0.064)	0.20 (0.058)	0.22 (0.072)	0.18 (0.047)	0.23 (0.062)	0.31 (0.066)	0.22 (0.053)	0.21 (0.040)	0.20 (0.043)	0.27 (0.034)	0.23(0.013)

Table II.1.3: Expected null allele frequencies calculated using the second method of Brookfield (1996) as implemented in MICRO-CHECKER. Standard errors for means are given in parentheses.

II.1.3.4. Population differentiation

The null hypothesis of no heterogeneity between sample pairs was rejected in 29 of 45 possible tests of allelic differentiation, and in 21 of 45 tests of genotypic differentiation, using Fisher's exact tests (Table II.1.4). Data corrected for null alleles resulted in fewer significant pairwise tests of both allelic (16 of 45) and genotypic (14 of 45) variation (Table II.1.5).

There were no significant differences in either allelic or genotypic proportions for any of the Irish, UK or Netherlands pairwise comparisons. The two samples from the Great Lakes did not differ from each other in either allelic or genotypic proportions, and neither was significantly differentiated from either the UK or Netherlands samples. However, the majority of Irish samples were significantly differentiated from the Great Lakes samples. Surprisingly, tests for genotypic differentiation showed no differentiation between samples from Romania and UK. However, tests for allelic differentiation and pairwise F_{ST} comparison revealed significant differentiation between the two sites.

Multilocus estimates of F_{ST} for population pairs (Table II.1.6) indicated an overall lack of significant differentiation between Irish, UK or Netherlands populations. There were only 8 significant estimates and seven of these involved pairwise comparisons between the Romanian population (PR) and all other European populations. It is noteworthy that no significant differentiation was observed between PR and the two Great Lakes samples. Overall, pairwise F_{ST} estimates were relatively low, -0.01 to 0.05 and –0.1 to 0.04 for data that were uncorrected or corrected for null alleles, respectively. A significant negative correlation was found between expected locus heterozygosity and F_{ST} estimates ($P = 0.03$, Fig. II.1.2) but no significant correlation was observed between allelic richness and F_{ST} values.

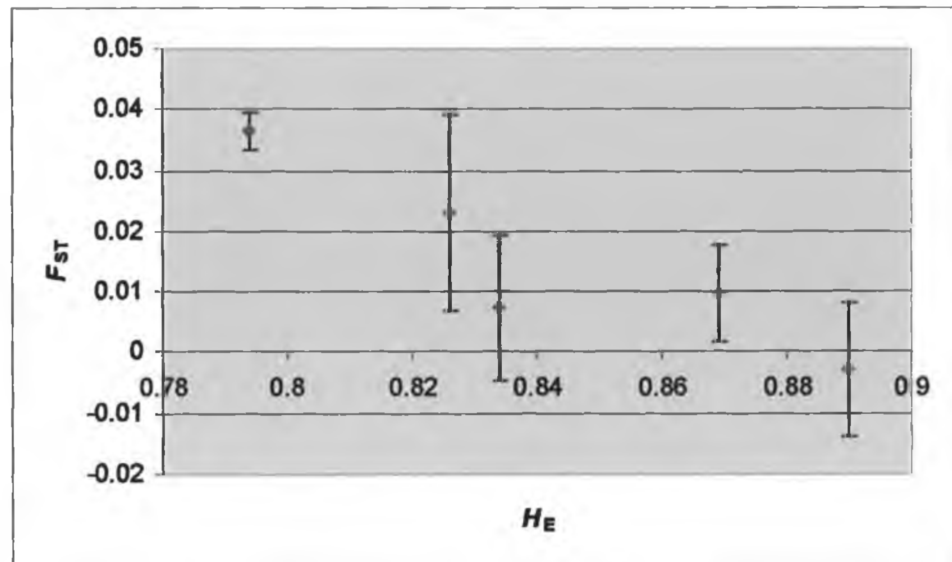

Fig. II.1.2: Single locus estimates of F_{ST} (± 95% CI) vs. expected heterozygosity for five microsatellite loci in 10 samples.

Regression of pairwise $F_{ST}/(1-F_{ST})$ values against ln geographical distance showed a significant correlation (R^2 = 0.63, P = 0.03). However, the correlation was no longer statistically significant when the most distant sample on the east coast of Ireland (DB) was excluded (P = 0.23) or when the DB sample was included and any one of the Shannon-Erne samples was excluded.

The dendrogram of relationships constructed using Nei *et al.* (1983) D_A distance (Fig. II.1.3) showed the Irish sites clustering together, with sites that are geographically close (LM and LD; AS and LK) more closely clustered than the single site on the east coast (DB).

The UK sample clustered with the Irish samples. Both Great Lakes populations clustered together but separately from the Irish, UK and Netherlands samples. The sample from Romania (PR) formed a distinct branch, separated from all other samples. The tree of

relationships based on Cavalli-Sforza and Edwards (1967) chord distance (D_C) showed a similar pattern.

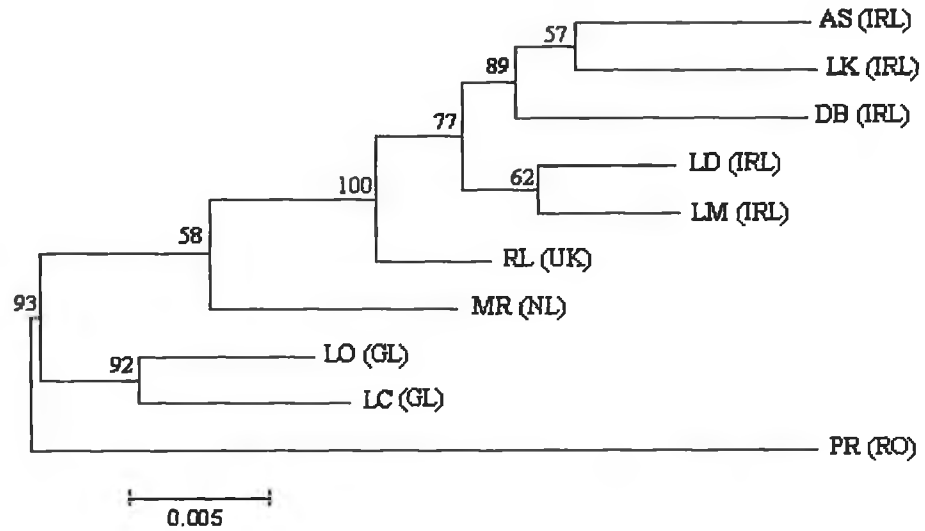

Fig. II.1.3: Dendrogram of relationships, using the neighbour-joining method of clustering, based on Nei *et al.* (1983) D_A distance. Bootstrap values have been computed by re-sampling loci and are given as percentages over 1000 replications. IRL: Ireland; UK: United Kingdom; NL: Netherlands; GL: Great Lakes; RO: Romania.

Population	**LM**	**LD**	**LK**	**AS**	**DB**	**RL**	**MR**	**PR**	**LO**	**LC**
LM	-	0.95	0.65	0.1	0.22	0.79	**0.004**	**<0.001**	**<0.001**	**0.002**
LD	0.61	-	0.16	0.006	0.64	0.91	0.23	**<0.001**	**0.002**	**0.003**
LK	0.11	0.007	-	0.37	0.22	0.69	**<0.001**	**<0.001**	**<0.001**	**<0.001**
AS	**0.002**	**<0.001**	0.07	-	0.15	0.55	**<0.001**	**<0.001**	**<0.001**	**<0.001**
DB	**<0.001**	0.17	**0.003**	**0.003**	-	0.83	0.14	**<0.001**	**0.004**	0.01
RL	0.15	0.39	0.08	0.10	0.13	-	0.53	**<0.001**	0.59	0.51
MR	**<0.001**	0.01	**<0.001**	**<0.001**	**<0.001**	0.01	-	**<0.001**	0.51	0.15
PR	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**	**<0.001**
LO	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.04	0.02	**<0.001**	-	0.99
LC	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.02	**<0.001**	**<0.001**	0.79	-

Table II.1.4: Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal). Values in bold indicate samples significantly different after sequential Bonferroni correction.

Population	LM	LD	LK	AS	DB	RL	MR	PR	LO	LC
LM	-	0.78	0.43	0.02	0.08	0.88	0.32	**<0.001**	0.03	0.01
LD	0.68	-	0.84	0.08	0.84	0.92	0.60	**<0.001**	**0.004**	**0.001**
LK	0.34	0.66	-	0.56	0.91	0.77	0.17	**<0.001**	**0.003**	**<0.001**
AS	0.04	0.07	0.60	-	0.78	0.07	0.03	**<0.001**	**<0.001**	**<0.001**
DB	0.04	0.72	0.73	0.73	-	0.90	0.79	**<0.001**	0.04	**<0.001**
RL	0.76	0.82	0.44	0.05	0.79	-	0.99	0.02	0.96	0.74
MR	0.25	0.41	0.04	0.008	0.54	0.97	-	**<0.001**	0.86	0.25
PR	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	-	**<0.001**	0.03
LO	0.03	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.88	0.44	**<0.001**	-	0.99
LC	0.01	**<0.001**	**<0.001**	**<0.001**	0.04	0.63	0.06	**0.004**	0.99	-

Table II.1.5: Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal) after correction for the presence of null alleles. Values in bold indicate samples significantly different after sequential Bonferroni correction.

Population	LM	LD	LK	AS	DB	RL	MR	PR	LO	LC
LM	-	-0.003	-0.003	0.009	0.009	-0.004	0.004	0.034***	0.011	0.012
LD	-0.009	-	-0.002	0.004	-0.007	-0.013	-0.0004	0.026***	0.010	0.009
LK	0.001	0.011	-	-0.006	-0.002	0.001	0.014	0.037***	0.015	0.015
AS	0.008	0.013	-0.002	-	-0.006	0.004	0.014	0.041***	0.019	0.020*
DB	0.012	0.002	0.015	0.010	-	-0.009	0.003	0.021***	0.005	0.003
RL	-0.004	-0.001	-0.001	-0.001	0.001	-	-0.008	0.020*	-0.007	-0.008
MR	0.015	0.007	0.035*	0.031	0.025	0.009	-	0.025***	-0.001	0.004
PR	0.015***	0.030***	0.054***	0.054**	0.032***	0.023***	0.034**	-	0.006	0.001
LO	0.009	0.009	-0.001	0.026	0.016	0.002	0.006	0.010	-	-0.012
LC	0.008	0.008	0.022	0.026	0.014	0.001	0.014	0.008	-0.004	-

Table II.1.6: Pairwise F_{ST} (Weir & Cockerham, 1984) using data uncorrected (below diagonal) and corrected (above diagonal) for the presence of null alleles

P* < 0.05, **P < 0.01, ***P < 0.001, from permutation tests in FSTAT program, after sequential Bonferroni correction

II.1.4. DISCUSSION

II.1.4.1. Genetic diversity

All studies on zebra mussels to date report high levels of genetic variability, irrespective of marker type or whether samples come from native or invasive populations (Stepien *et al.* 2002; Müller *et al.* 2002 and Chapter I.7). The present study shows high levels of variability in both invasive populations e.g., Great Lakes (observed heterozygosity: *Ho* 0.537-0.635; allele number: A 15), Netherlands (0.577; 13.4), UK (0.504; 13.2) and Ireland (0.517-0.608; 10-12.6) and in the native Romanian population (0.653; 15.2). *Ho* values for the newly established Irish populations are within the range observed for both native and well-established alien populations although the mean numbers of alleles per locus are significantly lower (Table II.1.2). Recently bottlenecked populations are likely to have lost rare alleles but may still contain substantial heterozygosity. However, there was no evidence that Irish populations had undergone a recent bottleneck. A bottleneck is most likely to be detectable when sampling 8-10 polymorphic microsatellite loci and 30 individuals (Luikart *et al.* 1998). While our mean sample size is 31±3, the number of sampled loci is low. In addition, the power of the bottleneck test (Cornuet & Luikart 1996) is such that about 20% of the time a bottleneck will not be detected even when a population has been recently bottlenecked (Luikart *et al.* 1998).

Müller *et al.* (2002), in the only published study on microsatellite variation in zebra mussels to date, observed higher levels of variability in European populations (*Ho*: 0.79-0.94; A: 30-35/locus) than we did for all populations, using four of the five microsatellite loci employed by us. The larger sample sizes (43±12) used by Müller *et al.* (2002) in comparison to ours makes it more likely that rare alleles would be detected in their samples. Sample size could also be a factor in the number of putative private alleles

observed in our study since it is unlikely, in recently established populations such as those in the Great Lakes, where eight such alleles were observed, that there has been sufficient time for new mutations to have become established.

II.1.4.2. Heterozygote deficits and null alleles

A deficit of heterozygotes relative to HWE is a common phenomenon in marine bivalves and has been well documented for allozyme loci since the early 1970s (Zouros & Foltz 1984; Gaffney 1990; Gosling 1992). Significant deficits have also been reported in zebra mussel populations (Marsden *et al.* 1996; Lewis *et al.* 2000; Elderkin *et al.* 2001). In contrast, there are only a few reports of heterozygote deficits at microsatellite loci for marine (Naciri *et al.* 1995; Bierne *et al.* 1998; McGoldrick *et al.* 2000; Hedgecock *et al.* 2004) or freshwater bivalves (Wilson *et al.* 1999), although the phenomenon is well documented in marine fish (O' Connell & Wright 1997). In our study, deficits of heterozygotes were recorded for all five loci and in all populations, although most of the significant values were recorded for *DpolA6*, *DpolB6* and *DpolB8* (27 out of 35 significant estimates). Several factors such as inbreeding, the Wahlund effect, selection or the presence of null alleles could account for the observed heterozygote deficits. In species with separate sexes partial inbreeding can occur through the mating of related individuals, thus producing a deficit of heterozygotes. There are two factors that argue against inbreeding as the causative agent for the observed deficits. First, the life cycle of zebra mussels, with high fecundity, external fertilization and an extended larval dispersal phase, makes mating of relatives unlikely. Second, inbreeding should affect all loci equally, generating uniform heterozygote deficiencies across loci. However, our data show that deficits (positive F_{IS} values in Table II.1.2) were heterogeneous among loci.

The Wahlund effect is observed when a sample comprises a mixture of individuals from two or more populations that differ in allele frequencies at a locus; it is manifested as an overall deficit of heterozygotes compared to the number predicted by the HWE. Apart for the Irish (DB) sample, where low densities of individuals did not allow collection from just a single patch, the remainder of the samples consisted of individuals collected within a few square meters. Nevertheless, it is possible that several cohorts of larvae that were spawned at different locations could have settled at the same sampling location. The lack of significant differentiation, however, in adults and spat collected from sites situated close together at Lough Key (see Chapter II.2) suggests that the Wahlund effect is unlikely to account for departures from HWE.

Another possible reason for heterozygote deficits, and one that has been most widely cited as the cause of deficits at allozyme loci, is selection. Microsatellites are generally considered as neutral markers (non-coding DNA regions), which means that selection cannot be invoked for observed deficits of heterozygotes, unless the microsatellite loci analysed are themselves linked to loci that are under selection. Since there was no evidence in our data that any pair of microsatellite loci were linked, this hypothesis would require that each microsatellite locus be linked to a specific locus (or loci) that is under selection.

Null alleles are a common problem with microsatellite loci and can lead to high observed deficits of heterozygotes (Brookfield 1996; Hoarau *et al.* 2002). Although Müller *et al.* (2002), in their study on European populations of zebra mussels, made no reference to departures from HWE in their data, Wilson *et al.* (1999) reported significant departures from HWE at six microsatellite loci in all eight samples of *Dreissena bugensis* analysed from the Great Lakes, which they attributed to the Wahlund effect. In our study it

is likely that heterozygote deficits are largely caused by null alleles. In a recent study Hedgecock *et al.* (2004) observed non-amplfying PCR-null alleles at 49 (51%) out of 96 microsatellite loci assayed in the marine bivalve, *Crassostrea gigas*, and average frequency of these alleles was 0.093. McGoldrick *et al.* (2000) for the same species reported null alleles at 16 (67%) out of 24 loci, with frequencies of 0.11-0.22, which are within the range observed in our study (0.00-0.46).

II.1.4.3. Population differentiation

The Shannon-Erne system is Europe's longest navigable leisure waterway (~750 km) with many different craft using the system (Sullivan *et al.* 2002). In addition to boat traffic moving up and down the system, angling boats are regularly transported overland between the different water bodies (Minchin *et al.* 2002). Therefore, dispersal of zebra mussels in the Shannon-Erne system is through a combination of downstream movement of larvae, movement of fouled craft traveling both upstream and downstream within the system, and through jump dispersal by overland movement of fouled craft. It is not surprising, therefore, that there is little evidence, from F_{ST} or exact tests, for significant differentiation between samples in the Shannon-Erne system.

Although a slightly significant correlation was found between pairwise F_{ST} estimates and geographic distance when all sites were considered, the correlation was not significant when any one of the samples was excluded. In our regression approach with pairwise values among five samples, only ten data points were available, leaving out a sample removes four out of ten points from the regression, hence seriously compromising the power of the analysis. However, the results do not provide strong support for any model of dispersal (island model of dispersal, where individuals or their gametes move from one

population to another with equal probability, or isolation-by-distance model where the probability of dispersal from site of origin to other sites declines with increasing geographic distance). An island model of dispersal would have been expected given the number of natural and human-mediated dispersal mechanisms (see Chapter I.2).

Outside of the Shannon-Erne system zebra mussels were recorded for the first time in 2002 at Ringsend, Dublin, the Grand Canal (Fig. II.1.1) and in several freshwater lakes in the west of Ireland, although documented densities were low (Minchin *et al.* 2002). Given that our DB sample was not significantly differentiated from any of the four Shannon-Erne samples, it is unlikely that DB mussels represent a new introduction into Ireland. In view of their low density it remains to be seen whether mussels at Ringsend will become established and spread to other sites in the Dublin port area, and westwards into the Grand Canal, where there are already several established breeding populations.

The lack of significant genetic differentiation between the British sample and Irish samples suggests a British origin for Irish zebra mussels. Pollux *et al.* (2003) came to the same conclusion from AFLP analysis of three Irish and two British samples, albeit based on few data. But why did it take until 1993 or 1994 for zebra mussel to reach Ireland? In 1993 value added tax was abolished on used boats purchased in the European Union, resulting in an increase in boat imports into Ireland (Pollux *et al.* 2003). The favourable exchange rate between the Irish and the British pound ensured that imported craft were predominantly from the UK. Field trials have shown that zebra mussels attached to craft that were lifted from British waters onto trailers, and transported to Ireland by ferry, survive and grow if re-immersed in Irish waters within a day (Pollux *et al.* 2003). These factors created an invasion window of opportunity that had not occurred in the previous 170 years.

Zebra mussels arrived in Britain in the early 1800s and by 1824 were well established in the River Thames, several years before they reached the Netherlands. Mussels were probably carried to both Britain and the Netherlands with imports of damp timber from Baltic ports (Minchin *et al.* 2002). This would explain the similarity between the RL and MR samples, and would also account for the genetic similarity between Irish samples (putatively introduced from Britain) and the MR sample.

It is generally believed that mussel larvae entered the Great Lakes via ballast water discharge from European shipping traffic (Hebert *et al.* 1989) but until recently there has been no information on possible founding source(s). Stepien *et al.* (2002), from an analysis of 63 randomly amplified polymorphic DNA loci, suggested that North American populations were probably founded by multiple introductions from north-western and north-central Europe, but not from south-central or eastern Europe. They found that samples from Lake Erie, Lake Ontario and Lake Huron clustered with samples from Poland and the Netherlands, but not with samples from the Dnieper or the Volga Rivers, which flow into the Black and Caspian Seas, respectively. We have already suggested that the close similarity between the UK and Netherlands samples probably reflects a Baltic origin for mussels in these countries. In turn, the lack of significant genetic differentiation (in exact tests) between the Great Lakes and the Netherlands and UK samples, in addition to the significant differentiation between the east European Prut sample and all other samples, certainly supports the hypothesis of Stepien *et al.* (2002) for a north-west European origin for North American zebra mussels.

F_{ST} values for pairwise comparisons were low (-0.01-0.054; Hartl & Clark 1997) with very few (8/45) significant values observed after sequential Bonferroni correction. In contrast, a larger number of significant values were obtained from exact tests of allelic

(16/45) and genotypic (14/45) proportions, reflecting the greater power of these tests to detect significant population structuring (Goudet *et al.* 1996; Balloux & Lugon-Moulin 2002).

In addition to the small number of significant F_{ST} values, we observed a significant negative correlation between F_{ST} and one measure of locus polymorphism, H_E (Fig. II.1.2), thus highlighting a limitation of highly polymorphic microsatellite loci to detect population differentiation. When less polymorphic loci such as allozymes are used, F_{ST} estimates are often considerably higher (1-2 fold) than for microsatellites (Lemaire *et al.* 2000; De Inocentiis *et al.* 2001; Dufresne *et al.* 2002; Olsen *et al.* 2004). The inverse relationship between F_{ST} and locus polymorphism is probably because of the potential of microsatellite loci to demonstrate size homoplasy, resulting from high rates of mutation and the assumption of a stepwise mutation process for microsatellite loci. The effects of homoplasy on reducing estimates of population differentiation are most marked when effective population sizes are large, mutation rates are high, and there are strong allele size constraints (Estoup *et al.* 2002). While it is difficult to comment on the latter without more data, it is likely that effective population sizes in zebra mussels are large because of the specie's life history. Mean mutation rates for microsatellite loci are high, in the range 5×10^{-3} to 5×10^{-5} (reviewed in Estoup & Angers 1998) compared to the rates of point mutations, which are of the order of 10^{-9} to 10^{-10} (Hancock 1999). In large populations (N_e >200) loci that mutate at the higher rate have a significant amount of homoplasious electromorphs; but at the lower rate, even for a N_e of several thousand individuals size homoplasy is greatly reduced (Estoup *et al.* 2002). Albeit based on few data numbers of alleles in zebra mussel populations are reasonably high (30-35/locus in Müller *et al.*

(2002) and 15-30/locus in the present study) similar to many marine fish. It seems likely that homoplasy probably contributed to the downward bias of F_{ST} estimates in our data.

II.2. MICRO-GEOGRAPHICAL VARIATION

II.2.1 INTRODUCTION

Since the introduction of highly polymorphic microsatellites, most population genetics studies focus on spatial patterns of allelic frequencies in natural populations. Such an approach is particularly useful in *Dreissena polymorpha* because the life cycle involves an extensive dispersal phase in environments that often lack natural boundaries. Allozyme markers have revealed little or no spatial differentiation in zebra mussel populations over large geographic distances (Marsden *et al.* 1995; Lewis *et al.* 2000), although genetic differentiation has been reported on a local scale within North American lakes (Lewis *et al.* 2000). In contrast, in European populations significant genetic structuring has been reported using both allozyme and microsatellite markers (Müller *et al.* 2001, 2002), although no evidence of genetic differentiation was reported in a recent microsatellite analysis of Irish zebra mussel populations (Astanei *et al.* 2005).

Because of technical difficulties associated with larval genetic screening, there are very few studies that have examined genetic variation between different life stages. In a study involving only one allozyme locus, Haag & Garton (1995) found significant genetic differences between planktonic larval stages and adult populations, citing selection as the main causative agent.

The aims of this study were to investigate genetic variability on a spatial, temporal and ontogenetic level in samples of mussels collected from a single location, Lough Key, Ireland. Spatial variability was investigated by sampling mussels from several sites within the lake at a single point in time, while temporal variation was examined by comparing variability in mussels collected from the same site within the lake, but two years apart. Ontogenetic variation was analysed by comparing newly settled spat and adults collected at the same time of year.

II.2.2. MATERIAL AND METHODS

II.2.2.1. Sample collection and DNA extraction

Lough Key (G830 060) is located on the Boyle River, which flows into the Shannon above Carrick-on-Shannon. The lake has a surface area of 9 km^2 and a mean depth of 5.1 m. Adult zebra mussels were collected in August 2004 from five locations within Lough Key (Figure II.2.1), Forest Park I (FP I; same location as sample collected in 2002, Chapter II.1), Forest Park II (FP II), Forest Park III (FP III), situated close together (within 100 m^2) and from Cillin (CL) on the NW shore of L. Key and Knockvicar (KV) on the NE shore. Mussels were collected from depths of 0.5 – 2.5 m by scraping vertical walls or rocky substrate. Samples were placed in 95% ethanol after collection and stored at room temperature. The phenol/chloroform method (Sambrook *et al.* 1989) was used to extract genomic DNA from whole body tissue.

A sample of newly settled juveniles (SJ) was also collected from the centre of the lake. These were sampled using 20 cm^2 PVC settlement plates. Three plates (held in place by cable ties) were suspended in the water column in July at depths of 2, 3 and 4 m, respectively. The rope was tied at the top to a navigational buoy. The plates were anchored to the substrate using a concrete weight. The plates were collected in August 2004, when the settled juveniles measured less than 1 mm in shell length. Once collected, the juveniles were kept alive in containers filled with lake water prior to DNA extraction. They were subsequently placed in individual 1.5 ml Eppendorf micro-centrifuge tubes (Eppendorf AG). Ten µl of lysis solution (7.5 mM Tris-HCl, pH 8.3; 3.75 mM NH_4Cl; 3.75 mM KCl; 1.5 mM $MgCl_2$; 2 µg proteinase K) (Sutherland *et al.* 1998) was added to each tube, which was then incubated at 37°C for 2 h. Samples were boiled for 10 min in a water bath

(Blanco) to inactivate proteinase K and were then either processed through the Polymerase Chain Reaction (PCR) procedure or stored at -20°C overnight.

Fig. II.2.1: Sampling sites in L. Key: FP I: Forest Park I; FP II: Forest Park II; FP III: Forest Park III; CL: Cillin; KV: Knockvicar; SJ: newly settled juvenile collection site.

II.2.2.2. Microsatellite analysis

Polymerase chain reaction (PCR) amplification of five trinucleotide microsatellite loci (Naish & Boulding 2001, Chapter II.1.2.3) was carried out using a reaction volume of 9 μl containing: 20 ng of template DNA, 1 ml 10X reaction buffer (Li-Cor Biosciences, UK), 1 mM $MgCl_2$, 0.2 mM each nucleotide, 0.5 pmol IRDye-labelled forward and reverse

primers (MWG Biotech), 0.5 units of *Taq* DNA polymerase (Sigma-Aldrich Co.). The thermocycling regime consisted of an initial denaturation step at 96°C for 3 min, followed by 30 cycles of denaturing at 96°C for 1 min, annealing at 58, 61, 50, 57 and 53 °C (for *DpolA6*, *DpolB6*, *DpolB8*, *DpolB9* and *DpolC5*, respectively), and extension at 72°C for 45s. PCR amplifications were performed in a Mastercycler gradient cycler (Eppendorf AG). Optimisations of PCR conditions were performed using an initial set of 10 cycles dropping from 5°C above the annealing temperature in 0.5°C increments.

Amplified products were visualized on a Li-Cor infrared DNA automatic analyser (Li-Cor Biosciences), using an internal lane standard and SAGA^{GT} software for determination of allele size. A set of reference samples was rerun on each gel to ensure reliability of allele scoring.

Statistical analysis for adults and juveniles were similar to those described in Chapter II.1.2.4.

II.2.3. RESULTS

II.2.3.1. Genetic diversity

All five loci were polymorphic in all samples, the number of alleles per locus (Table II.2.1) varying from 10 (*DpolC5*) to 19 (*DpolB6*), which is higher than the number of alleles previously recorded for L. Key (7-13 alleles/locus, see Chapter II.1.3.1). This is most likely due to different sample sizes in the two studies (30 in LK sample, compared to 171±4 in the present L. Key total sample), although the FP I sample (mean N = 33.8±1.9), collected from the same location as the L. Key sample collected two years earlier, also exhibited a higher number of alleles (10-16 alleles/locus). The highest allele frequency for any individual locus in any one population did not exceed 0.44, a value very similar to that observed in the macro-geographic survey of zebra mussels (0.51; see Chapter II.1.3.1).

Between 74% and 90% individuals were expected to be heterozygous in each sample across all loci. However, observed heterozygosities (H_O) were lower for all samples, ranging between 0.441 and 0.742 (Table II.2.1). Overall, the juvenile sample exhibited lower H_O values than any of adult samples (Table II.2.1). Expected and observed heterozygosities calculated for FP I (mean H_E = 0.859±0.013; mean H_O = 0.648±0.032) were generally higher than the estimates recorded for the L. Key sample collected two years earlier (mean H_E = 0.798±0.027; mean H_O = 0.573±0.086).

Locus/ Sample	FP I	FP II	FP III	CL	KV	SJ	Mean
DpolA6							
N	32	37	37	34	31	32	33.8 (1.1)
A	12	13	12	11	11	9	11.3 (0.6)
H_E	0.852	0.774	0.741	0.773	0.814	0.798	0.792 (0.016)
H_O	0.719	0.595	0.595	0.441	0.581	0.531	0.577 (0.037)
F_{IS}	0.171	0.245	0.21	**0.442**	**0.302**	**0.348**	
DpolB6							
N	40	39	35	35	34	36	36.5 (1.0)
A	16	17	17	15	14	14	15.5 (0.6)
H_E	0.881	0.889	0.891	0.844	0.86	0.9	0.878 (0.009)
H_O	0.725	0.641	0.714	0.686	0.588	0.556	0.652 (0.028)
F_{IS}	**0.189**	**0.291**	**0.212**	**0.201**	**0.330**	**0.393**	
DpolB8							
N	36	34	35	38	34	37	35.7 (0.7)
A	14	13	13	14	12	12	13.0 (0.4)
H_E	0.889	0.881	0.887	0.894	0.871	0.883	0.884 (0.003)
H_O	0.583	0.529	0.571	0.579	0.588	0.541	0.565 (0.010)
F_{IS}	**0.356**	**0.412**	**0.368**	**0.364**	**0.338**	**0.399**	
DpolB9							
N	31	31	34	32	32	33	32.2 (0.5)
A	11	11	11	11	10	9	10.5 (0.3)
H_E	0.859	0.851	0.857	0.845	0.85	0.842	0.851 (0.003)

H_O	0.581	0.742	0.677	0.688	0.719	0.546	0.659 (0.032)
F_{IS}	**0.339**	**0.144**	**0.225**	**0.202**	0.17	**0.366**	
DpolC5							
N	30	31	36	32	33	34	32.7 (0.9)
A	10	9	8	8	8	6	8.2 (0.5)
H_E	0.812	0.806	0.775	0.761	0.811	0.737	0.784 (0.013)
H_O	0.633	0.645	0.694	0.688	0.697	0.647	0.667 (0.012)
F_{IS}	**0.236**	**0.215**	**0.117**	0.113	0.155	**0.136**	
All loci							
N	33.8 (1.9)	34.4 (1.6)	35.4 (0.5)	34.2 (1.1)	32.8 (0.6)	34.4 (0.9)	34.2 (0.4)
A	12.6 (1.1)	12.6 (1.3)	12.2 (1.5)	11.8 (1.2)	11 (1.0)	10 (1.4)	11.7 (0.4)
H_E	0.859 (0.013)	0.840 (0.022)	0.830 (0.030)	0.823 (0.025)	0.841 (0.012)	0.832 (0.030)	0.838 (0.005)
H_O	0.648 (0.032)	0.630 (0.035)	0.650 (0.028)	0.616 (0.049)	0.635 (0.030)	0.564 (0.021)	0.624 (0.013)
F_{IS}	**0.258**	**0.261**	**0.226**	**0.264**	**0.259**	**0.328**	**0.226**

Table II.2.1: Sample size (N), number of alleles (A), expected (H_E) and observed (H_O) heterozygosity, and deviations from HWE (F_{IS}) according to Weir & Cockerham (1984). Values in bold indicate samples which deviate significantly from HWE ($P < 0.05$) after sequential Bonferroni correction.

II.2.3.2. HWE and null alleles

Significant departures from HWE, randomly distributed across loci, were observed in 24 of the 30 single locus exact tests after sequential Bonferroni correction (Table II.2.1). The juvenile sample was the only one that exhibited significant deficits of heterozygotes at all five loci.

The second method of Brookfield (1996), as implemented in MICRO-CHECKER, was used to calculate the expected frequency (r) of null alleles per locus. Frequencies ranged from 0.2 to 0.49 (Table II.2.2), with mean frequencies per locus varying from 0.30 to 0.36. No evidence of upper allele dropout and/or stuttering during PCR amplification was found. When data were corrected for the presence of null alleles, the number of significant departures from HWE dropped from 24 to 2.

Genotypic data were also tested for linkage disequilibrium, but no significant disequilibrium was detected for any locus pair in any of the populations analysed ($P > 0.1$ in every case).

The BOTTLENECK results indicated no significant excess or deficit of heterozygosity under the SMM, TPM or IAM mutation models either for uncorrected data or for data corrected for null alleles. Allele frequencies followed an L-shaped distribution i.e., no skews of allele frequencies towards intermediate values were observed in any of the populations, as expected in a non-bottlenecked population at mutation-drift equilibrium (Luikart *et al.* 1998).

Locus/ Sample	FP I	FP II	FP III	KV	CL	SJ	Mean
DpolA6	0.42	0.29	0.25	0.42	0.43	0.42	0.37 (0.032)
DpolB6	0.20	0.24	0.30	0.30	0.36	0.32	0.28 (0.023)
DpolB8	0.38	0.43	0.37	0.27	0.36	0.29	0.35 (0.024)
DpolB9	0.49	0.41	0.34	0.39	0.35	0.40	0.39 (0.023)
DpolC5	0.49	0.44	0.25	0.37	0.33	0.30	0.36 (0.036)
Mean	0.36 (0.054)	0.30 (0.040)	0.35 (0.023)	0.36 (0.028)	0.34 (0.016)	0.35 (0.026)	0.34 (0.009)

Table II.2.2: Expected null allele frequencies calculated using the second method of Brookfield (1996) as implemented in MICRO-CHECKER. Standard errors for means are given in parentheses.

II.2.3.3. Population differentiation

Multilocus F_{ST} population pairwise comparisons (Table II.2.3) revealed low levels of differentiation, with a global F_{ST} value of -0.0006 ± 0.003. None of the estimates was significant after sequential Bonferroni correction.

The lack of genetic differentiation was confirmed by Fisher's tests of genotypic differentiation, where none of the tests was significant after sequential Bonferroni correction (Table II.2.4). Tests for allelic differentiation showed significant differentiation only between CL and SJ (Table II.2.4). However, the differentiation between the two samples was no longer significant after correction for null alleles.

Sample	FP I	FP II	FP III	KV	CL	SJ
FP I	-	-0.005	0.002	-0.005	-0.003	0.002
FP II	-0.002	-	-0.007	-0.005	-0.003	-0.004
FP III	0.003	-0.007	-	-0.003	0.001	-0.003
KV	0.006	-0.005	-0.006	-	-0.003	-0.005
CL	0.004	0.003	0.0005	0.002	-	0.003
SJ	-0.001	-0.004	-0.007	-0.003	0.005	-

Table II.2.3: Pairwise F_{ST} estimates (Weir & Cockerham 1984) using data uncorrected (below diagonal) and corrected (above diagonal) for the presence of null alleles

Sample	FP I	FP II	FP III	KV	CL	SJ
FP I	-	0.216	0.112	0.009	0.020	0.026
FP II	0.701	-	0.993	0.845	0.118	0.189
FP III	0.468	0.990	-	0.850	0.139	0.116
KV	0.198	0.988	0.987	-	0.221	0.028
CL	0.228	0.532	0.535	0.579	-	**0.001**
SJ	0.242	0.722	0.530	0.295	0.029	-

Table II.2.4: Probability values for Fisher's exact test of allelic differentiation (above the diagonal) and genotypic differentiation (below the diagonal). Value in bold indicates sample significantly different after sequential Bonferroni correction.

Pairwise multilocus F_{ST} comparisons between two samples collected two years apart from the same location (LK and FP I) indicated no significant difference between them (F_{ST} = 0.006, P = 0.14). Significant differentiation was observed for Fisher's exact tests of allelic (P = 0.002), but not genotypic proportions (P = 0.026).

Summarising, there was no evidence for ontogenetic, spatial or temporal genetic differentiation between samples.

II.2.4. DISCUSSION

Higher levels of genetic variability, in terms of number of alleles and heterozygosity values, were observed in this study compared to the L. Key sample analysed two years earlier (Chapter II.1). New introductions of zebra mussels with higher genetic diversity may explain these results. New introductions can take place actively by swimming of larvae or passively by dozens of leisure boats, which arrive in L. Key every year (Lucy & Sullivan 2001). The use of an automatic DNA analyser for the 2004 samples may also be a factor in that this method is more sensitive in detecting polymorphisms than the autoradiography of the dried gels (technique which was used in 2002).

There was no evidence for significant differentiation between the two samples collected from the same site two years apart, indicating a lack of temporal genetic variation. Neither was there evidence for spatial differentiation between samples collected from different sites within L. Key. This lack of spatial differentiation on a local scale is at variance with results reported by Lewis *et al.* (2000), who found significant genetic differentiations, using allozymes, among zebra mussel populations within North American lakes, which they saw as the product of dynamic population demography rather than natural selection. However, another allozyme study (Soroka *et al.* 1997) found no significant genetic differentiation between samples of zebra mussels collected from sites within a Polish lake.

In the only published study to date, in which genetic variation was examined in larvae and adult zebra mussels, Haag & Garton (1995) observed significant differences in genotype frequencies between larvae and adults at an allozyme locus, which they attributed to selection. Using putatively neutral markers, the present study revealed no

evidence for differences between newly settled and adult zebra mussels. None of the samples analysed in the present study (including the juveniles) was in HWE. Departures from HWE in the form of heterozygote deficits have been widely reported in studies involving bivalves, and possible factors that may account for the observed departures from HWE in zebra mussel populations have been described in the Chapter II.1.4.2. However, the lack of significant heterogeneity between sites supports the hypothesis that null alleles, rather than the Wahlund effect, are responsible for the significant deficit of heterozygotes observed at all loci. Furthermore, correcting the data for null alleles reduced the number of significant departures from HWE from 24 to only 2, strongly supporting this hypothesis.

CHAPTER III

ALLOZYME VARIATION

III.1. INTRODUCTION

Electrophoresis is the separation of macromolecules in an aqueous solution when exposed to an electric field. In protein electrophoresis, different polypeptides are separated on the basis of their structure. If crude protein extracts from an organism are placed in a starch, cellulose acetate or polyacrylamide gel matrix, the proteins will be separated based on the strength of attraction to the opposite polarity and the ease at which the molecule can move through the gel matrix. Specific stains can be used to visualize the enzymes on a gel and the banding pattern produced for an individual can be used to infer the genotype at a locus. The method has offered a cheap and quick method for the analysis of single locus variation in natural and artificial populations and has proved to be an important tool in studies involving the adaptive significance of protein polymorphisms, taxonomy and systematics and analysis of population structure.

The basis of electrophoretic analysis of proteins was laid down in 1957, when isozymes were discovered (McMillin 1983). In 1959 isozymes were defined as the different molecular forms in which protein may exist with the same enzymatic specificity (Buth 1984). This means that different variants of the same enzyme have identical or similar functions and are present in the same individual. Nevertheless, isozymes played a minor role in genetics studies, until Lewontin & Hubby (1966) and Harris (1966) revealed their considerable polymorphism, following studies on *Drosophila pseudobscura* and humans. The term "allozyme" was introduced to describe different electrophoretic forms of an enzyme, which are the products of alternative alleles segregating at a single locus. Not to be confused with isozymes, which are alternative forms of an enzyme produced by different loci. Using allozyme analysis in population surveys it was then possible to make precise quantitative estimates of genetic variability based upon one parameter of the

molecular structure of the primary products of the genes themselves. Subsequently, numerous studies carried out on a range of organisms revealed a high degree of allozyme polymorphism present in most animals and plant species (reviewed in Ferguson 1980).

Most studies on the population genetic structure of zebra mussels have been based on allozyme data. Results of these studies have shown high levels of genetic variability, both in European and North American populations (see Chapter I.7). Although Boileau & Hebert (1993) and Elderkin *et al.* (2001) reported significant genetic heterogeneity between North American populations, Marsden *et al.* (1995) and Lewis *et al.* (2000) failed to find significant population differentiation on a large scale in North America. However, Lewis *et al.* (2000) found significant genetic heterogeneity on a local scale within lakes. In Europe, two major clusters corresponding to the Danube and Northern Europe (from the Netherlands to the Volga River) have been revealed using allozymes (Marsden *et al.* 1995; Müller *et al.* 2001). Construction of the Main-Danube canal in 1992, linking the Rhine with the Danube, provided the opportunity for the two major phylogroups to mix extensively. Müller *et al.* (2001, 2002) have provided supporting evidence, from allozyme and microsatellite markers, that such an exchange is indeed occurring.

To date, there have been no studies on the population genetic structure of zebra mussels in the British Isles using allozyme markers. Therefore, this study provided the opportunity of analyzing genetic variability at eight such loci in recently-established populations of the zebra mussel in Ireland. Another goal of the present study was to compare estimates of differentiation from allozymes with those obtained for the same populations using microsatellite markers. The selective neutrality of allozymes has often been questioned (Hilbish and Koehn 1985; DiMichele *et al.* 1991; Karl & Avise 1992; Pogson *et al.* 1995; Latta & Mitton 1997; Lemaire *et al.* 2000; De Innocentiis *et al.* 2001;

Dufresne *et al.* 2002; Riginos *et al.* 2002), and balancing or directional selection may affect estimates of population differentiation. A direct comparison of population differentiation obtained with the two classes of molecular markers could be a way of assessing the respective roles of selection and gene flow in the genetic structuring of zebra mussel populations in Ireland.

III.2. MATERIAL AND METHODS

III.2.1. Sampling and electrophoresis

Zebra mussels were collected between February and April 2002 from five sites (Fig. III.1): Limerick Docks (LM, R 575 573), Lough Derg (LD, R 813 860), Lough Key (LK, G 849 041), Assaroe, on Lower Lough Erne (AS, G 891 609) and Ringsend, Dublin (DB, O 174 338). Mussels were taken from depths of 0.5 - 2.5 m from the sides of permanent or floating docks. Apart from the DB sample, where low densities did not allow collection from just a single patch, the remainders of the samples were collected from a single point using a net bag attached to a scraping device on a long pole.

Samples were frozen at -80°C prior to electrophoresis. Defrosted, whole body tissue was homogenized in tissue extracting buffer (6.05 gl^{-1} Tris, 1.23 gl^{-1} $MgSO_4$), adjusted to pH 7.8 with 1 N HCl. Samples were run on 13 % starch gels using two different buffer systems: Tris Citrate pH 6 (TC 6; Gel buffer: 0.008 M Tris, 3.0 mM Citric acid, 3.0 mM NaOH; Electrode Buffer: 0.22 M Tris, 0.1 M Citric acid, 0.05 M NaOH) and Tris-EDTA-Maleic pH 7.4 (TEM 7.4, 0.1 M Tris, 0.1 M Maleic acid, 0.01 M Na_2EDTA, 0.02 M $MgCl_2$, 0.12 M NaOH) for eight polymorphic loci: malate dehydrogenase, EC 1.1.1.37 (*MDH*; TC 6); isocitrate dehydrogenase, EC 1.1.1.42 (*IDH-2*; TC 6); D-octopine dehydrogenase, EC 1.5.1.11 (*OPDH*; TC 6); aconitate hydratase, EC 4.2.1.3 (*ACO-1* and *ACO-2*; TC 6); phosphoglucomutase, EC 5.4.2.2 (*PGM*; TEM 7.4); glucose-6-phosphate isomerase, EC 5.3.1.9 (*GPI*; TC 6); esterase-D, EC 3.1.1 (*EST-D*; TEM 7.4). Loci are numbered in order of increasing anodal mobility. Gel buffers, stain recipes and electrophoretic procedure are according to Murphy *et al.* (1996). Alleles were designated according to mobility and direction from the most common (100) allele.

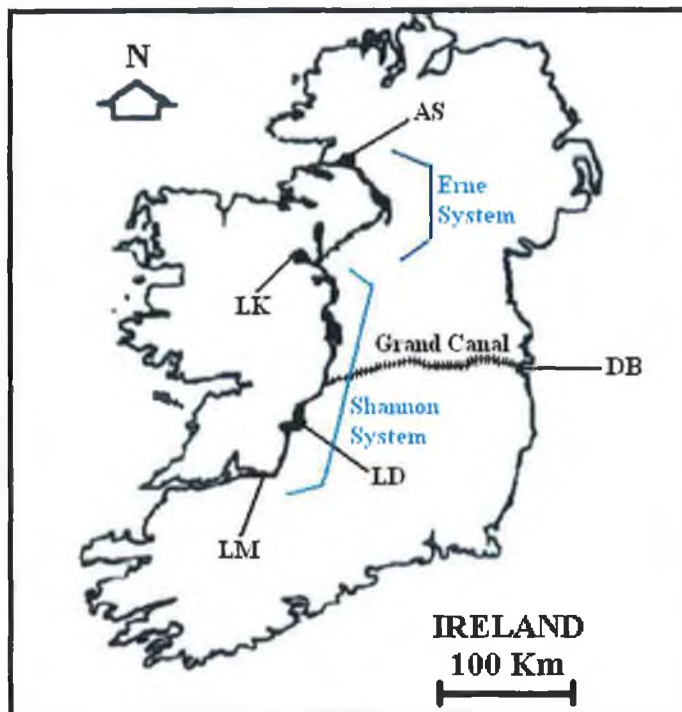

Fig. III.1: Location of the sampling sites. LM: Limerick, LD: Lough Derg, LK: Lough Key, AS: Assaroe, DB: Dublin.

III.2.2. Data analysis

Allelic distribution for each locus, expected (H_E) and observed (H_O) heterozygosity estimates for each population were obtained using the GENETIX v. 4.01 computer program (Belkhir *et al.* 2000). Conformance to Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP v 3.1 program (updated version of Raymond & Rousset 1995), with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. The same program was used to test for linkage disequilibrium between locus pairs. Exact tests of allelic and genotypic distributions between pairs of populations were conducted using

GENEPOP. Also, the level of genetic differentiation among populations was quantified using F_{ST} (Weir & Cockerham 1984), the significance of which was determined using permutation tests as implemented in the FSTAT program (Goudet 2001). Significance of multiple pairwise comparisons was determined using the sequential Bonferroni method (Rice 1989).

Isolation by distance, the correlation between genetic and geographical distance, was tested by regressing $F_{ST}/1 - F_{ST}$ on the natural logarithm of geographical distance (Rousset 1997), using the Mantel test (Mantel 1967) with 1000 permutations, as implemented in GENEPOP.

Genetic relationships among populations were examined by constructing trees of relationships using the neighbour-joining method of clustering implemented in the PHYLIP program (Felsenstein 1989). Tree topology was determined using the standard distance of Nei (1978). Bootstrap values were computed by resampling loci over 1000 replications.

To test for selection we used a modified form of the Lewontin & Krakauer (1973) test to compare mean F_{ST} estimates from two classes of markers (allozymes and microsatellites, Pogson *et al.* 1995), calculating:

$$\chi^2_{(n-1)} = (n-1)(F_{ST}(\text{microsatellites}) / F_{ST}(\text{allozymes})$$

where n = number of populations and the F_{ST} for each marker class are the weighed means.

To test which allozyme loci may be affected by selection we treated the ratio of the F_{ST} for each allozyme locus to the mean F_{ST} for microsatellites as a variance ratio F-test and performed a two-tailed test of significance (Barker *et al.* 1997).

III.3. RESULTS

III.3.1. Genetic diversity

Loci were considered as polymorphic when the frequency of the most common allele was $\leq$0.95. Numbers of alleles per locus varied from 2 (*ACO-1*, *ACO-2*, *MDH* and *PGM*) to 5 (*EST-D*), with an average of 2.7 alleles per locus. *GPI* and *EST-D* were the most polymorphic loci, with 4 and 5 alleles respectively, and no population exhibited less than 4 alleles (Table III.1). The frequency of the most common allele ranged from 0.21-1.00 across all populations.

Mean observed heterozygosity (H_O) values across loci ranged from 0.275 to 0.413, while expected mean heterozygosity (H_E) values were between 0.376 and 0.440.

Out of 40 tests for Hardy-Weinberg equilibrium, for each locus and population, 28 showed deficits of heterozygotes (positive F_{IS} values) but only 7 were significant after sequential Bonferroni correction, 4 of these at the *EST-D* locus (Table III.1). Although 11 negative F_{IS} values were observed, none of these were significant after sequential Bonferroni correction. In one sample (LK) no heterozygotes were observed at the *ACO-2* locus, most likely due to small sample size (N = 8). Permutation tests on F_{IS} values across loci revealed significant heterozygote deficits in 4 (LM, LD, LK, AS) of the 5 populations (Table III.1), largely due to the significant F_{IS} estimates for *EST-D*. Although a significant disequilibrium was initially detected between *OPDH* and *GPI* (P = 0.04), this was no longer significant after sequential Bonferroni correction.

Locus/ Population	LK	LM	LD	AS	DB	All populations
ACO-1						
Allele						
95	0.438	0.214	0.573	0.740	0.568	
100	0.563	0.786	0.427	0.260	0.432	
N	8	49	48	48	22	35 (8.5)
H_E	0.492	0.337	0.489	0.385	0.491	0.439 (0.033)
H_O	0.625	0.184	0.479	0.271	0.318	0.375 (0.079)
F_{IS}	-0.207	**0.463**	0.031	0.306	0.372	**0.235**
F_{ST} over all populations						0.190
ACO-2						
Allele						
96	0.750	0.704	0.344	0.277	0.205	
100	0.250	0.296	0.656	0.723	0.796	
N	8	49	48	47	22	34.8 (8.4)
H_E	0.375	0.417	0.451	0.400	0.326	0.394 (0.021)
H_O	0.000	0.306	0.563	0.340	0.318	0.305 (0.090)
F_{IS}	1.000	0.275	-0.237	0.16	0.045	0.099
F_{ST} over all populations						0.196
EST-D						
Allele						
100	0.338	0.363	0.271	0.260	0.525	
104	0.225	0.137	0.135	0.125	0.025	
112	0.070	0.073	0.083	0.063	0.000	
114	0.183	0.194	0.323	0.271	0.225	
120	0.183	0.234	0.188	0.281	0.225	
N	71	62	48	48	20	49.8 (8.6)
H_E	0.763	0.752	0.762	0.76	0.623	0.732 (0.027)
H_O	0.254	0.532	0.438	0.458	0.800	0.496 (0.088)

F_{IS}	**0.672**	**0.300**	**0.434**	**0.406**	-0.261	**0.418**
F_{ST} over all populations						0.007
GPI						
Allele						
100	0.437	0.367	0.417	0.333	0.318	
103	0.143	0.141	0.125	0.188	0.114	
108	0.333	0.414	0.344	0.396	0.296	
112	0.087	0.078	0.115	0.083	0.273	
N	63	64	48	48	22	49 (7.6)
H_E	0.670	0.668	0.680	0.690	0.724	0.686 (0.010)
H_O	0.556	0.547	0.667	0.542	0.546	0.571 (0.024)
F_{IS}	0.179	0.189	0.029	0.225	0.269	0.170
F_{ST} over all populations						0.0006
IDH-2						
Allele						
100	0.571	0.214	0.740	0.615	0.750	
106	0.429	0.778	0.260	0.385	0.250	
108	0.000	0.008	0.000	0.000	0.000	
N	42	63	48	48	22	44.6 (6.6)
H_E	0.490	0.349	0.385	0.474	0.375	0.415 (0.028)
H_O	0.381	0.286	0.312	0.354	0.500	0.367 (0.037)
F_{IS}	0.234	0.189	0.199	0.262	-0.313	0.174
F_{ST} over all populations						0.202
MDH						
Allele						
85	0.104	0.273	0.375	0.688	0.705	
100	0.896	0.727	0.625	0.313	0.296	
N	48	64	48	48	22	46 (6.8)
H_E	0.187	0.397	0.469	0.43	0.416	0.380 (0.050)

H_O	0.208	0.328	0.292	0.375	0.500	0.341 (0.048)
F_{IS}	-0.106	0.182	**0.387**	0.138	-0.179	0.157
F_{ST} over all populations						0.239
OPDH						
Allele						
97	0.031	0.008	0.042	0.094	0.227	
100	0.953	0.984	0.948	0.906	0.773	
104	0.016	0.008	0.010	0.000	0.000	
N	64	64	48	48	22	49.2 (7.7)
H_E	0.090	0.031	0.100	0.170	0.351	0.148 (0.055)
H_O	0.063	0.031	0.104	0.104	0.182	0.097 (0.025)
F_{IS}	**0.315**	-0.004	-0.035	0.396	0.500	**0.306**
F_{ST} over all populations						0.059
PGM						
Allele						
87	0.074	0.031	0.104	0.000	0.068	
100	0.926	0.969	0.896	1.000	0.932	
N	61	64	48	48	22	48.6 (7.4)
H_E	0.137	0.061	0.187	0.000	0.127	0.102 (0.032)
H_O	0.115	0.063	0.208	0.000	0.136	0.104 (0.035)
F_{IS}	0.168	-0.024	-0.106	0.000	-0.050	0.009
F_{ST} over all populations						0.023
All loci						
N	45.6 (8.8)	59.9 (2.4)	48 (0.0)	47.9 (1.2)	21.8 (0.2)	44.6 (6.2)
Mean no. of alleles	2.8 (0.4)	2.9 (0.4)	2.8 (0.4)	2.5 (0.4)	2.5 (0.3)	2.7 (0.1)
H_E	0.401 (0.088)	0.376 (0.089)	0.44 (0.078)	0.414 (0.087)	0.429 (065)	0.412 (0.011)

H_O	0.275 (080)	0.285 (0.067)	0.383 (0.066)	0.306 (0.063)	0.413 (0.076)	0.332 (0.028)
F_{IS}	**0.282**	**0.196**	**0.088**	**0.237**	0.048	**0.226**
F_{ST}						0.115

Table III.1: Allele frequencies, sample size (N), expected (H_E) and observed (H_O) heterozygosity, deviations from HWE (F_{IS}) according to Weir & Cockerham (1984) and F_{ST} estimates over five populations. Values in bold indicate samples which deviated significantly from HWE after sequential Bonferroni corrections. Standard errors for means are given in parentheses.

III.3.2. Population differentiation

F_{ST} estimates varied substantially across loci with an overall multilocus F_{ST} estimate across the five populations of 0.115 ± 0.045. Four loci, *ACO-1*, *ACO-2*, *IDH-2* and *MDH* had high F_{ST} values of 0.190, 0.196, 0.202 and 0.239, respectively (Table III.1). Multilocus estimates of F_{ST} for population pairs (Table III.2) indicated highly significant genetic differentiation in 8 of the 10 pairwise comparisons. Not surprisingly, Fisher's exact tests of allelic and genotypic distributions also revealed highly significant differentiation for all pairwise comparisons (Table III.3). When the four loci with the highest across-population F_{ST} values were excluded from the analysis no significant differentiation was observed for either F_{ST} pairwise comparisons, or for pairwise comparisons of allelic or genotypic proportions, with the exception of the DB-LK and DB-AS (allelic), and DB–AS (genotypic) comparisons. Regression of pairwise $F_{ST}/(1-F_{ST})$ values against ln geographical distance indicated no significant correlation (P = 0.80).

Regression remained nonsignificant when *ACO-1*, *ACO-2*, *IDH-2* and *MDH* loci were excluded from the analysis (P = 0.30).

Sample	LK	LM	LD	AS	DB
LK	-	0.048***	0.065***	0.160***	0.177***
LM		-	0.139***	0.195***	0.217***
LD			-	0.036***	0.043
AS				-	0.022

Table III.2: Pairwise F_{ST} values (Weir & Cockerham, 1984). $***P < 0.001$, from permutation tests in FSTAT program, after sequential Bonferroni correction.

Sample	LK	LM	LD	AS	DB
LK	-	<0.001	<0.001	<0.001	<0.001
LM	<0.001	-	<0.001	<0.001	<0.001
LD	<0.001	<0.001	-	<0.001	0.002
AS	<0.001	<0.001	<0.001	-	0.008
DB	<0.001	<0.001	<0.001	0.001	-

Table III.3: Probability values for Fisher's exact test of allelic (below the diagonal) and genotypic (above the diagonal) differentiation after sequential Bonferroni correction.

The dendrogram of relationships constructed using standard distance of Nei (1978) based on all eight loci (Fig. III.2.A) showed no geographic pattern to the clustering, i.e., sites that were geographically closer, e.g., LM and LD, or AS and LK, clustered separately

from one another. However, when the four loci with the highest across-population F_{ST} values were excluded from the analysis, the pattern of clustering changed and sites situated geographically close (AS and LK) clustered together (Fig. III.2.B). Nevertheless, other geographically close sites (LD and LM) still clustered separatelly.

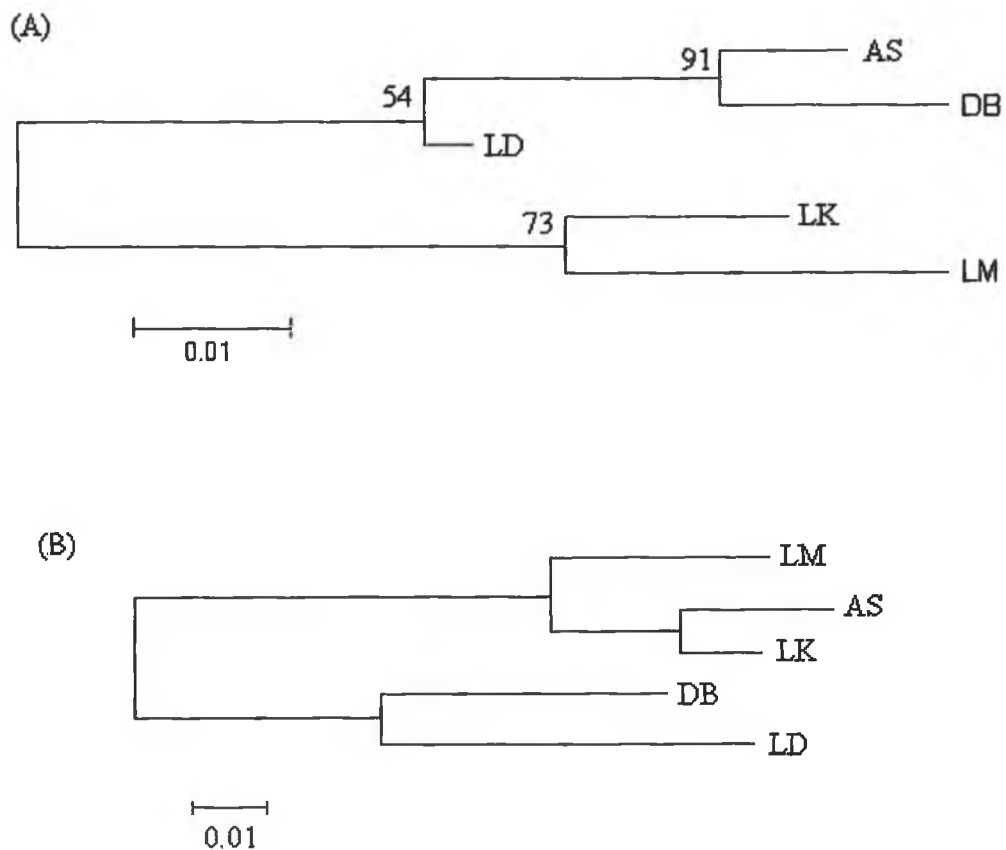

Fig. III.2: Dendrograms of relationships using the standard distance of Nei (1978), based on: (A) all loci; and (B) *EST-D, GPI, OPDH* and *PGM* loci. Bootstrap values have been computed by resampling loci and are given as percentages over 1000 replications.

III.4. DISCUSSION

III.4.1. Genetic variability

Irish samples of zebra mussels show somewhat reduced levels of genetic variability (H_O: 0.28-0.41) compared to those reported for European (0.27-0.49) and North American (0.30-50) populations (references in Chapter I.7). However, when the mean number of alleles for Irish populations (2.7 ± 0.1) was compared with the mean, using the same loci, for European populations there was no significant difference (Marsden *et al.* 1995: 2.7), although the difference was significant (Mann-Whitney U test $P < 0.05$) when North American and Irish samples were compared (Marsden *et al.* 1995: 5.3).

There was an overall deficiency of heterozygotes at all sites but these deficits were only significant in 7 out of 40 cases (Table III.1). Heterozygote deficits are a common occurrence in marine bivalves (Zouros & Foltz 1984; Zouros *et al.* 1988) and have frequently been reported in *Dreissena polymorpha* (Boileau & Hebert 1993; Marsden *et al.* 1995; Soroka *et al.* 1997; Lewis *et al.* 2000; Elderkin *et al.* 2001). Some studies, however, have reported no departures from HWE (Hebert *et al.* 1989; Garton & Haag 1991; Müller *et al.* 2001). Several factors, such as inbreeding, the Wahlund effect, selection or the presence of null alleles could account for the observed heterozygote deficits. There are two factors that argue against inbreeding as the causative agent for the observed deficits. First, the life cycle of zebra mussels, with high fecundity, external fertilization and an extended larval dispersal phase, makes mating of relatives unlikely. Second, inbreeding should affect all loci equally, generating uniform heterozygote deficiencies across loci. However, the data show that deficits (positive F_{IS} values in Table

III.1) were heterogeneous among loci; four of the 7 significant F_{IS} values concerned the *EST-D* locus.

The Wahlund effect is observed when a sample comprises a mixture of individuals from two or more populations that differ in allele frequencies at a locus; it is manifested as an overall deficit of heterozygotes compared to the number predicted by the HWE. Apart for the Irish (DB) sample, where low densities of individuals did not allow collection from just a single patch, the remainder of the samples consisted of individuals collected within a few square meters. Nevertheless, it is possible that several cohorts of larvae that were spawned at different locations could have settled at the same sampling location. However, results from a microsatellites study on five samples of adults and spat collected within Lough Key (Chapter II.2) failed to find significant differentiation between any of the subpopulations, strongly arguing against the presence of a Wahlund effect.

Null alleles, those with reduced or absent expression of allozymes, have been reported as possible causes for departures from HWE (Gardner 1992; Gaffney 1994). Lewis *et al.* (2000) considered null alleles as a likely factor causing significant heterozygote deficiencies at some loci in North American populations of zebra mussels. However, high mutation rates or strong selection in favor of null heterozygotes is needed to maintain null alleles at even low frequencies in a population (Zouros *et al.* 1988). Considering the low number of individuals that failed to stain and the fact that no two-banded patterns (if the null allele is able to dimerize) were observed for the dimeric enzyme *EST-D*, for which the highest number of significant F_{IS} values was recorded, it is unlikely that null alleles are primarily responsible for heterozygote deficiencies.

Selection is the factor most widely cited as the cause of heterozygote deficits in marine bivalves. Zouros and Foltz (1984) proposed a model whereby selection operates

against heterozygotes at the pre-settlement stage (due to higher food demand associated with higher growth rate) but favours heterozygotes at the adult stage. Since only adult mussels were analysed in this study, and because deficits were unevenly spread across loci and populations, it is unlikely that selection is responsible for the deficits.

It is difficult to establish whether one, or combinations of these four factors are responsible for heterozygote deficits in Irish zebra mussel populations.

III.4.2. Population differentiation

Significant differentiation was observed between Irish populations, with pairwise multilocus F_{ST} estimates significant in 8 out of 10 comparisons (Table III.2) and allelic and genotypic distributions significantly different for all pairwise comparisons (Table III.3). Other workers have also reported significant differentiation between zebra mussel populations. For example, in mainland Europe results, although based on just three allozyme loci, revealed two genetically distinct clusters, the Main and the Danube, with mixing occurring across the newly constructed Main-Danube canal (Müller *et al.* 2001). Significant genetic heterogeneity has also been observed between North American populations (Boileau & Hebert 1993; Elderkin *et al.* 2001), although for Great Lakes populations Marsden *et al.* (1995) and Lewis *et al.* (2000) failed to find significant population differentiation between lakes or lake complexes.

The Shannon-Erne system is Europe's longest navigable leisure waterway (~750 km) with many different craft using the system (Sullivan *et al.* 2002). In addition to boat traffic moving up and down the system, angling boats are regularly transported overland between the different water bodies (Minchin & Moriarty 2002). Therefore, dispersal of zebra mussels in the Shannon-Erne system is through a combination of downstream

movement of larvae, movement of fouled craft traveling both upstream and downstream within the system, and through jump dispersal by overland movement of fouled craft. It is surprising, therefore, that samples in the Shannon-Erne system are genetically differentiated. Also, there was no relationship between amount of differentiation and geographic distance; mussels from geographically adjacent sites e.g. LM and LD, or LK and AS were more genetically different from each other than samples that were further apart (Fig. III.2).

These results contrast sharply with results from five microsatellite loci on the same samples (Chapter II.1). The overall multilocus F_{ST} estimate across the five populations was 0.015±0.007 for microsatellites with pairwise F_{ST} values between -0.01 and 0.01, while the equivalent F_{ST} estimates for allozymes were 0.115±0.045, and 0.02-0.22. Not all allozyme loci contributed equally to genetic differentiation. Four loci *ACO-1*, *ACO-2*, *IDH-2* and *MDH*, had F_{ST} values across populations of between 0.190 and 0.239 while estimates for the other four loci were between 0.001 and 0.06 (Table III.1).

Microsatellite loci evolve considerably faster than allozyme loci and have mutation rates of 5×10^{-3} - 5×10^{-5} (reviewed in Estoup & Angers 1998) compared to mutation rates of 10^{-6}-10^{-9} for allozymes (Ayala 1976). The higher mutation rate of microsatellite loci is reflected in their higher polymorphism, and their greater potential to detect population differentiation than less variable markers. This has indeed been shown for several studies on aquatic species (Bentzen *et al.* 1996; Seeb *et al.* 1998; Estoup *et al.* 1998; Shaw *et al.* 1999; Ruzzante *et al.* 1999; Wirth & Bernatchez 2001) in which microsatellites revealed a finer resolution of genetic structuring than allozymes. However, other studies on aquatic species have shown the opposite: greater geographic differentiation with allozymes than

with microsatellites (Lemaire *et al.* 2000; De Innocentiis *et al.* 2001; Dufresne *et al.* 2002).

Several factors may lead to differences in F_{ST} estimates between marker types. When a marker displays a F_{ST} value significantly greater than another marker, this could be due to balancing selection acting on the marker showing significantly lower differentiation, or to differentiating selection acting on the marker exhibiting significantly higher differentiation (Lemaire *et al.* 2000). Microsatellites are, however, believed to behave neutrally, unless they are linked to loci under selection. In the Irish samples tests for linkage disequilibrium between microsatellite loci revealed no such disequilibria, and it is highly unlikely that each of the five microsatellite loci was linked with a locus (or loci) that was itself under selection (Astanei *et al.* 2005).

Another explanation is homoplasy generated by allele size constraints and high mutation rates at microsatellite loci, leading to an underestimate of genetic differentiation (O'Reilly *et al.* 2004). The effects of homoplasy on reducing estimates of population differentiation are most marked when effective population sizes are large, mutation rates are high, and there are strong allele size constraints (Estoup *et al.* 2002). While it is difficult to comment on the latter without more data, it is likely that effective population sizes in zebra mussels are large because of the species life history. Mean mutation rates for microsatellite loci are high and in large populations (N_e >200) loci that mutate at the higher rate have a significant amount of homoplasious electromorphs; but at the lower rate, even for a N_e of several thousand individuals size homoplasy is greatly reduced (Estoup *et al.* 2002). Another factor tending to lower microsatellite F_{ST} estimates is the dependence of this statistic on mutation rates when mutation (μ) is high relative to migration (m) (at equilibrium $F_{ST} \approx 1 / 1+4N(m + \mu)$). This is unlikely to apply to Irish

zebra mussel populations which have colonized Irish waterways in a short period of time, greatly assisted through larval dispersal and boat traffic (see above). In fact, the low F_{ST} estimates for microsatellites are probably a reflection of considerable gene flow between populations. All things considered it is probably reasonable to assume that microsatellite markers can be used as a neutral baseline with which to compare variation at allozyme loci. If allozyme loci exhibit significantly more geographic variation than microsatellites for the same populations, then this is evidence for the effects of natural selection (McDonald 1994).

The modified form of the Lewontin & Krakauer (1973) test comparing mean F_{ST} estimates from microsatellite and allozyme markers (Pogson *et al.* 1995) revealed significant difference between the two means ($\chi^2_{(4)} = 31.01$, $P < 0.001$). Barker *et al.* (1997) suggested that the above tests should also be applied for the variances of F_{ST} from the two classes of markers, as the mean F_{ST} estimates may not differ, while variances do, or vice versa. Results of modified Lewontin & Krakauer tests using variances of F_{ST} estimates once again revealed highly significant differentiation ($\chi^2_{(4)} = 176.15$, $P < 0.001$). These findings clearly indicate that selection may be acting on allozyme loci (assuming the microsatellite loci are neutral).

Significant variance ratios were obtained for loci *ACO-1, ACO-2, IDH-2* and *MDH* when treating the ratio of the F_{ST} for each allozyme locus to the mean F_{ST} for microsatellites as a variance ratio F-test and performing a two-tailed test of significance (Barker *et al.* 1997).

The question now is whether selection is acting on the mentioned allozyme loci, or on loci in linkage disequilibrium with them. Lemaire *et al.* (2000) have argued that the latter would imply high linkage disequilibrium, not just with the loci under selection, but also

between the individual marker loci. Also, maintaining such disequilibrium would be difficult in a species with high effective population size unless the selected loci were tightly linked to the marker loci. Since there was no evidence of linkage disequilibrium between any locus pair, it seems reasonable to suggest that directional selection is probably acting on the allozyme loci themselves. An increasing number of papers report evidence for directional selection on allozyme loci (Hilbish & Koehn 1985; DiMichele *et al.* 1991; Lemaire *et al.* 2000; De Innocentiis *et al.* 2001; Dufresne *et al.* 2002; Riginos *et al.* 2002). This is not unexpected since many of them function in metabolic pathways. Is it noteworthy that three enzymes, Aconitase, Malate dehydrogenase and Isocitrate dehydrogenase, putatively subject to selection in Irish populations, function in the high-energy yielding tricarboxylic (Krebs) cycle, while three (Glucosephosphoisomerase, Phosphoglucomutase and D-octopine dehydrogenase) of the other four are part of the anaerobic, low-energy yielding glycolytic cycle.

Locus	Variance ratio
ACO-1	12.87*
ACO-2	13.25*
EST-D	0.47
GPI	0.04
IDH-2	13.65*
MDH	16.17**
OPDH	4.01
PGM	1.55

Table III.4: Variance ratios from Barker *et al.* (1997) test of selection for individual allozyme loci.

$P^* < 0.05$; $^{**}P < 0.01$.

CHAPTER IV

HETEROZYGOSITY-FITNESS CORRELATION

IV.1. INTRODUCTION

The association between fitness-related traits and mean heterozygosity, as measured by electrophoresis across a number of polymorphic enzyme-coding loci, has been under study for almost three decades. Allozyme-associated heterosis has been reported in a wide variety of organisms (see Desharnais *et al.* 1985; Skibinski & Roderick 1989 and Zouros & Pogson 1994; see David 1998 for review).

The relationship between multiple-locus heterozygosity (MLH) and fitness traits has been particularly well documented in mollusc species (Mitton & Grant 1984; Zouros *et al.* 1988; Bush & Smouse 1992; Pogson & Zouros 1994). Heterozygosity was found to correlate positively to viability in populations of *Crassostrea virginica* (Singh 1982; Zouros *et al.* 1983) and *Mytilus edulis* (Diehl & Koehn 1985). Similarly, heterozygosity was linked to adult fecundity in *Mytilus edulis* (Rodhouse *et al.* 1986). Positive correlations between heterozygosity and growth rates have been recorded in populations of *Crassostrea virginica* (Singh & Zouros 1978; Zouros *et al.* 1980, 1983; Fujio 1982), *Mytilus edulis* (Koehn & Gaffney 1984; Diehl & Koehn 1985; Diehl *et al.* 1986; Gaffney 1990), *Mulinia lateralis* (Garton *et al.* 1984; Koehn *et al.* 1988; Gaffney *et al.* 1990), *Ostrea edulis* (Alvarez *et al.* 1989) and *Placopecten magellanicus* (Pogson & Zouros 1994). Heterozygous individuals of the same age were on average larger than the homozygotes.

However, the genetic and metabolic interrelations that determine those benefits are not yet fully understood. Experiments have been carried out to investigate the physiological processes that may help to explain positive relationships between heterozygosity and growth. Koehn & Shumway (1982) suggested that the physiological

mechanism by which heterozygotes achieve higher growth rates could be a lowered cost of standard or basal metabolism. They demonstrated that weight-specific basal oxygen uptake declined significantly with increasing heterozygosity in starved oysters, *Crassostrea virginica*, increasing the net energy available for growth and reproduction. Using starved oysters, Rodhouse & Gaffney (1984) further demonstrated that the increased metabolic efficiency associated with increasing heterozygosity was reflected in reduced rates of dry weight loss. Negative correlations between multiple locus heterozygosity and standard metabolic rate have also been recorded in *Thais haemastoma* (Garton 1984), *Mytilus edulis* (Diehl *et al.* 1986) and *Mulinia lateralis* (Garton *et al.* 1984). In experiments with *Mytilus edulis*, Hawkins *et al.* (1986, 1989) demonstrated that less heterozygous individuals had high intensities of protein turnover (synthesis and breakdown) with associated high-energy requirements, which ultimately reduced the energy available for growth and reproduction. Subsequently, Hawkins (1991) and Bayne & Hawkins (1996) found that protein turnover accounted for a major proportion of maintenance metabolic energy loses and that the energy saved by heterozygotes resulted in increased ingestion and absorption efficiency.

Several studies have failed to find a positive correlation between MLH and a fitness trait. Most of these studies involve offspring from restricted matings in hatcheries (Beaumont *et al.* 1983; Adamkewich *et al.* 1984; Gaffney & Scott 1984; Mallet *et al.* 1986; Beaumont 1991; Saavedra & Guerra 1996), although some also involve outbred populations (Foltz & Zouros 1984; Beaumont *et al.* 1985; Diehl & Koehn 1985; Gosling 1989; Fevolden 1992). One explanation for the failure to observe the relationship in hatchery samples is the limited genetic background of animals resulting from hatchery spawnings (Gaffney & Scott 1984; Mallet *et al.* 1986). Mitton & Grant (1984) suggested

that the lack of a positive correlation in hatchery crosses could be the result of the supposedly unstressed and uncompetitive conditions of the hatchery. Such an environment is unlikely to highlight any advantages heterozygous individuals may have over homozygotes. Green *et al.* (1983), in their study on *Macoma balthica* provide support for this suggestion, reporting a significant positive correlation between heterozygosity and growth rate for midshore individuals, but not for low shore clams, which presumably experience more homogenous environmental conditions. Lack of a significant association between allozyme heterozygosity and phenotypic traits has also been reported in populations of snails (Booth *et al.* 1990; Elliott & Pierce 1992), the fruit fly, *Drosophila melanogaster* (Houle 1989), beetles (Whitlock 1993), and pines (Savolainen & Hedrick 1995).

Despite these exceptions there is a general consensus that fitness parameters are correlated with multiple locus heterozygosity (Mitton & Grant 1984; Zouros & Foltz 1987; Mitton 1997; David 1998). Britten (1996), using statistical tools called meta-analyses to combine results from several studies, concluded that although fitness is not uniformly dependent on allozyme variability, the association between heterozygosity and fitness traits is on the whole significant and positive.

Several hypotheses have been proposed to explain heterozygosity-fitness traits correlations but only the two main ones will be described. For a detailed treatment of the topic see Zouros & Mallet (1989), Gaffney *et al.* (1990), Koehn (1990), (Zouros 1990), Beaumont (1991), Gosling (1992) and Zouros & Pogson (1994).

The first, the direct involvement hypothesis, suggests that the scored loci themselves are directly responsible for the correlation either by true overdominance (heterozygotes are superior to homozygotes at each locus) or by multiple-locus dominance (heterozygotes

at many scored loci are on average superior to homozygotes). Support for this hypothesis was provided by Koehn *et al.* (1988), who found that of 15 loci surveyed in the coot clam, *Mulinia lateralis*, only those loci coding for enzymes involved in glycolysis or protein catabolism had large and significant effects on growth rate. However, there have been few subsequent papers supporting the direct involvement hypothesis (see Gosling, 1992).

The second, the inbreeding or associative overdominance hypothesis is based on the assumption that electrophoretic homozygosity is an index of homozygosity for recessive alleles with deleterious effects on the fitness-related trait in question. Individuals scored as multiple homozygotes have a lower fitness compared to multiple-locus heterozygotes because of homozygosity for deleterious genes at loci in linkage disequilibrium (Zouros & Mallet 1989). This effect will be more evident in highly homozygous inbred populations, but may also be observed in outbred populations, when multiple-locus homozygotes are sampled. There is a lot of support for this hypothesis (Zouros 1987, 1993; Zouros & Mallet 1989; Gaffney 1990; Gaffney *et al.* 1990). In addition, the associative overdominance hypothesis is supported by the observation that when a positive association between heterozygosity and growth rate is reported, there is no consistency in the relative effects of particular loci across populations. However, despite greater support for the associative overdominance hypothesis, there is still no consensus on the genetic mechanism underlying fitness trait-heterozygosity correlations (Britten 1996; David 1998). Furthermore, Pogson & Zouros (1994) provided evidence against this hypothesis by reporting differences between the effects of allozyme heterozygosity (which correlated significantly with growth rate) and RFLP heterozygosity (for which no positive correlation with growth rate was observed) in a cohort of *Placopecten magellanicus*.

On balance, there is sizeable evidence that more heterozygous individuals generally grow faster, have a lower respiration rate, a greater efficiency for protein synthesis and a higher scope for growth than individuals that are more homozygous (references above).

Numerous studies of natural populations of bivalves have revealed general and often significant departures from Hardy-Weinberg equilibrium (HWE) in the form of heterozygote deficits (Zouros & Foltz 1984; Gaffney 1990; Gosling 1992; Marsden *et al.* 1996; Lewis *et al.* 2000; Elderkin *et al.* 2001). The co-occurrence of heterozygote/fitness correlation (HFC) and heterozygote deficiencies has been reported in electrophoretic studies on natural populations of bivalves (Zouros *et al.* 1980; Gaffney *et al.* 1990; Beaumont 1991). However, no single plausible explanation has been proposed to account for the association.

Scope for growth (SFG) represents the energy available for growth and reproduction, expressed as the difference between absorbed energy and energy lost through metabolic processes (Bayne & Newell 1983). SFG can be increased by increasing the metabolic efficiency (reduction of metabolic costs) and/or by increasing the energy absorption (increasing feeding rate or absorption efficiency). Previous studies (Koehn & Shumway 1982; Diehl *et al.* 1985; Rodhouse & Gaffney 1984; Garton *et al.* 1984; Hawkins 1991; Bayne & Hawkins 1996; Hawkins & Day 1996, 1999) have shown that increased metabolic efficiency might explain differences in growth associated with heterozygosity. However, the experiments in many of these studies involved starved animals and the conclusions are based on extrapolations from starved animals to active, growing animals.

Feeding rate is also an important factor affecting growth rate. In studies involving oyster drills (*Thais haemastoma* and *Thais lamellosa*), Garton (1984) and Garton &

Stickle (1984) showed that feeding rates were the most important factor contributing to differences in scope for growth among different heterozygosity classes.

Zebra mussels (*Dreissena polymorpha*), like most bivalves, are suspension feeders that can pump considerable volumes of water through their gills and filter out small particulate food items from the water column, being capable of removing abiotic as well as biotic material from water. There is a substantial literature on various aspects of feeding rate and growth in zebra mussels (Walz 1978 *a*, b, c; Reeders *et al.* 1989; Dorgelo & Kraak 1993; Aldridge *et al.* 1995; Lei *et al.* 1996). In spite of this, there have been no attempts to date to correlate genetic and physiological variation. The purpose of the present study was to determine the relationship between SFG and the degree of individual multiple locus heterozygosity as determined by starch electrophoresis, in an Irish population of zebra mussels. Correlations between individual parameters of the SFG equation (clearance rate, respiration rate and faeces disposal rate) and shell length were also examined.

IV.2. MATERIAL AND METHODS

IV.2.1. Sample collection

A sample of mussels was collected in September 2003 from Lough Key (G 849 041, Fig. II.1.1) from depths of 0.5-1.5 m. A 4 m extendable pole with an attached 15 cm scraper-blade and net pocket was used to scrape vertical surfaces. Mussels were transported in insulated containers with ice packs. Approximately 150 mussels of various sizes (shell length between 13-28 mm) were selected. Care was taken to avoid deformed shells thereby excluding potentially damaged or sick animals from the experiments. After cleaning the shells of epibionts and sediments, the zebra mussels were tagged, weighed (on an analytical balance, to the nearest 1 mg) and measured (with digital callipers to the nearest 0.01 mm). Subsequently, mussels were placed in a tank of aerated L. Key water at 15°C and adapted to the experimental food. This was a mixture of marine phytoplankton (*Nannochloropsis, Tetraselmis, Isochrysis* sp.) called Phytoplex (Kent Marine Inc., USA). Phytoplex cells, measured with a particle-counter analyser (Sysmex CDA-500) were in the size range 1.26-5 µm with a mean size of 2.13 µm. A food concentration of 16 000-20 000 cells ml^{-1} was used, to prevent the production of pseudofaeces, and to ensure maximum filtration rate (O'Toole 2001). Food concentration was chosen to be below the threshold of pseudofaecal production, which for *Dreissena polymorpha* was experimentally determined to be above a particle concentration of 20 000 cells ml^{-1}.

IV.2.2. Scope for Growth

Growth represents a balance between processes of energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion). The energy equation for scope for growth (SFG) (Widdows & Staff 1998) integrates the experimentally determined components, namely, consumption, assimilation and respiration into the energy balance as follows:

$$SFG = A - R,$$

$$A = C - F$$

Or

$$A = C \times AE; \quad (AE = 1 - F/C)$$

Where: SFG = scope for growth;

A = absorbed food energy;

R = respiratory energy expenditure;

C = total consumption of food energy;

F = faecal energy loss;

AE = absorption efficiency.

The physiological responses C, F and R were experimentally determined, converted into energy equivalents (Jh^{-1}) and the energy available for growth and reproduction (SFG) was calculated. The method provides a rapid and quantitative assessment of the energy status

of the animal and assesses the contribution of the individual components to energy balance.

IV.2.2.1. Clearance rate (CR)

Filtration or pumping rate is defined as the volume of water flowing through the gills per unit time. The clearance rate is that volume of water that is completely cleared of particles per unit time. When all particles presented to the gill are cleared from suspension, then the clearance rate is the same as the filtration rate.

The clearance rate was estimated by measuring the removal of algae cells added to micro-filtered lake water, according to the equation for static systems (Widdows & Staff 1998):

$$CR\ (l\ h^{-1}) = \text{Volume of water} \times (\log_e C_1 - \log_e C_2)/\text{time interval in h}$$

A hundred and twenty ml beakers were used to create static (closed) systems. Hypodermic needles connected to an oxygen pump served for aeration, and ensured an evenly distributed food concentration. The experimental temperature was set at 15°C. Twelve hours before the experiment the mussels were placed in micro-filtered lake water (GFC Whatman membrane filter, pore size 0.2 µm).

The beakers were filled with 110 ml of a mixture of micro-filtered lake water and Phytoplex solution (16 000-20 000 cells ml^{-1}). The initial concentration in each beaker (t_0) was taken at the beginning of the experiment, using a particle-counter analyser. A 50 µm aperture tube for cell size ranges 1.25 to 5 µm was used. The mussels were placed individually into the beakers and left for 10 min to allow for opening of the valves and for the mussel to start feeding. The mussels were removed from the beakers after 30 min

(production of faeces begins after 30 min). The beakers were then closed and shaken to ensure an even mixture of the suspension.

Measurements of the Phytoplex particle concentrations for each beaker (t_1) were then taken. The obtained data for t_0 and t_1 were corrected using blank readings for filtered lake water. The clearance rate was calculated as the decrease in cell concentration between the initial time (t_0) and the final time (t_1), at 30 min.

The energy content of the food was determined using the Dry Combustion technique. The method consists of oxidizing the Phytoplex under pressure, followed by the determination of the evolving CO_2 using a carbon dioxide sensitive infrared detector in a CNS-1000 Leco elemental analyzer (Leco Co., USA). The results (expressed as percentages of carbon) were converted into joules using the conversion factor of 1 g organic carbon = 42 kJ (McLusky 1989).

IV.2.2.2. Respiration rate (RR)

The energy loss by respiration was measured by monitoring the oxygen uptake, following the method of Wilson & McMahon (1981). The rates of oxygen consumption by individual mussels were measured at 15°C in glass jacketed respiration chambers, which were filled with freshly filtered lake water. Volumes of filtered water between 3 and 8 ml were poured into each chamber, depending on the size of the animal. Zebra mussels were placed individually into the respiration chambers on plastic mesh stages and allowed sufficient time to open the valves. The temperature in the respirometers was kept constant by a temperature controlled water bath. A magnetic stirrer was placed in each chamber to ensure the circulation of the water. An oxygen electrode (Clark-type, silver-platinum, polarographic) and an YSI oxygen monitor (model 53) connected to a JWS writer

(modelgraphic 5301) were used to measure the oxygen saturation in the chambers during the experiment. The respiration chambers were carefully closed with the lid and oxygen electrode, and the eventual air bubbles trapped within the chamber were removed.

The oxygen saturation (measured as a percentage during the experiment) was converted into µl O_2 anim^{-1} h^{-1}, using the following formula:

$$VO_2\,(\mu l\ anim^{-1}\ h^{-1}) = \frac{Sol_{O2}\,(\mu l\ ml^{-1}) \times Vwater\,(ml) \times \%O_2 \times BP}{T\,(h) \times 1000}$$

Where: VO_2 = volume of O_2 consumed;

Sol $_{O2}$ = Solubility of O_2 in water in equilibrium with air at 760 mmHg pressure and 100% relative humidity;

Vwater= volume of filtered lake water used;

$\%O_2$ = Percentage of O_2 consumed;

BP = Barometric pressure on day of the experiment;

T = Time in hours.

The respiration rate (expressed as µl O_2 anim^{-1} h^{-1}) was converted into energy units (joules) using the conversion factor 20.2 kJ per ml O_2 (Ivlev 1934).

IV.2.2.3. Faeces disposal rate (FDR)

The faeces disposal rates of zebra mussels were measured in groups, in order to collect sufficient material for the determination of the energy value in the faeces. The faecal energy loss was then averaged for an individual.

Four groups of 15-20 mussels of similar sizes (size range of 12-15, 15-20, 20-23 and 23-26 mm) were placed separately into 10 l containers of filtered lake water containing the same food concentration of Phytoplex (16 000-20 000 cells ml^{-1}) as in the filtration/ clearance rate experiments.

The large volume of water was chosen in order to provide sufficient food over the ingestion period. The mussels were allowed to feed for 12 hours and were then transferred into micro-filtered lake water (GFC Whatman membrane filter, pore size 0.2 μm) for 6 hours to discharge faeces. The micro-filtered water with the discarded faeces was subsequently filtered on pre-weighed and pre-ashed GFC filters. The collected faeces and filters were dried in an oven at 90°C for 24 hours and then re-weighed.

The content of carbon in the collected faeces was measured by dry combustion, using a Leco elemental analyzer. The results (expressed as percentages of carbon) were converted into energy units (joules) using the conversion factor of 42 kJ $mg^{-1}C$ (McLusky 1989).

IV.2.3. Allozyme electrophoresis

Following physiological analysis, the mussels were frozen alive and kept at -80°C before electrophoresis. The mussels were defrosted and removed from their shell before use. The allozyme genotype of each individual was obtained for seven polymorphic gene loci: malate dehydrogenase, (*MDH*), isocitrate dehydrogenase (*IDH-2*); aconitate hydratase

(*ACO-1* and *ACO-2*), phosphoglucomutase (*PGM*), glucose-6-phosphate isomerase (*GPI*); esterase-D, (*EST-D*). Octopine dehydrogenase (*OPDH*) was not included in the list due to poor resolution of the bands obtained. Electrophoretic protocols have already been described in Chapter III.2.1.

IV.2.4. Genetic data analysis

Allelic distribution for each locus, expected (H_E) and observed (H_O) heterozygosity estimates for each population were obtained using the GENETIX v. 4.01 computer program (Belkhir *et al.* 2000). Conformance to Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP v 3.1 program (updated version of Raymond & Rousset 1995), with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. The same program was used to calculate F_{IS} estimates (Weir and Cockerham 1984). Significance levels for multiple tests were adjusted using the sequential Bonferroni correction (Rice 1989). Individual multilocus heterozygosity (MLH) was calculated as the number of loci at which an individual was a heterozygote divided by the number of loci examined (7).

All data were checked for normality and logarithmic transformations were conducted if necessary before parametric statistical analysis. Regression analyses were performed between MLH values and SFG and energy budget parameters (CR, RR and FDR) to test for possible heterozygosity-fitness correlations. Individual heterozygosities were also regressed against SFG for each locus separately. Correlation analysis was also carried out on body size (shell length) and SFG and energy budget parameters. Individual mussels were grouped into four size classes (<16 mm, 16-19 mm, 19-22 mm, >22 mm) and correlation analysis between MLH and SFG was performed for each size class. All

analyses were performed in PopTools program (available at: http://office.microsoft.com/assistance/9798/sr2fact.aspx).

IV.3. RESULTS

IV.3.1. Fitness parameters

Out of the 150 zebra mussel individuals used to initiate the experiment, 110 survived the experimental period and the manipulations necessary for determining energy budgets (26.7% mortality). Natural populations of *Dreissena polymorpha* are characterized by high growth rates and high individual mortality rates (Minchin *et al.* 2002).

The carbon content of a Phytoplex food cell was established as 1.95×10^{-7} g C cell^{-1}, corresponding to a food concentration of 2.9-3.9 mg C l^{-1}. SFG values ranged between 0.79 J g^{-1} h^{-1} and 55.78 J g^{-1} h^{-1}. As expected, SFG estimates were significantly and positively correlated with shell lengths (r^2 = 0.12, P < 0.05, Fig. IV.1). Assimilation efficiency (AE) values were high and consistent, varying between 0.27 and 0.71 (with a mean of 0.56). No significant relationship was observed between AE and body size (r^2 = 0.07, P = 0.06, Fig. IV.2, Table IV.1).

Clearance rate (CR) values ranged between 3.22 J h^{-1} and 14.18 J h^{-1}, whereas respiration and faeces estimates ranged between 0.80-1.78 J h^{-1} and 2.33-4.12 J h^{-1}, respectively. Log CR and log FDR were significantly, positively correlated with log transformed shell lengths, log SL (Fig. IV.3, Fig. IV.5, Table IV.1). However, log RR was not significantly correlated with log SL (Fig. IV.4, Table IV.1). Details on the mean estimates for energy budget parameters and regression of energy budget components and assimilation efficiency on size (shell length) are given in Table IV.1.

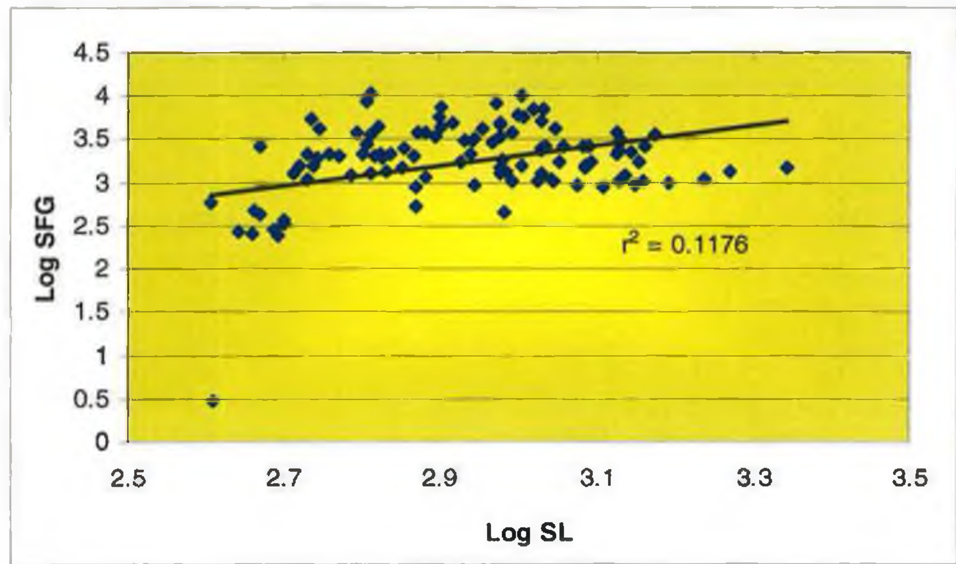

Fig. IV.1: Relationship between scope for growth (SFG) and shell length (SL). Data for the regression were log transformed.

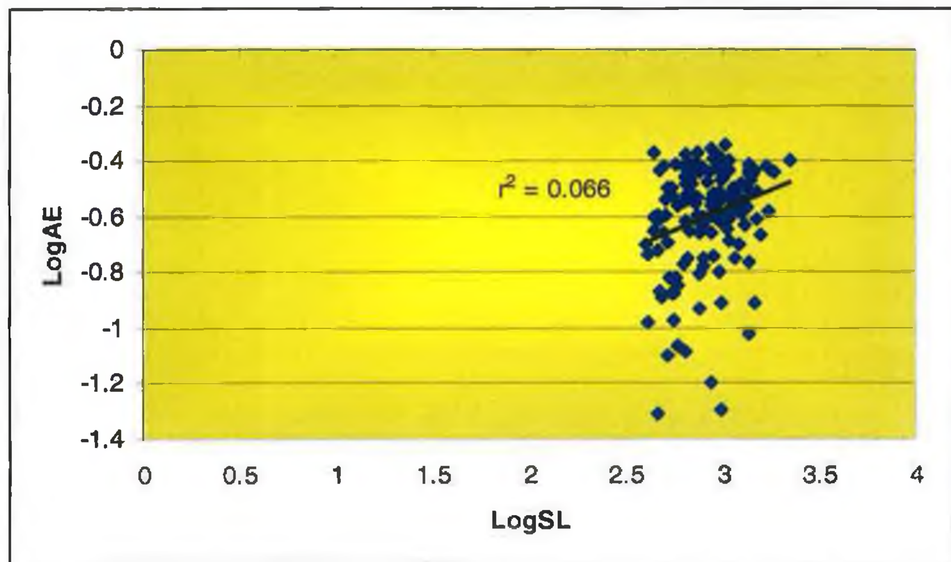

Fig. IV.2: Relationship between assimilation efficiency (AE) and shell length (SL). Data for the regression were log transformed.

Parameter	Mean	Regression equation	r^2	P-value
CR	7.26±0.21	-5.2019+0.6562(SL)	0.86	<0.001
RR	0.70±0.04	0.0276+0.0344(SL)	0.06	0.24
FDR	2.99±0.04	0.6888+0.1212(SL)	0.99	<0.001
SFG	27.92±0.98	16.566+0.5981(SL)	0.12	<0.05
AE	0.56±0.008	3.0529+0.2221(SL)	0.066	0.06

Table IV.1: Mean values for energy budget parameters and regression of energy budget components and assimilation efficiency on measured shell length (CR: clearance rate; RR: respiration rate; FDR: faeces disposal rate; SFG: scope for growth; AE: assimilation efficiency). Data for the regressions were log transformed.

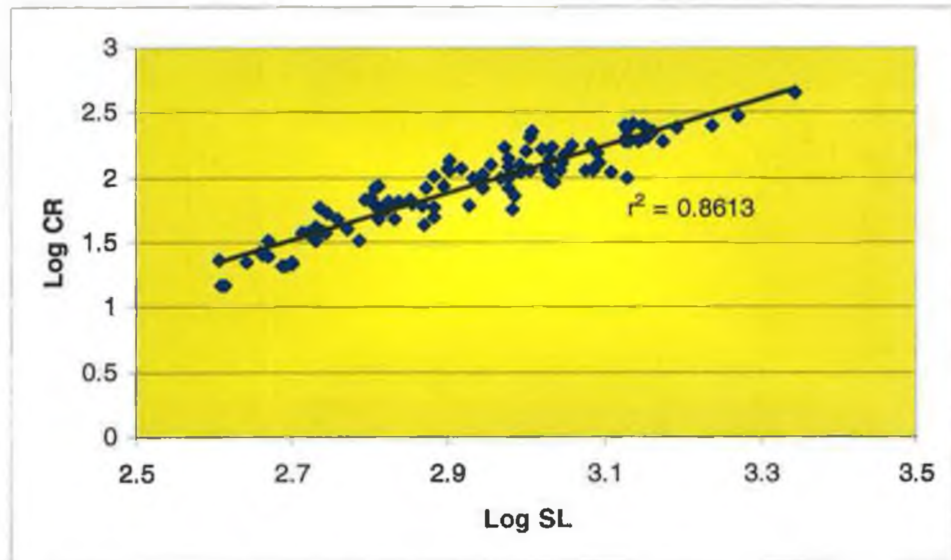

Fig. IV.3: Relationship between clearance rate (CR) and shell length (SL). Data for the regression were log transformed.

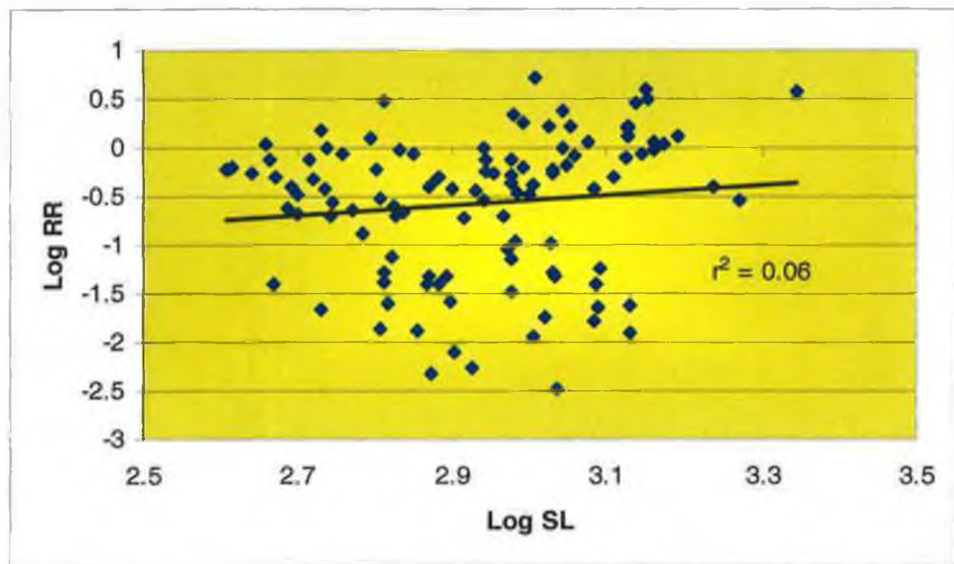

Fig. IV.4: Relationship between respiration rate (RR) and shell length (SL). Data for the regression were log transformed.

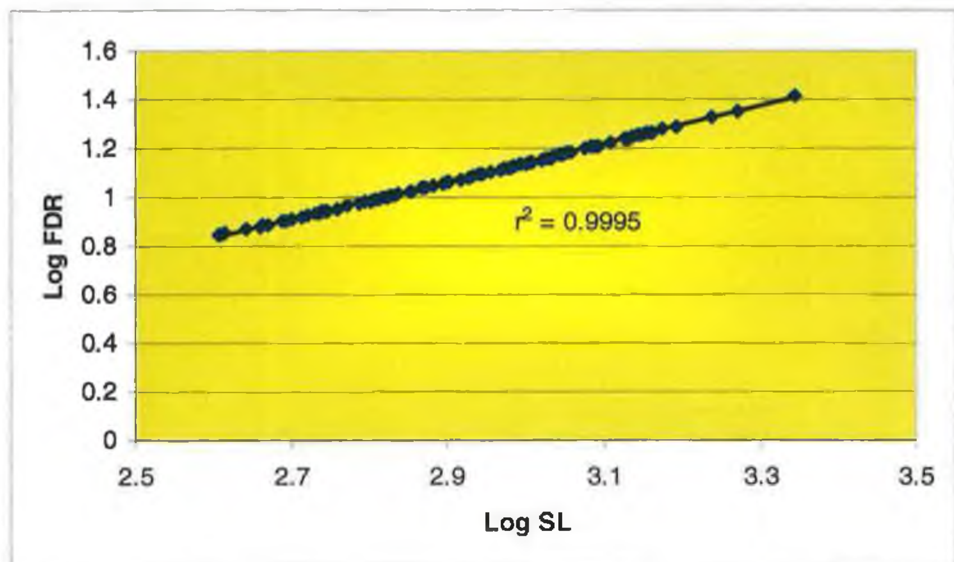

Fig. IV.5: Relationship between faeces disposal rate (FDR) and shell length (SL). Data for the regression were log transformed.

IV.3.2. Allozyme electrophoresis

The most polymorphic loci were *EST-D* (five alleles) and *GPI* (four alleles), with observed heterozygosities of 0.234 and 0.708, respectively (Table IV.2). The least polymorphic locus was *PGM*, with 2 alleles and a H_O value of 0.071, lower than those recorded for other loci. Individual multilocus heterozygosity ranged from 0 to 85.7%. Linkage disequilibrium tests revealed no dependence between loci pairs.

Tests for Hardy-Weinberg equilibrium (Table IV.2) revealed significant deficits of heterozygotes at three loci (*ACO-2*, *EST-D* and *IDH-2*). Weak heterozygote excesses were recorded for *GPI* and *PGM* loci, but none were significant even before Bonferroni correction.

No significant correlation was observed between MLH and size (SL) ($P = 0.39$, Fig. IV.6). When individual mussels were grouped into size classes, no significant correlation was observed between MLH and size in any of these classes ($P > 0.05$ in every case). Tests for HWE revealed more pronounced heterozygote deficiencies among smaller individuals than larger ones (Table IV.3).

Multilocus pairwise F_{ST} estimates revealed significant differentiation ($F_{ST} = 0.035$, $P < 0.001$) between the L. Key sample analysed in this chapter and the L. Key sample analysed two years earlier, in 2002 (Chapter III). Similarly, Fisher's exact tests of genic (allelic) differentiation and genotypic differentiation showed significant differentiation between the two samples. However, the much smaller sample size in 2002 (46±9), compared to 2004 (105±3), and the different number of loci analyzed (8 and 7 for 2002 and 2004, respectively) may be responsible for the significant differentiation observed.

Locus	Allele	Allele frequency	N	H_E	H_O	F_{IS}
ACO-1	95	0.25	92	0.375	0.304	0.194
	100	0.75				
ACO-2	96	0.47	96	0.498	0.333	**0.335**
	100	0.53				
EST-D	100	0.47	109	0.687	0.234	**0.429**
	104	0.11				
	112	0.16				
	114	0.02				
	120	0.23				
GPI	100	0.44	106	0.676	0.708	-0.042
	103	0.06				
	108	0.27				
	112	0.23				
IDH-2	100	0.59	103	0.491	0.447	**0.095**
	106	0.40				
	108	0.01				
MDH	85	0.17	115	0.287	0.261	0.096
	100	0.83				
PGM	87	0.04	112	0.069	0.071	-0.033
	100	0.97				
Mean			104.7 (3.1)	0.440 (0.082)	0.337 (0.075)	**0.153** (0.067)

Table IV.2: Allele frequency, number of individuals scored (N), expected (H_E) and observed (H_O) heterozygosity, and deviations from HWE (F_{IS}) according to Weir & Cockerham (1984). Values in bold indicate significant deviations from HWE after sequential Bonferroni correction. Standard errors for means are given in parentheses.

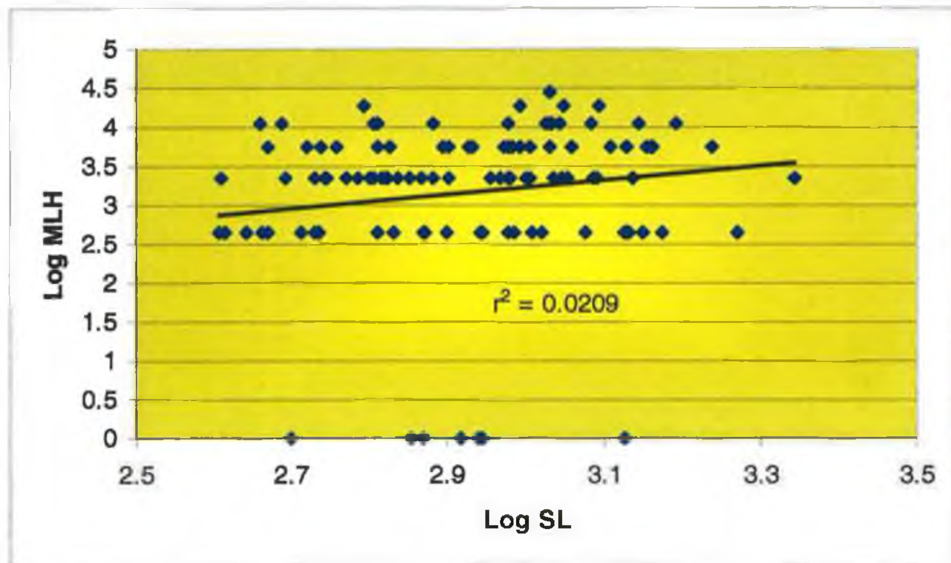

Fig. IV.6: Relationship between shell length (SL) and multiple locus heterozygosity (MLH). Data for the regression were log transformed.

Locus/Shell length class (mm)	<16	16-19	19-22	>22
ACO-1	**0.489**	0.333	-0.027	0.072
ACO-2	**0.416**	0.270	0.372	0.233
EST-D	**0.689**	**0.283**	**0.447**	**0.225**
GPI	0.131	-0.151	0.013	-0.151
IDH-2	0.161	**0.281**	0.063	-0.066
MDH	-0.042	0.205	0.044	0.237
PGM	-0.042	-	-	-0.036
Mean	0.257	0.204	0.152	0.073

Table IV.3: Deviations from HWE (F_{IS} values) in four shell length classes. Values in bold indicate significant deviations from HWE after sequential Bonferroni correction.

IV.3.3. Heterozygosity and energy budget parameters

There was no significant relationship between MLH and SFG (P > 0.05, Fig. IV.7). Similarly, no significant correlations were evident between MLH and any energy budget parameter: CR (r^2 = 0.016, P = 0.18), RR (r^2 = 0.0005, P = 0.82), FDR (r^2 = 0.025, P = 0.09) and AE (r^2 = 0.030, P = 0.07). None of the regressions were significant when individual heterozygosity was regressed against SFG for each locus separately. When individual mussels were grouped into size classes no significant correlation was observed between MLH and SFG in any of these classes (P > 0.05 in every case).

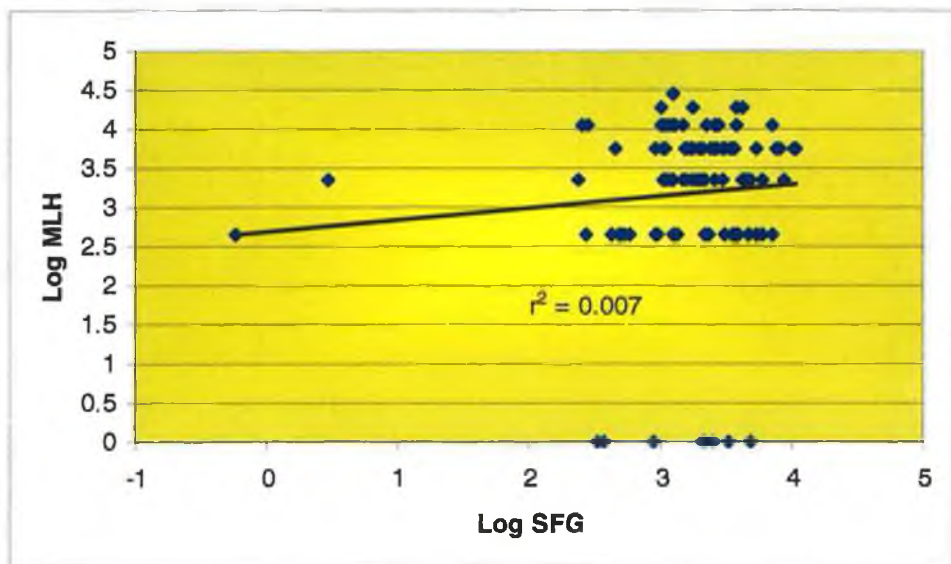

Fig. IV.7: Relationship between scope for growth (SFG) and multiple locus heterozygosity (MLH). Data for the regression were log transformed.

No significant correlations were evident between MLH and SFG and any energy budget parameters when only the four loci susceptible of selection (*ACO-1*, *ACO-2*, *IDH-2* and *MDH,* see Chapter III.4.2) were considered (P > 0.1 in every case).

The most important finding in the present study is the absence of a significant correlation between genetic variation (as expressed by MLH) and physiological variation (SFG and SFG parameters). However, some parameters (SFG, CR and FDR) were positively correlated with the body size (shell length). It is noteworthy that smaller individuals exhibited more pronounced heterozygote deficiencies than larger individuals.

IV.4. DISCUSSION

IV.4.1. Growth aspects

SFG, the number of calories remaining after the metabolic demands of the organism have been met, can be positive or negative. All SFG values recorded in the present study were positive, between 0.79 and 55.78 J g^{-1} h^{-1} (mean SFG = 27.9±0.8 J g^{-1} h^{-1}), suggesting that in the experiments zebra mussels were not exposed to stressful conditions. In a series of publications on various stages of food uptake on *Dreissena polymorpha* (filtration, ingestion and assimilation efficiency rates), Walz (1978*a*) observed a mean assimilation efficiency (AE) of 40.1% for a food concentration of 2 mg C l^{-1} and Stańczykowska *et al.* (1975) and Aldridge *et al.* (1995) obtained similar AE values. However, in Irish mussels a higher mean AE estimate of 56% was observed. This is most likely a consequence of the higher food concentration used in our study (2.9 - 3.9 mg C l^{-1}). Although higher than previously reported, the food concentration used in the present study was chosen to be below the threshold of pseudofaecal production. However, AE estimates also depend on the type of food. Although natural seston (Stańczykowska *et al.* 1975), pure cultures of *Chlorella vulgaris* (Aldridge *et al.* 1995) and *Nitzschia sp.* (Walz 1978*a*) have been used by others, a mixture of marine phytoplankton (*Nannochloropsis, Tetraselmis, Isochrysis* sp.) was used in the present study. Pure algae cultures are the ideal type of food for use in such physiological experiments (Jørgensen *et al.* 1984). However, pure cultures of algae could not be grown in the lab.

No significant correlation was established between AE and the body size (SL) in our study. Although Winter (1969) reported a significant decrease in AE with increasing size in the bivalve *Arctica islandica*, our results are consistent with the findings of Vahl (1973)

and Walz (1978*a*), who revealed no significant relationship between AE and body size in populations of *Mytilus edulis* and *Dreissena polymorpha*, respectively. As expected, the present study showed significant relationships between two energy budget parameters (CR and FDR) and body size. Respiration rate was the only SFG parameter which was not significantly correlated with body size. Although care was taken to ensure that respiration rate was recorded while the valves were opened, the results obtained could have been due to the valves not being fully open for short periods during which respiration rate is lower (Frischer *et al.* 2000). It is often claimed that the relative maintenance oxygen requirement is independent of weight (Kelso 1972; Walz 1978*b*).

IV.4.2. Heterozygosity-SFG correlations

There was no evidence for a positive relationship between MLH and the fitness measure (SFG) in *Dreissena polymorpha*. Neither was there a positive correlation between MLH and assimilation efficiency, or between MLH and any of the energy budget parameters: consumption rate, respiration rate and faeces disposal rate. These results are at variance with Britten (1996), who in a review of MLH and fitness trait correlations on 22 species, concluded that, on balance, MLH-fitness correlations were on the whole significant. This was despite numerous studies that reported non-significant correlations in bivalves (Beaumont *et al.* 1983, Adamkewicz *et al.* 1984; Foltz & Zouros 1984; Gaffney & Scott 1984; Beaumont *et al.* 1985; Diehl & Koehn 1985; Mallet *et al.* 1986; Zouros *et al.* 1988; Gosling 1989; Beaumont 1991; Fevolden 1992; Saavedra & Guerra 1996; Carissan-Lloyd *et al.* 2004), or on other species (Houle 1989; Booth *et al.* 1990; Elliott & Pierce 1992; Whitlock 1993; Savolainen & Hedrick 1995).

It is possible that the literature reflects a biased reporting in favor of significant correlations, because significant findings are more likely to be published. Begg & Berlin (1988) emphasized that biased reporting of only positive results can severely affect our view of reality. The lack of significant correlations in many studies involving a variety of species suggests that the correlation between MLH and fitness traits is not universal (Chakraborty 1987). Most of the studies reporting significant associations are based on samples of hundreds and give values of r^2 from 0.01 - 0.05 which border on significance. According to David (1998), the sample sizes used in these studies are not sufficient to draw such conclusions, as the signal yielded is not much higher than the noise. The vast majority of papers reporting positive correlations are based on populations of marine bivalves (*Mytilus edulis, Crassostrea virginica, Mulinia lateralis*). David (1998) pointed out that researchers have focused attention on particular species, knowing that only some species show significant heterozygosity-fitness trait correlations.

Similar to the majority of allozyme surveys on bivalves, heterozygote deficiencies were recorded for some of the loci in our study. While deficits at the *EST-D* locus were recorded in all four size classes, smaller mussels, up to 19 mm SL, also had deficits at three additional loci, *ACO-1*, *ACO-2* and *IDH-2*. The various factors that may explain deficits of heterozygotes obtained in allozyme studies involving bivalve populations are discussed in Chapter III.4.1. Although no significant association was established between MLH and SL, more pronounced deficits of heterozygotes were recorded in smaller size classes in our study. Similar deficits have been frequently reported in wild and laboratory-reared juvenile mussels (reviewed in Gosling, 1992). Selection is the factor most widely cited as the cause of such deficits. Zouros & Foltz (1984) proposed a model where selection acts against heterozygotes (underdominance) at the pre-settlement stage but is

later compensated by heterozygote superiority (overdominance) in adults. Although the present data are consistent with this model, the model requires large selection pressures and a reversal in the direction of selection between the larval and adult stages. It is difficult to imagine what these selective factors might be, and whether they might be the same for wild and laboratory-reared animals.

Data from a study on the coot clam (*Mulinia lateralis*) showed a significant correlation between heterozygote deficiency exhibited by particular loci and their contribution to the heterozygosity/growth correlation (Gaffney *et al.* 1990). Loci exhibiting the largest heterozygote deficits were those with the greatest positive effect on growth rate. However, Gaffney (1990), who reviewed data from a number of different studies on the marine mussel, *Mytilus edulis,* found little evidence to support this. Subsequently, in a study involving pair mating of *Mytilus edulis*, Beaumont (1991) showed that the two phenomena (the occurrence of heterozygote deficiencies and the positive heterozygosity/growth correlation) did not occur in offspring from restricted matings, and therefore may have distinctly separate causes. Our data lend support for this hypothesis. Although significant deficits of heterozygotes were recorded at three loci in our study (*ACO-2*, *EST-D* and *IDH-2*), there was no evidence for a heterozygosity-growth correlation at any of the loci.

Our failure to observe a positive correlation between MLH and scope for growth may be due to several factors. The first could be the rather low sample size in our study (105±3). Ideally, sample sizes should be in the thousands in order to detect a significant correlation (David 1998). However, analysing such large sample sizes requires a long period during which mussels have to be kept alive, under standard laboratory conditions. It has been suggested that the strength of the correlation within a population can vary over

time (Gaffney 1990). This suggestion is supported by the results obtained by Carissan-Lloyd (2004), who identified summer/winter variation in the strength of the relationship between a measure of fitness (percentage of basophils, an immune system parameter) and multilocus heterozygosity in *Mytilus edulis*. While the correlation was significant for the winter months, it was not significant for the summer months. We cannot exclude the possibility that this might be the case for zebra mussels also. Gentili & Beaumont (1988) observed a significant correlation between MLH and growth rate in a laboratory population of *Mytilus edulis* exposed to environmental stress. However, the correlation was no longer significant when the same population was held in less stressful conditions. In our study the zebra mussels were maintained in optimal conditions, so as to avoid stress as much as possible. The high SFG values in our study (between 0.79 - 55.78 J g^{-1} h^{-1}; mean SFG = 27.9±0.8 J g^{-1} h^{-1}) suggest that the zebra mussels were not experiencing a stressful environment. The observation of a significant correlation may also depend on the life stage examined. Danzmann *et al.* (1987) found a positive correlation between MLH and growth (length and weight) in rainbow trout aged up to six months. However, negative correlations were found between MLH and length for one-year-old individuals, and between MLH and length and weight in maturing adults (Ferguson 1990, 1992). The sample of zebra mussels was comprised of individuals of various ages and this may be yet another reason for the non-significant correlation obtained. However, when zebra mussels were grouped into size classes, the correlations between MLH and SFG remained non significant in all the classes. Outside of these factors it may be that the allozyme loci that were selected for study do not affect SFG, or that the loci are not good indicators of the level of heterozygosity of the entire genome, or that heterozygosity and fitness traits are not necessarily correlated.

CHAPTER V

CONCLUSIONS

V.1. MICROSATELLITES

Levels of allelic diversity and mean expected heterozygosity were high for Irish, European and North American populations, with a mean number of alleles/locus and H_E of 10-15.2 and 0.79-0.89, respectively. Although a significantly lower number of alleles were observed in Irish populations, there was no evidence that these populations had undergone a recent bottleneck event.

Significant departures from Hardy-Weinberg equilibrium in the form of heterozygotes deficits were recorded in all populations and for all loci. Of the various factors that contribute to deficits in populations e.g., null alleles, inbreeding, selection, and the Wahlund effect, null alleles were the most likely cause.

Analysis of population differentiation indicated no significant differentiation between Irish samples. There was no strong support in favor of a single model of dispersal, although the island model of dispersal, where individual mussels or larvae move from one population to another with equal probability, seems more likely.

Comparisons between Irish zebra mussels and populations from Europe and North America showed that Irish and British samples were not significantly differentiated. It is likely that zebra mussels in Ireland have come from Britain, favoured by the abolition, in 1993, of value added tax on used boats purchased in the European Union, resulting in an increase in boat imports into Ireland. The similarity between samples from Britain and Netherlands is probably due to a shared Baltic origin, and the similarity, in turn, between these populations and the two Great Lakes samples suggest a north-west rather than a central or east European source for North American mussels.

The inverse relationship observed between F_{ST} and locus polymorphism suggests that homoplasy may have contributed to the downward bias of F_{ST} estimates in the data.

There was no evidence of significant differentiation on a local spatial scale when samples collected from five sites within L. Key in Ireland were compared. Neither were there differences between newly settled spat and adults collected at the same time of year, or between samples collected from the same site within the lake, analysed two years apart.

V.2. ALLOZYMES

Irish samples of zebra mussels showed somewhat reduced, but not significantly different, levels of genetic variability compared to those reported for European and North American populations.

Pairwise multilocus F_{ST} estimates and Fisher's exact tests revealed significant differentiation between Irish samples. This was surprising given that dispersal of mussels in the Shannon-Erne system is through a combination of downstream movement of larvae, movement of fouled craft traveling both upstream and downstream within the system, and through jump dispersal by overland movement of fouled craft. The results contrasted with those observed for microsatellites on the same populations. Assuming that microsatellites act as neutral markers, a comparison of mean F_{ST} estimates for the two types of markers indicated that directional selection was probably acting on several of the allozyme loci.

V.3. HETEROZYGOSITY-FITNESS CORRELATIONS

Significant positive correlations were observed between clearance rate (CR) and faeces disposal rate (FDR), and body size, whereas no significant relationship was found between assimilation efficiency (AE) or respiration rate (RR), and body size.

Results were unequivocal with no evidence for heterozygosity/fitness correlations: no significant relationships were found between mean locus heterozygosity (MLH) for seven allozyme markers and size, or between MLH and any of the energy parameters: scope for growth (SFG), AE, CR, RR, or FDR. Factors that may account for this are small sample size, lack of environmental stress, dependence of the MLH correlation on life-stage of experimental animals and choice of allozyme loci.

Significant deficits of heterozygote were observed at three allozyme loci, with more pronounced deficits in smaller size classes. This agrees with a model of selection (Zouros & Foltz 1984), which acts against heterozygotes (underdominance) at the pre-settlement stage, but is later compensated by heterozygote superiority (overdominance) in adults.

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