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The effect of hybridisation on mitochondrial DNA inheritance in the Mytilus edulis complex.

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The Effect of Hybridisation on Mitochondrial DNA Inheritance in the *Mytilus edulis* Complex

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SUMMARY

The *Mytilus edulis* complex comprises three closely related marine mussel species (*M. edulis*, *M. galloprovincialis* and *M. trossulus*). In areas where species distributions overlap a varying degree of hybridisation occurs, yet genetic differences between allopatric populations are maintained. These mussels have an unusual mode of mitochondrial DNA (mtDNA) inheritance termed Doubly Uniparental Inheritance (DUI). Females are homoplasmic for the F mitotype which is inherited maternally, whereas males are heteroplasmic for this and the paternally inherited M mitotype. Observations from natural populations and previous laboratory experiments suggest that DUI may be disrupted by hybridisation, giving rise to heteroplasmic females and homoplasmic males. To investigate this, controlled laboratory crosses were carried out to produce pure species and hybrid larvae of known parentage. Species identification was confirmed using the Me 15/16 nuclear DNA marker which amplifies part of the adhesive protein gene sequence. This marker reliably identified European *M. edulis* and *M. galloprovincialis* and their hybrids, but results for Canadian *M. edulis* and for *M. trossulus* from Canada and the Baltic Sea contradicted those from allozyme loci. MtDNA markers were used to follow the fate of the F and M mitotypes through larval development. The results confirmed that the M mitotype enters all eggs at fertilisation in *M. edulis*, *M. galloprovincialis* and their hybrids and is later eliminated in a proportion of pure species larvae. Disruption of the mechanism which determines whether the M mitotype is retained or eliminated occurred in an estimated 40% of *M. edulis* x *M. galloprovincialis* hybrid larvae, a level double that previously observed in adult mussels from natural *M. edulis* x *M. galloprovincialis* populations. Furthermore, reciprocal hybrid crosses exhibited contrasting types of DUI disruption. The results indicate that disruption of DUI in hybrid mussels may be a factor in the maintenance of genetic integrity for each species.

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Chapter 1. General Introduction

1.1 The genus *Mytilus*

1.11 Background on mussels

Mussels of the genus *Mytilus* Linnaeus, 1758, are among the commonest of marine molluscs and are found on rocky shores and in shallow sublittoral waters in boreal and temperate regions (Hayward and Ryland 1995). These mussels are sedentary, filter feeding bivalves which attach themselves to firm substrata by proteinaceous byssal threads secreted from glands in the foot (Yonge 1976). They have been the focus of a wide range of scientific investigations and a vast body of data is available from ecological, physiological, biochemical and genetic studies. *Mytilus* species are important economically as food and fouling organisms and as biomonitors of coastal water quality.

1.12 Reproduction and life cycle

Like many boreal and temperate water bivalves, *Mytilus* has a seasonal pattern of reproduction, with gametes being released directly into the sea, usually in spring or summer. The precise time and duration of spawning varies among populations both spatially and temporally. Apart from a few hermaphrodites, the sexes in *Mytilus* are separate and most populations contain approximately equal numbers of males and females (Seed 1976; Sastry 1979; Brousseau 1983; Sprung 1983; Fisher and Skibinski 1990; Skibinski *et al.* 1994). Following spawning and external fertilisation a mussel embryo begins to swim after 4-5 h and the first larval shell begins to form approximately 24-48 h after fertilisation. This shell, the prodissoconch I (Bayne 1976), is 'D'-shaped with a length of 100-120 μm (Jablonski and Lutz 1980; Sprung 1984). The second shell, the prodissoconch II, forms shortly afterwards and this planktotrophic veliger stage remains in the plankton for 1-4 weeks. The veliger larvae drift passively in water currents, often over considerable distances, before developing a foot, at which point they are termed "pediveligers", and becoming capable of

metamorphosis and settlement. At settlement the pediveliger secretes byssus threads which it uses to attach itself to the substrate and then metamorphosis follows. Secretion of the adult dissoconch shell begins within 48 h of pediveliger attachment (Lutz and Kennish 1992).

1.13 Systematics, taxonomy and hybridisation

The genus *Mytilus* belongs to the family Mytilidae which is thought to have originated some 400 million years ago in the Devonian period (Soot-Ryen 1969). The genus itself is of relatively recent origin with no records older than the Pliocene epoch, 3 to 12 million years ago (Soot-Ryen 1969). The widespread distribution of the genus, together with the variation in shell shape under different environmental conditions, has resulted in a long history of confused taxonomy. However, *Mytilus* taxa can be separated into smooth- and ribbed- shelled groups. The two ribbed-shelled *Mytilus* species can be easily identified by morphological features: *Mytilus californianus* Conrad, 1837 has radiating ribs on the shell (Soot-Ryen 1955) and *M. coruscus* Gould, 1861 has a thick shell with small crenulations on the ventral margin near the apex (Kira 1962). The smooth-shelled taxa are much more difficult to separate using morphological characteristics. In a review of *Mytilus* taxonomy, Lamy (1936) recognized seven distinct smooth-shelled species, namely: *Mytilus edulis* Linnaeus, 1758, *M. galloprovincialis* Lamarck 1819 from the Mediterranean Sea, *M. trossulus* Gould, 1850 from the Pacific coast of North America, *M. chilensis* Hupe, 1854 from Chile, *M. platensis* Orbigny, 1846 from Argentina, *M. planulatus* Lamarck, 1819 from Australia and *M. desolationis* Lamy, 1936 from the Kerguelen Islands. Powell (1958) added to the list with a description of *M. aoteanus* from New Zealand. However, others considered most of the above named taxa to be subspecies of *M. edulis* (Soot-Ryen 1955; Fleming 1959). A further two subspecies, *M. edulis kussakini* and *M. edulis zhurmunskii*, were described from the Pacific coast of Asia by Scarlato and Starobogatov (1979). More recently, the results from electrophoretic surveys of allozyme loci have to some extent clarified the relationships between the smooth-shelled mussel taxa described above. Allozyme electrophoresis is a method for exploring genetic

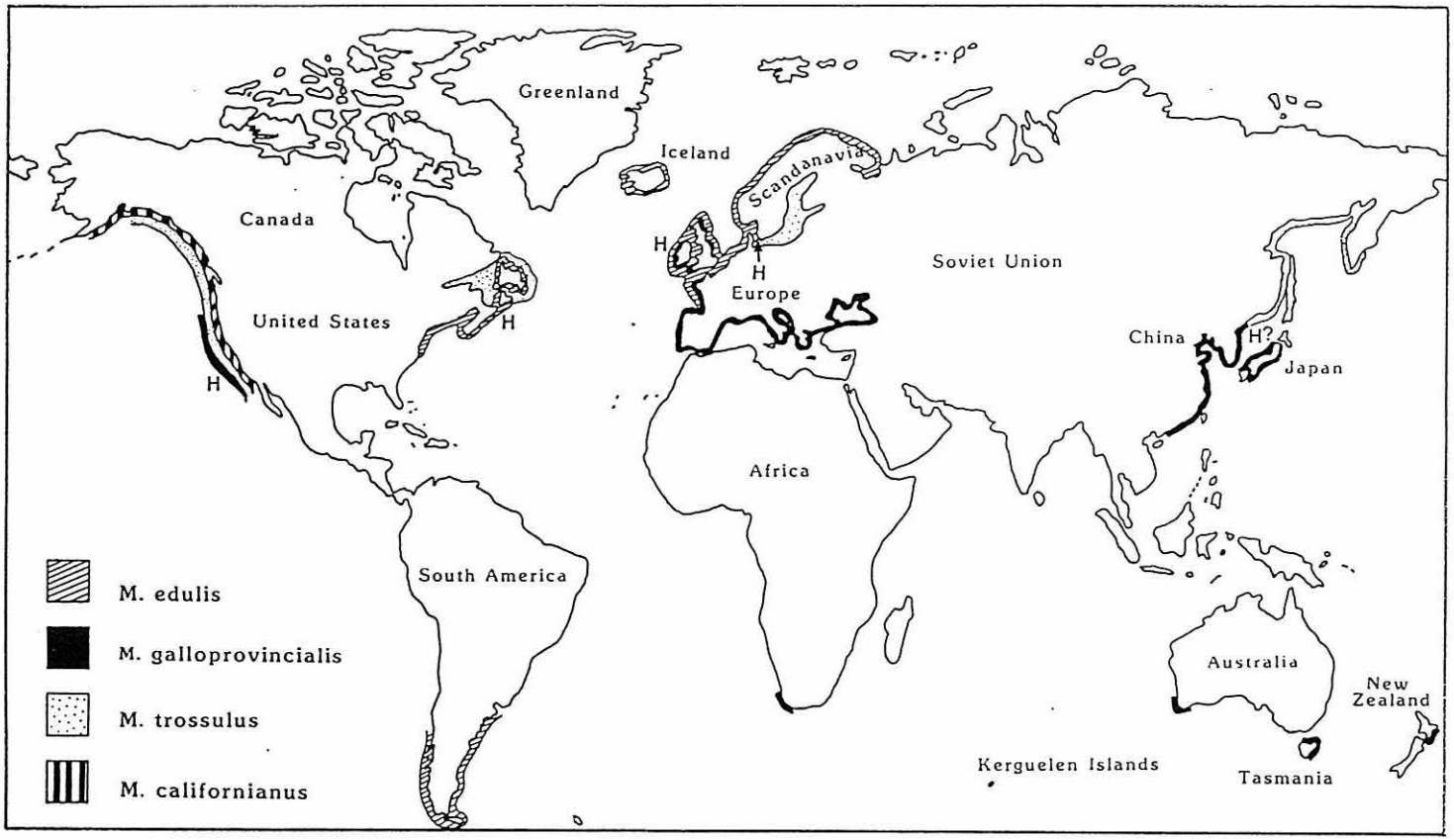
variation at the protein level. The principles of electrophoresis are described in Section 1.2. McDonald *et al.* (1991) examined morphological characteristics and allozyme variation in samples of smooth-shelled mussels taken from 45 locations in the northern and southern hemispheres. In agreement with the results of other less extensive allozyme studies (Grant and Cherry 1985; Blot *et al.* 1988; Bulnheim and Gosling 1988; McDonald and Koehn 1988; Beaumont *et al.* 1989; McDonald *et al.* 1990) McDonald *et al.* (1991) concluded that three electrophoretically distinguishable species existed in the northern hemispere: *M. edulis* from western Europe and eastern North America, *M. galloprovincialis* from the Mediterranean Sea, western Europe, California and eastern Asia and *M. trossulus* from the Baltic Sea, eastern Canada, western North America and the Pacific coast of Siberia. In the southern hemisphere, they suggested that mussels from South America, the Falkland Islands and the Kerguelen Islands previously described as *M. chilensis*, *M. desolationis* and *M. platensis* should be included in *M. edulis*, and those from Australia and New Zealand previously described as *M. planulatus* and *M. aoteanus*, respectively, should be included in *M. galloprovincialis*. The present day distributions of *M. edulis*, *M. galloprovincialis* and *M. trossulus* are thought to be as shown in Figure 1.1.

In areas where two of the taxa are present in sympatry, a varying degree of hybridisation has been found to occur (Gosling 1992; McDonald *et al.* 1991). The hybrid zones of *Mytilus* studied fall into three groups according to geographical location: Europe, the Atlantic coast of North America, and the Pacific coast of North America. In Europe, hybridisation between *Mytilus edulis* and *M. trossulus* occurs in the transition zone between the Baltic and North Seas (Vainola and Hvilsom 1991), and between *M. edulis* and *M. galloprovincialis* on the Atlantic coast of France and Portugal (McDonald *et al.* 1991) and in the British Isles (Skibinski *et al.* 1983). *M. edulis* and *M. trossulus* hybridize in eastern Canada on the Atlantic coast of North America (Saavedra *et al.* 1996) and *M. galloprovincialis* and *M. trossulus* hybridize in California on the Pacific coast of North America (Rawson and Hilbish 1995a). Despite the widespread hybridisation the three taxa maintain morphological and genetic differences and their taxonomic status has long been a topic of debate. To date there is no

consensus on whether these three taxa merit full specific status or should instead be considered as semi-species or subspecies, but to avoid further confusion they are referred to as *M. edulis*, *M. galloprovincialis* and *M. trossulus* and collectively as the *M. edulis* complex (Gosling 1992).

Little information is available on the evolution of the *M. edulis* complex but it is generally accepted that *M. edulis* is the ancestral species from which the other forms have evolved. *M. galloprovincialis* is believed to have diverged from *M. edulis* during a Pleistocene ice age when the Mediterranean Sea was cut off from the Atlantic Ocean (Barsotti and Meluzzi 1968). As the ice receded *M. galloprovincialis* was able to extend its range northwards onto the Atlantic coasts of western Europe. Varvio *et al.* (1988) suggested a northern origin for *M. trossulus*, in the Pleistocene epoch, due to its confinement to northerly latitudes and its presence on both sides of the Atlantic and Pacific Oceans. It perhaps evolved through an increased adaptation to reduced temperatures, which would have allowed it to persist in higher latitudes during glacial periods (Gosling 1992). To colonize the Baltic Sea *M. trossulus* must have been present in the Kattegat area approximately 7000 years ago when the Baltic (then a freshwater lake) became connected with the North Sea (Winterhalter 1981) and the reason for its current absence from the North Sea is unknown.

Figure 1.1 Global distribution of species in the *Mytilus edulis* complex. H = areas of contact and hybridisation. (Taken from Gosling 1992).



1.2 Tools for DNA analysis

A wide range of DNA techniques are now available that allow biologists to manipulate and analyse genetic material. DNA techniques include sequencing (Maxam and Gilbert 1977; Sanger *et al.* 1977), restriction fragment length polymorphisms (RFLPs), the polymerase chain reaction (PCR)(Mullis *et al.* 1986; Saiki *et al.* 1988) and PCR-related applications such as minisatellites (Jeffreys *et al.* 1985), microsatellites (Tautz 1989), random amplified polymorphic DNA (RAPD)(Welsh and McClelland 1990; Williams *et al.* 1990), and amplified fragment length polymorphisms (AFLPs)(Vos *et al.* 1995). There have been numerous reviews concerning the principles and uses of the many techniques available (e.g. Hillis *et al.* 1996; Avise 1994; Skibinski 1994; Park and Moran 1995; Hoelzel 1998; Silva and Russo 2000).

PCR is a method for the amplification of random or specific segments of DNA from a complex template such as genomic DNA and has become a mainstay of molecular ecology and population genetic research in the brief time since its invention. The method involves the use of short single-stranded molecules of DNA (primers) and a DNA polymerase enzyme to generate copies of the template sequence (see Appendix A). If the DNA sequence of the region to be amplified is known then specific primers can be designed to amplify only this region. If sequence information is not available then random primers can be used to detect genetic variation between individuals.

Genetic polymorphisms can be visualised by electrophoresis of the PCR products in a gel matrix. The PCR products are introduced at a specific point in the gel and an electrical field is applied. Differences in the size or conformation of the PCR products as a result of mutation in the corresponding gene cause differences in migration rate. As a consequence, PCR products derived from different alleles at a particular locus may be detected by differing migration rates in the gel. The potential for rapid generation of high quantities of DNA allows relatively easy visualisation of PCR products by methods such as ethidium bromide or silver staining. The high sensitivity of PCR makes it

particularly applicable to small organisms such as larvae which often provide insufficient amounts of DNA for other methods of analysis. However, this sensitivity necessitates the rigorous use of precautions to prevent contamination of PCR mixtures with extraneous DNA.

Further variation in PCR products may be detected by treatment with restriction endonucleases. These enzymes cut DNA at specific sequences, called restriction sites, which occur fairly randomly within DNA. Mutations may result in the creation or destruction of restriction sites or in length variation of the segments of DNA between restriction sites. When restriction fragments are separated by electrophoresis and visualised, this variation is detected as restriction fragment length polymorphisms (RFLPs).

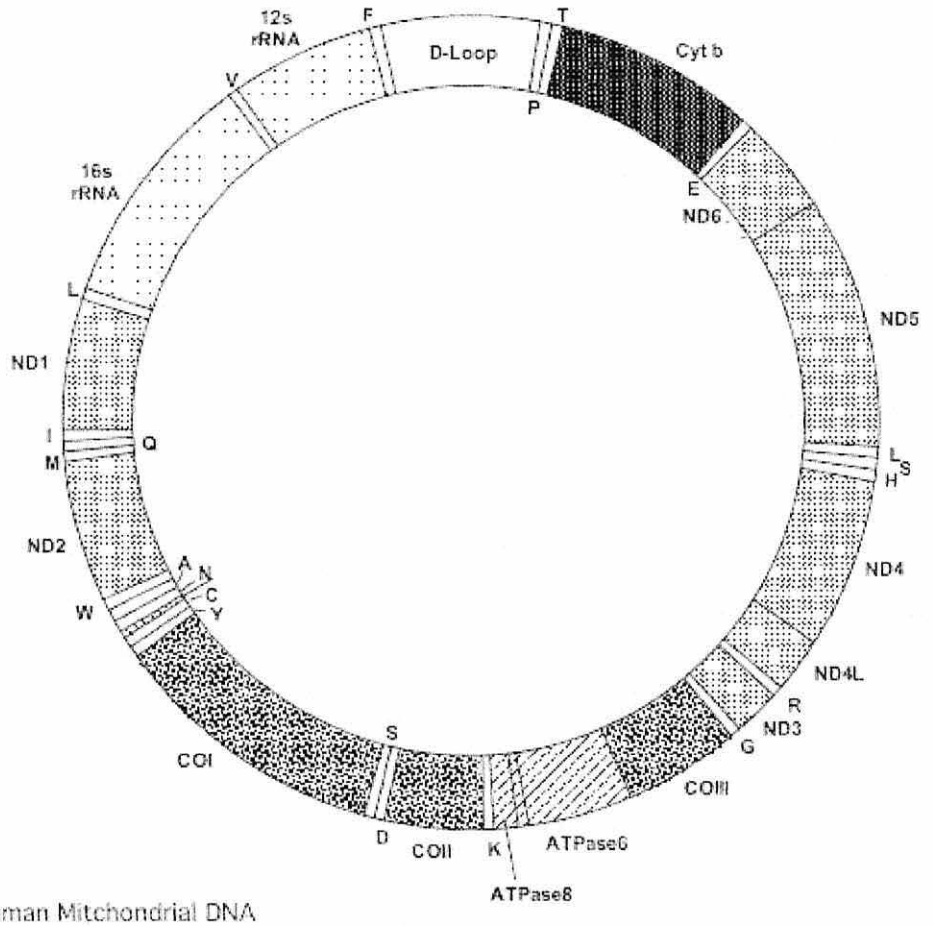
1.3 Mitochondrial DNA

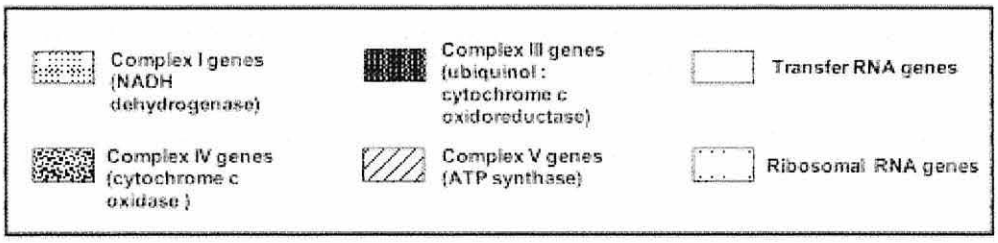
1.31 Structure, function and evolution

The mitochondrial DNA (mtDNA) of metazoans is a closed circular molecule (Maynard-Smith 1989), ranging in size from approximately 14 to 39 kilobases (kb) (Moritz *et al.* 1987; Sederoff 1984; Snyder *et al.* 1987). This size variation is, in most cases, due to differences in non-coding DNA sequence, which does not code for any gene product (Harrison 1989). Generally, metazoan mtDNA gene content and arrangement is highly conserved, comprising 13 protein coding genes or messenger RNA genes, 22 transfer RNA genes to translate the mitochondrial genetic code, and 2 ribosomal RNA genes for the small and large subunit ribosomal RNAs (Hoffmann *et al.* 1992; Maynard-Smith 1989). A map of the human mtDNA molecule is shown in Figure 1.2.

Molluscs and nematodes present exceptions to the conservation of gene arrangement observed in other taxa examined (Hoffmann *et al.* 1992). Both phyla have members which differ from the typical metazoan pattern, and both show variation in gene order between subgroups within the phylum. The mtDNA of the mussel *Mytilus edulis* is of average metazoan length (17.1 kb) but has a gene arrangement different to any other known, in coding for an extra transfer RNA (for methionine) and in lacking a protein coding gene for ATPase subunit 8 (Hoffmann *et al.* 1992; Maynard-Smith 1989; see Figure 1.3.). The gene for ATPase subunit 8 is also missing in the nematodes for which mtDNA gene content is known (Hoffmann *et al.* 1992). Information on the mtDNA of other bivalves is scarce, but some data on the gene sequence of an individual from the genus *Lasaea* and the Japanese scallop *Patinopecten yessoensis* suggests that the gene order in these species is not identical to that of *M. edulis* (Hoffmann *et al.* 1992). Boore and Brown (1994) determined the mtDNA sequence for the polyplacophoran *Katharina tunicata* and found it to differ substantially from that for *M. edulis*. The *K. tunicata* mtDNA included a gene for ATPase subunit 8 and was more similar to mtDNA from the genus *Drosophila* than to *M. edulis* (Boore and Brown 1994).

Figure 1.2 Diagram to show the gene content of the human mitochondrial genome. Modified from MITOMAP http://www.gen.emory.edu/mitomap.html



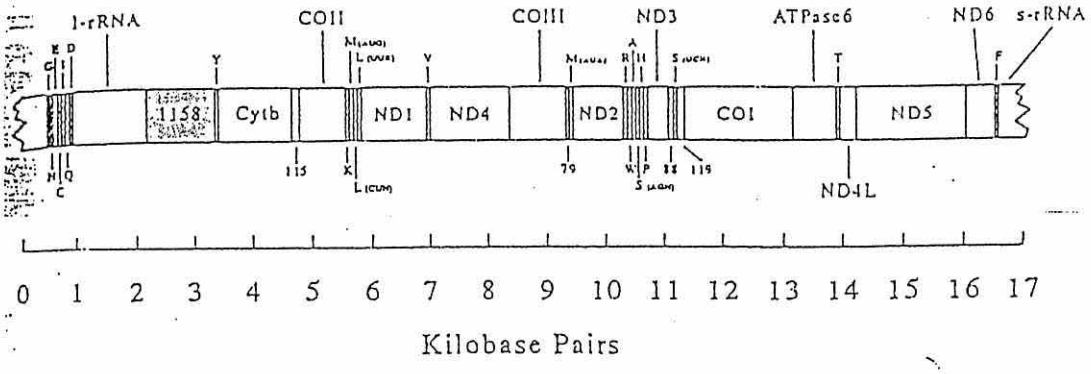


13 protein coding genes: Cyt b, ND6, ND5, ND4, ND4L, ND3, COIII, ATPase 6, ATPase 8, COII, COI, ND2, ND1.

22 transfer RNA genes (marked by single letter amino acid codes):
A,C,D,E,F,G,H,I,K,2*L,M,N,P,Q,R,2*S,T,V,W,Y.

2 ribosomal RNA genes: 12srRNA, 16srRNA.

Figure 1.3 Diagram to show the gene content of a *Mytilus edulis* mtDNA molecule. (Modified from Hoffmann *et al.* 1992). Segments labelled with numbers are unassigned intergenic regions, the number indicates the size in nucleotides. NB. There is no ATPase 8 gene & there are 2 transfer RNA genes for methionine (labelled M).



Although the rate of gene order change seems to be very slow for animal mtDNA, the rate of nucleotide substitution is much greater than in nuclear DNA (Maynard-Smith 1989). Again *Mytilus* mtDNA is unusual, with a rate of nucleotide substitution more than twice that for human or *Drosophila* mtDNA (Hoeh *et al.* 1996a).

1.32 MtDNA inheritance

Mitochondria are cytoplasmic organelles and are thus inherited cytoplasmically. As the cytoplasm of a fertilized egg is derived mainly from the mother, in most animals mtDNA is thought to be predominantly maternally inherited (Avise and Lansman 1983). Hence individuals are usually homoplasmic, containing only one type of mtDNA: that derived from their mother (Maynard-Smith 1989). A low level of paternal mtDNA transmission has been found to occur in some animals, for example: mice (Gyllensten *et al.* 1991), anchovy (Magoulas and Zouros 1993), *Drosophila* sp. (Satta 1988); and in some plants (Neale *et al.* 1989; Reboud and Zeyl 1994). Where the father's mtDNA differs from that of the mother, this can result in low levels of heteroplasmy (up to 5%); the occurrence of more than one type of mtDNA within an individual (Clark 1988).

1.33 Doubly uniparental inheritance of mtDNA

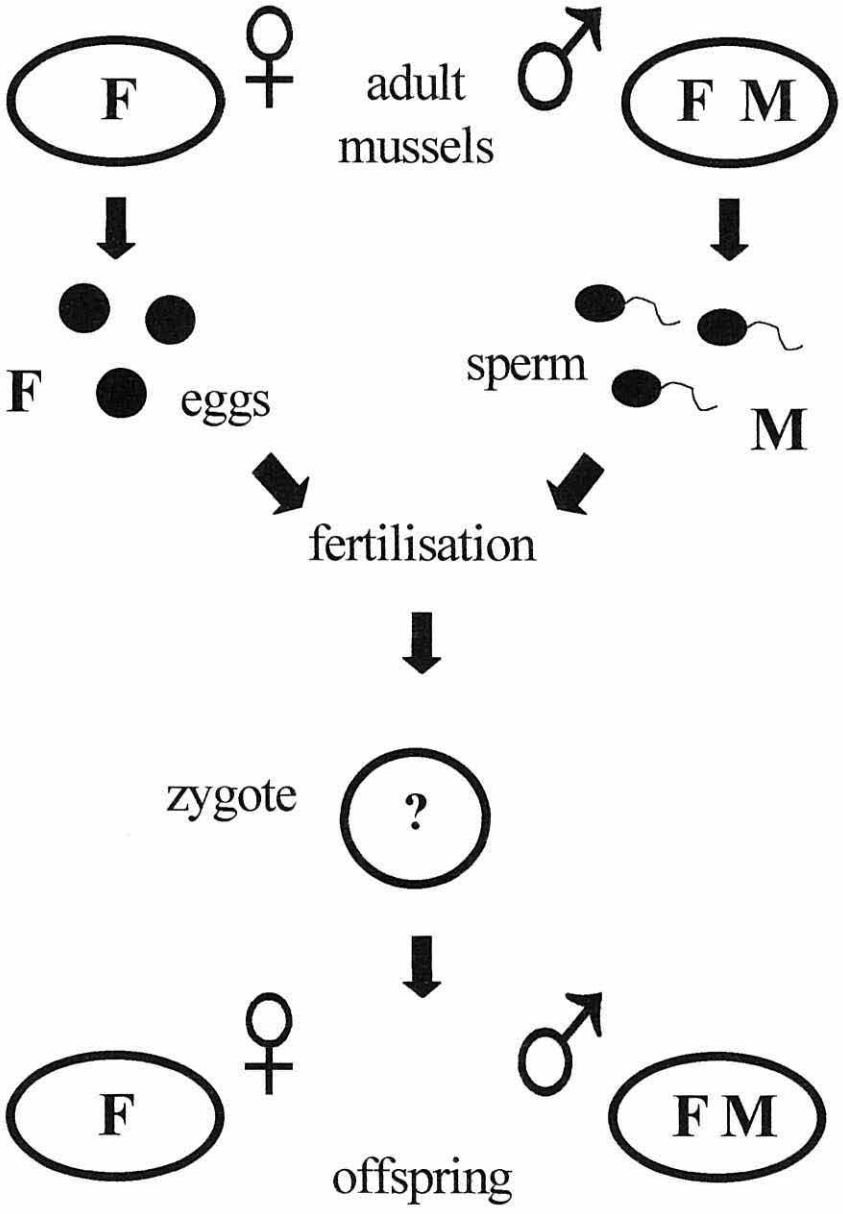
In *Mytilus* relatively high levels of heteroplasmy (up to 60%) have been observed (Fisher and Skibinski 1990; Hoeh *et al.* 1991). Furthermore the occurrence of heteroplasmy is associated with sex (Fisher and Skibinski 1990; Skibinski *et al.* 1994; Zouros *et al.* 1992; Zouros *et al.* 1994). Female mussels are homoplasmic for a genome termed "F" by Fisher and Skibinski (1990) whereas males are heteroplasmic for this and a second genome, termed "M". However, the gametes of both sexes are homoplasmic, in females for F and in males for M. The theory is that males inherit F from their mother and M from their father, but transmit only M in sperm to their sons. Females inherit only F from their mother and transmit this to both sons and daughters. Hence the two

genomes maintain separate transmission routes, a phenomenon termed "doubly uniparental inheritance" (DUI) by Zouros *et al.* (1994) (see Figure 1.4).

Most incidences of mtDNA heteroplasmy in animals detected to date seem to be associated with length variation (Clark 1988). In *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus californianus*, sequence heteroplasmy exists, with the F and M genomes showing nucleotide sequence divergence of between 9 and 31% (Beagley *et al.* 1997; Fisher and Skibinski 1990; Hoeh *et al.* 1991; Quesada *et al.* 1996; Skibinski *et al.* 1994). In contrast *Mytilus trossulus* exhibits length heteroplasmy; the two genomes differ in length by up to 3 kilobases (Wenne and Skibinski 1995).

The freshwater mussel *Pyganodon* (*Anodonta*) *grandis* (Bivalvia: Unionidae) also has gender-specific mtDNA, with an estimated sequence divergence of 6.8% between the male and female types (Liu *et al.* 1996). No evidence of heteroplasmy was found by Liu *et al.* (1996) but only gonadal tissue was used for DNA extraction therefore the occurrence of heteroplasmy in somatic tissues, as in male *Mytilus* spp., cannot be ruled out. Hoeh *et al.* (1996b) observed gender-associated mtDNA lineages in three other bivalve species: the unionids *Pyganodon fragilis* and *Fusconaia flava* and the mytilid *Geukensia demissa*. Although this is a characteristic feature of DUI of mtDNA, DUI was not directly demonstrated for these species. Gender-associated mitotypes have also been detected in the venerid clam *Tapes philippinarum* (Passamonti and Scali 2001), in which male and female gonads were homoplasmic for mitotypes with approximately 15% sequence divergence. Male *T. philippinarum* somatic tissues were heteroplasmic for both these mitotypes. The observations in *T. philippinarum* show strong similarity with the DUI system in *Mytilus* and support the hypothesis that DUI may be an ancestral character in the Bivalvia.

Figure 1.4 Doubly Uniparental Inheritance of the female (F) and male (M) mitotypes in *Mytilus*. The F mitotype is inherited maternally and the M mitotype paternally. See text for explanation. (Modified from Hurst and Hoekstra 1994).



1.34 Evolution of mussel mtDNA and the origin of DUI

DUI results in two distinct gender-associated lineages (M and F). The M mitotype has been found to be more divergent and to evolve at a faster rate than the F mitotype for several parts of the *Mytilus* mtDNA genome in independent studies. For example, the CO III gene and the ND2 gene (Skibinski *et al.* 1994), the 16S rRNA gene (Rawson and Hilbish 1995b), the CO III gene (Stewart *et al.* 1995) and a region of mtDNA with no assigned function (Stewart *et al.* 1996). Male mitotypes were also observed to be more divergent than female mitotypes in *Pyganodon grandis* (Liu *et al.* 1996) and in *Tapes philippinarum* (Passamonti and Scali 2001). Greater divergence in itself suggests faster evolution for the M mitotype, assuming the two lineages are of equal age. It has been suggested that the difference in evolution rate between the M and F mitotypes could be explained by a difference in the degree of selection operating on these molecules. Some evidence for this may be found by examining nucleotide substitutions occurring within the M and F mitotypes. Two categories of nucleotide substitutions may be considered: synonymous and non-synonymous. Synonymous substitutions are those which do not change the amino acid coded by the sequence, non-synonymous substitutions do change the amino acid coded for. Hence selection might act upon non-synonymous substitutions but not synonymous ones. Stewart *et al.* (1996) found that *Mytilus* M and F mitotypes accumulate synonymous substitutions at similar rates, but the M mitotype accumulates non-synonymous substitutions at a higher rate than the F mitotype. This suggests that either M is under relaxed selection or F is under relatively strong selection (Stewart *et al.* 1996).

The high sequence divergence between M and F is consistent with little or no recombination and long term maintenance of the heteroplasmic state (Skibinski *et al.* 1994). No evidence has yet been found for recombination between these two lineages (Rawson and Hilbish 1995b), and indeed the high sequence divergence (normally > 20%) found between the M and F mitotypes is considered to be prohibitive for homologous recombination in other organisms (Rayssiguier *et al.* 1989; Selva *et al.* 1995). However, in a minority of males, an

F mitotype may invade the M transmission route, resulting in newly established M molecules (M^F) whose sequences are initially similar to F but diverge rapidly. In males from natural populations which are heteroplasmic for the M^F and F mitotypes the sequence divergence has been estimated to be about 4% (Hoeh *et al.* 1997). Ladoukakis and Zouros (2001) have provided direct evidence that mtDNA recombination between F and M^F mitotypes occurs at a high rate in males from a Black Sea population of *M. galloprovincialis*.

Within the *M. edulis* complex (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) the divergence between M and F lineages is greater than that between species within lineages (Rawson and Hilbish 1995b, Stewart *et al.* 1995). Rawson and Hilbish (1995b) looked at variation in the 16S rRNA gene in all three species and found four mitotypes: one associated with males of all three species and three associated with females but with differing species distributions. Sequence analysis produced two major mtDNA clades associated with gender (M and F), within which sequences from *M. edulis* and *M. galloprovincialis* were more closely related to each other than to those from *M. trossulus* (Rawson and Hilbish 1995b). This is supported by Stewart *et al.* (1995) who looked at CO III gene sequences from *M. edulis* and *M. trossulus*. They also found that the M and F mtDNA sequences were grouped into two distinct lineages, irrespective of species origin. Both groups of workers concluded that the time of the M/F split, and the origin of DUI, is likely to predate the formation of these three species.

1.35 Is DUI linked to sex determination in some way?

The gender-associated properties of the M and F genomes suggest an interaction between mtDNA and sex determining factors (Skibinski *et al.* 1994; Zouros 1995). Little is known about the mechanism of sex determination in *Mytilus*, or for molluscs in general (Guo and Allen 1994).

Saavedra *et al.* (1997) investigated sex ratio and mtDNA inheritance in pair matings of *M. galloprovincialis*. They found that sex ratio varied widely between families and noticed a pattern in this variation. Families from the same

mother had similar sex ratio, whereas those from the same father but different mothers showed no pattern (Saavedra *et al.* 1997). This suggests that sex ratio is under maternal control. It is possible that the sex, or the probablity of becoming either sex, is determined in an egg before fertilisation (Saavedra *et al.* 1997). However, the sex ratio variance between families from the same mother was significantly greater than zero for one of the five mothers, indicating that other factors must play a part in determining sex ratio (Saavedra *et al.* 1997).

Within Saavedra *et al.* (1997)'s study, 50 male progeny which lacked the M mitotype were observed. Of these, 44 shared the same father, which only had one son containing the M mitotype. No difference was found between the sperm of this male and that of the other fathers: as expected it contained M but not F (Saavedra *et al.* 1997). The M-negative sons had F mitotypes corresponding to those of their mothers therefore it seems that they did not receive (or retain) F from their father (Saavedra *et al.* 1997). However the gonads of the M-negative sons did contain the F genome, whether this would be passed on to their offspring is unknown (Saavedra *et al.* 1997). The remaining 6 M-negative sons were fathered by two other males which did show some evidence of the F mitotype in their sperm. Possibly some sperm from these males had no M mitotype, and resulted in the M-negative sons. However no evidence of the father's F mitotype was found in these sons (Saavedra *et al.* 1997). A further observation made by Saavedra *et al.* (1997) was that M-negative sons occurred more frequently with those mothers which gave a female biased sex ratio.

The occurrence of heteroplasmic females and/or homoplasmic males in interspecific (Zouros *et al.* 1994) and intraspecific (Saavedra *et al.* 1997) crosses of *Mytilus* rules out any direct causal role for mtDNA in sex determination. It has been suggested that interactions between mtDNA and factors associated with a nuclear switch may be likely (Skibinski *et al.* 1994). Possibly a sperm-derived mitochondrial factor is necessary in early germ cells to induce production of a male gonad but continuous presence of this factor is not necessary to maintain maleness (Saavedra *et al.* 1997). The probability of the sperm's mtDNA entering the germ line might be dependent on the mother's

genotype, it would be small in eggs produced by female biased mothers and high in those from male biased mothers (Saavedra *et al.* 1997).

Saavedra *et al.* (1997) propose a model with a paternally coded mitochondrial factor which provides a replicative advantage, and a maternally coded egg factor which suppresses this advantage to varying degrees. They suggest that in female biased families the sperm mtDNA would only enter the germ line in a few eggs and would be vulnerable to elimination from these, which would give rise to M-negative sons. Saavedra *et al.* (1997) observed the similarity between their model and the system by which maternal inheritance of mtDNA is maintained in mice (Kaneda *et al.* 1995; Shitara *et al.* 1998). In mice, however, the elimination of paternal mitochondria seems to depend on the presence of a species-specific nuclear-encoded factor, rather than a mitochondrial factor (Kaneda *et al.* 1995). In hybrid mouse crosses the recognition of the sperm factor by the egg factor is not perfect therefore elimination of paternal mtDNA is less efficient, but paternal leakage appears to be limited to the first hybrid generation only (Shitara *et al.* 1998).

1.36 What do we know about the mechanism of DUI?

The sperm's contribution to the mtDNA pool of the zygote would be minimal compared to that of the egg (Avise 1991). Indeed Skibinski *et al.* (1994) estimated that there would be about 10 000 times as many F molecules as M in a fertilized egg. However in the somatic tissue of adult *Mytilus* males Skibinski *et al.* (1994) often found a predominance of M over F. This suggests that the M genome has a replicative advantage over the F genome in males (Fisher and Skibinski 1990; Skibinski *et al.* 1994; Zouros *et al.* 1994). In females this must be suppressed, or the M genome must be either excluded or eliminated from an egg destined to become a female (Skibinski *et al.* 1994).

Sutherland *et al.* (1998) suggested the possibility that, in *Mytilus*, sperm mtDNA may only enter eggs destined to become males and not those destined to become females, which would explain why male, but not female, adult mussels possess

the mtDNA of their father. During the fertilisation of most molluscs the sperm mitochondria enter the egg (Lambert and Battaglia 1993). However in some cases the sperm mitochondria are excluded, for example in the brackish water bivalve *Laternula limicola* (subclass Anomalodesmata) the single sperm mitochondrion remains outside the egg during fertilisation (Lambert and Battaglia 1993). The *M. edulis* spermatoazoan contains five mitochondria which are larger than those seen in somatic cells (Longo and Dornfeld 1967). Longo and Anderson (1969) observed that the *M. edulis* sperm is wholly incorporated into the egg during fertilisation, and that during the formation of the male pronucleus the five, enlarged sperm mitochondria seem to decrease in size until they become indistinguishable from the maternal mitochondria. It has been suggested that the transformed sperm mitochondria may exist as functional units throughout embryogenesis (Longo 1973), but Longo and Anderson (1969) observed an apparent disintegration and swelling in some portions of the sperm mitochondria, which is suggestive of a limited influence of the sperm mitochondria on embryogenesis.

To investigate this further Sutherland *et al.* (1998) produced a series of families of *M. edulis* from parents which had previously produced only female offspring, or a combination of both sexes. They sampled progeny at 18, 24 and 48 hours after fertilisation and also after 14 days, 3 and 6 months, and examined them individually for presence of M mtDNA. All the 18 hour old larvae showed presence of M mtDNA at a relatively high frequency, but some of the 24 and 48 hour old larvae had no traces of M mtDNA. This suggests that sperm mitochondria do enter the egg irrespective of which sex it will become, confirming the observations of Longo and Anderson (1969), and may be eliminated in females between 18 and 24 hours after fertilisation (Sutherland *et al.* 1998).

The faster replication of M and its preferential transmission to sperm implies a fitness advantage (Skibinski *et al.* 1994), or some mechanism of replication control, of the M genome over the F genome. Hastings (1992) suggested that *Mytilus* might possess 'selfish' mitochondria, which might possibly result in a

sex ratio bias in favour of males, thus further increasing the fitness of the M genome (Hurst 1993). The theoretical sex ratio for truly gonochoristic bivalves is 1:1 (Mackie 1985; Morton 1991) and studies of natural populations of *Mytilus* species have not found any deviation from this (e.g. Sastry 1979; Brousseau 1983; Fisher and Skibinski 1990; Skibinski *et al.* 1994). One reason suggested for the evolution and maintenance of anisogamy and maternal organelle inheritance is that it prevents the spread of deleterious cytoplasmic genomes outside the maternal lineage in which they arise. The system of DUI in *Mytilus* supports this, with not one but two uniparental transmission routes (Hurst and Hoekstra 1994).

1.4 Is DUI affected by hybridisation in the *M. edulis* complex?

Mussels of the *M. edulis* complex are found at temperate latitudes throughout the Northern and Southern Hemispheres (McDonald *et al.* 1991). In areas where two of the taxa are present in sympatry, a varying degree of hybridisation has been found to occur (McDonald *et al.* 1991; Gosling 1992). Where backcrossing of hybrids with parental taxa takes place, it can lead to introgression of nuclear and/or mitochondrial DNA from one taxon to the other (Harrison 1991; Arnold 1992). Introgression of mtDNA often occurs separately from nuclear alleles, as a result of differing selective pressures and because mitochondria segregate independently of chromosomes (Barton and Hewitt 1989; Harrison 1989). The doubly uniparental mtDNA inheritance in *Mytilus* and the tissue-specific distribution of M and F in males may impose greater requirements for compatibility between nuclear and mitochondrial genomes than in species with standard maternal inheritance (Saavedra *et al.* 1996). For introgression to occur, compatibility of one species' nuclear DNA with the other species' mtDNA would be required in addition to compatibility of one species' F mitotype with the other species' M mitotype in hybrid males (Saavedra *et al.* 1996).

Most hybrid zones are narrow relative to the species range and to their dispersal range. It is generally thought that selection against hybrids is more important than environmental factors in maintaining hybrid zones (Barton and Hewitt 1989). However, for *Mytilus*, there is a variety of evidence indicating that the fitness of hybrid mussels is not inferior to that of parental taxa (Gardner 1994). Little is known about how *Mytilus* hybrid zones are maintained in this group of taxa which have a prolonged larval stage facilitating dispersal over great distances. No evidence for gamete incompatibility between species has been found (Zouros *et al.* 1992; Beaumont *et al.* 1993), indicating that post-zygotic factors may limit introgression where species distributions overlap. If the system of DUI is disrupted by hybridisation, then the level of introgression among *Mytilus* species may be affected, which could be an important factor in the maintenance of genetic integrity for each species (Rawson, Secor and Hilbish 1996).

Rawson, Secor and Hilbish (1996) studied variation in the mitochondrial 16S rRNA gene sequence in a hybrid population of *Mytilus edulis* and *M. galloprovincialis* in South-West England, using allozyme analysis to determine species/hybrid genotype. They found no significant disruption of DUI or variation from a 1:1 sex ratio among the hybrids. Edwards and Skibinski (1987) and Rawson and Hilbish (1998) observed that *M. galloprovincialis* has some mitotypes common to *M. edulis*, in addition to mitotypes unique to *M. galloprovincialis*. Edwards and Skibinski (1987) interpreted this as an asymmetrical flow of mtDNA from *M. edulis* to *M. galloprovincialis*, which could be a result of asymmetrical dispersal patterns of larvae of the two species. Rawson and Hilbish (1998) investigated this and an alternative explanation, that the pattern observed could result from incomplete lineage extinction, given the relatively recent separation of the two species. They examined 16S rRNA gene sequences from maternal and paternal mtDNA lineages for divergence between the species, and found strong support for the introgression hypothesis. This asymmetrical mtDNA introgression could be generated by selection in favour of mussels with *M. galloprovincialis* nuclear alleles, which has been shown to exist in hybrid populations (Skibinski *et al.* 1983; Gardner and Skibinski 1987; Skibinski and Roderick 1991; Gardner 1994). This selection would tend to remove mussels with mainly *M. edulis* nuclear genotypes which carry introgressing *M. galloprovincialis* mtDNA, but not those with mainly *M. galloprovincialis* nuclear genotypes and *M. edulis* mtDNA (Rawson and Hilbish 1998). Rawson and Hilbish (1998) also found evidence of introgression of *M. edulis* mtDNA into populations of *M. trossulus* in the Baltic Sea, as did Quesada, Wenne and Skibinski (1995).

In contrast to the findings in Europe, Saavedra *et al.* (1996) found no evidence for introgression of mtDNA in a natural population of coexisting and hybridizing *Mytilus edulis* and *M. trossulus* in Nova Scotia, Canada. Their results suggested that introgression is blocked fairly early in the hybridisation process, indicating that there could be some degree of incompatibility between mtDNA from one species and nuclear genes from the other (Saavedra *et al.*

1996). To investigate compatibility between the M and F mitotypes of different species, independently of compatibility between mtDNA and nuclear genes, Saavedra *et al.* (1996) used male F_2 hybrids. Although only four mussels in their sample could be classified as such, all of these possessed M and F mitotypes from the same species, suggesting a possible incompatibility between M and F mitotypes of different species (Saavedra *et al.* 1996). All the hybrid males studied by Saavedra *et al.* (1996) were heteroplasmic for M and F and the females were homoplasmic for F, indicating no breakdown of DUI had occurred in hybrids of *M. edulis* and *M. trossulus*. However, in laboratory reared mussels from *M. edulis*/*M. trossulus* crosses, Zouros *et al.* (1994) did find some evidence of breakdown of DUI, with the occurrence of several M-negative males.

Rawson and Hilbish (1995a) examined geographic variation in the 16S rRNA gene for *Mytilus trossulus* and *M. galloprovincialis* along the Pacific coast of North America using methods which detected both M and F mitotypes in both species. Their results confirmed the species distributions detected by a previous allozyme study (Sarver and Foltz 1993). There was evidence of only a limited amount of introgression between the two species in the zone of contact (Rawson and Hilbish 1995a). One site, Bodega Bay, gave contrasting results for the allozyme (Sarver and Foltz 1993) and mtDNA (Rawson and Hilbish 1995a) studies. Sarver and Foltz (1993) found mainly *M. trossulus* nuclear alleles and Rawson and Hilbish (1995a) found mainly *M. galloprovincialis* mitotypes. This could be explained by the introgression of *M. galloprovincialis* mtDNA into *M. trossulus*, but very few mussels were found to be heteroplasmic for M and F mitotypes from different species, so this explanation seems unlikely (Rawson and Hilbish 1995a). In addition, Rawson and Hilbish (1995a) state that an ongoing study of nuclear alleles in the Bodega Bay population indicates the presence of mostly *M. galloprovincialis* genotypes. Rawson and Hilbish (1995a) suggest that Bodega Bay may receive cohorts of larvae of different species over time, or that there may be microgeographic separation of the two species within the bay, but further study would be required to evaluate the likelihood of these possible explanations.

Rawson, Secor and Hilbish (1996) carried out a more detailed study of mtDNA in a single hybridizing population of *Mytilus galloprovincialis* and *M. trossulus* in San Francisco Bay, California. Both M-negative hybrid males and M-positive hybrid females were found, indicating that DUI is disrupted in male and female hybrids of these two species. In addition, within the hybrid mussels, the sex ratio was biased with approximately four females to one male (Rawson, Secor and Hilbish 1996). Such a strong bias suggests the possibility that hybridisation between *M. galloprovincialis* and *M. trossulus* may be associated with a bias towards either the production or the survival of females (Rawson, Secor and Hilbish 1996).

It seems that the disruption of DUI in hybrid crosses depends, at least partly, on the species involved. There is little evidence for disruption of DUI in *M. edulis*/*M. galloprovincialis* hybrids (Rawson, Secor and Hilbish 1996), limited breakdown of DUI seems to occur in some *M. edulis*/*M. trossulus* hybrid populations (Zouros *et al.* 1994) but not others (Saavedra *et al.* 1996), and in *M. galloprovincialis*/*M. trossulus* hybrids disruption of DUI appears to be more common (Rawson, Secor and Hilbish 1996). There is always the possibility that the breakdowns of DUI observed in these studies are unrelated to hybridisation, particularly in view of the breakdown of DUI observed in intraspecific crosses (Saavedra *et al.* 1997). On the other hand, it could be that disruption of DUI is more common in hybrid crosses, but if disruption of DUI has a negative effect on the fitness of a mussel then it may not survive to adulthood and the true extent of disruption might not be detected in natural hybrid populations. However, the pattern of disruptions implies that *M. edulis* is more closely related to *M. galloprovincialis* than either is to *M. trossulus*, and that *M. trossulus* is closer to *M. edulis* than to *M. galloprovincialis*, where the regulation of M mitotype inheritance is concerned. These relationships are consistent with results of allozyme analysis and phylogeny of the M and F mitotypes for the three species (Rawson, Secor and Hilbish 1996), thus it appears that phylogenetic distance may have some effect on the occurrence of disruption of DUI in hybrid mussel populations.

1.5 Aim

The aim of this study is to assess the resilience of DUI to hybridisation in the *M. edulis* complex by following the inheritance and fate of the F and M mitotypes in pure species and F_1 generation hybrid larvae produced in controlled laboratory crosses.

Previous studies have detected disruption of DUI to varying degrees in mussels from natural hybrid populations (Rawson, Secor and Hilbish 1996; Saavedra *et al.* 1996). However, due to uncertainties about the precise genetic and geographic background of mussels from hybrid zones, and to unknown variation in environmental conditions between studies, the conclusions that can be drawn from the results of these studies are limited. A laboratory based approach, performing crosses between mussels from allopatric populations of known species identity, has more power to quantify the extent of disruption to DUI in hybrid mussels in comparison to that in pure species mussels. Zouros *et al.* (1992, 1994) studied DUI in laboratory crosses between *M. edulis* and *M. trossulus* from eastern Canada but there have been no published studies of DUI in laboratory produced hybrids between *M. edulis* and *M. galloprovincialis* or between *M. galloprovincialis* and *M. trossulus*.

Since the previous studies mentioned above, several advances have been made in our knowledge of the DUI system through the work of Saavedra *et al.* (1997) and Sutherland *et al.* (1998). The results of Saavedra *et al.* (1997)'s study are important because they suggest that it is valid to assume that families which share the same mother should have the same sex ratio. If this assumption is made then more inferences can be drawn from studies of larval or juvenile mussels which are too young to be sexed. Sutherland *et al.* (1998)'s results, which show that the M mitotype is inherited in all larvae and eliminated in a proportion of larvae between 18 and 24 h after fertilisation, suggest that any disruption to DUI is likely to occur in the early stages of development. Sutherland *et al.* (1998)'s study was restricted to pure *M. edulis* larvae and it is

not yet known if the results also apply to *M. galloprovincialis* or *M. trossulus*, or to hybrid larvae.

Laboratory based crosses can produce larvae of known parentage and offer an opportunity to investigate inheritance and elimination of the M mitotype in pure species and hybrid larvae which share the same mother and should therefore have similar sex ratios (Zouros *et al.* 1994; Saavedra *et al.* 1997). This will enable direct comparison of pure species and hybrid larvae with respect to the inheritance and fate of the F and M mitotypes. This approach has the potential to determine whether it is the inheritance or the elimination of the M mitotype which is disrupted in hybrid mussels, a distinction which has not been possible in previous studies of DUI.

Assessment of the extent of disruption of DUI in hybrid mussels is necessary to determine whether disrupted mtDNA inheritance is important in maintaining genetic differences among *Mytilus* species. Such information will increase understanding of the causes of limited introgression of nuclear alleles and mtDNA genomes across species contact zones. The study of disruption of DUI in hybrid mussels may also provide insights into the mechanisms that regulate DUI and the relationship between DUI and sex determination in *Mytilus*, about which little is known.

Chapter 2. General Materials and Methods

2.1 Sources of Mussels

Adult mussels were obtained from six populations, four of which are thought to contain pure species based on previous allozyme analysis (Skibinski *et al.* 1983; McDonald *et al.* 1991; Gosling 1992; Wenne and Skibinski 1995). *Mytilus edulis* populations used were from the Menai Strait in North Wales (32 ppt salinity) and Loch Etive in Scotland (32 ppt salinity). The *Mytilus galloprovincialis* were collected near to Montpellier on the Mediterranean coast of France (32 ppt salinity) and *Mytilus trossulus* were obtained from the Gulf of Gdansk in the Baltic Sea (10 ppt salinity). Putative *M. edulis* were also collected from an aquaculture facility in the Bay of Lunenburg, Nova Scotia, Canada (31 ppt salinity), where they had been grown from seed collected in Lameque, New Brunswick, Canada. The Bay of Lunenburg contains both *M. edulis* and *M. trossulus*, but the Lameque area is known for its low frequency of *M. trossulus*. Putative *M. trossulus* were collected from a different area of the Bay of Lunenburg and were selected on the basis of shell morphometry. This method of species identification has previously shown a high level of concordance with allozyme studies in this area.

Mussels were transported live and held in a Ministry of Agriculture, Fisheries and Food approved flowing seawater quarantine system, at their source salinity (at 32 ppt for Bay of Lunenburg mussels) and at approximately 6°C, with added microalgae, until required for hybridisation trials. For trials involving *Mytilus trossulus* from the Baltic Sea, all potential parents were gradually acclimatised over a two week period to an intermediate salinity of 25 ppt.

2.2 Hybridisation Trials and Larval Rearing

Eleven hybridisation trials were performed between 1998 and 2000 (Table 2.1). Between 20 and 50 mussels from each species to be used were removed from the holding tanks and induced to spawn by the following method. 2 ml of 0.5 M

KCl was injected into the mantle cavity of each mussel, followed by a period of 1-2 h out of water at 14°C. Seawater was passed through a 0.2 μm filter and UV treated prior to use in the trials, larval rearing and sampling. Each mussel was placed in a glass jar of seawater so that gametes for each individual could be collected. If the required number of individuals did not spawn within 3-4 h, jars were placed in a water bath at 20°C to attempt to induce spawning.

Once spawning had occurred, the required number of parent mussels were selected. The eggs from individual females were transferred into 1l measuring cylinders in 1l of seawater, mixed using a stirring rod and samples were taken to check for morphological normality and for counting in order to estimate the total number of eggs in the cylinder. The spermatozoa were checked for motility and the sperm concentration for each male was compared by eye.

Fertilisations were carried out by mixing eggs from individual females and spermatozoa from individual males in separate containers. Embryos were checked for the presence of polar bodies and then divided into 2 or 3 separate 1 litre crystallizing dishes as pseudo-replicates for each cross. Approximately 100 000 eggs were used per dish and the spermatozoan to egg ratio was between 10 and 100:1. Fertilised eggs were allowed to develop undisturbed at 14°C for 3 days, when assessments of larval survival and level of morphological abnormality were made.

Table 2.1. Hybridisation trials performed in 1998 - 2000. E, *Mytilus edulis*; G, *Mytilus galloprovincialis*; T, *Mytilus trossulus*. f, female; m, male. Me15/16 genotype is given only where this differed from expectation. xh, larvae sampled x hours after fertilisation.

Trial	Date	Species	Location	Salinity (ppt)	Family no.	Parents	Me15/16 genotype	Samples of larvae obtained (N=normal, A=abnormal) 3h	6h	8h	15h	18h	21h	24h	48h	72h
0	03/07/98	E	N. Wales	32	1	Ef1 Em1		N		N		N				N
					2	Ef1 Em2		N		N		N				N
					3	Ef1 Em3		N		N		N				N
					4	Ef2 Em1		N		N		N				N
					5	Ef2 Em2		N		N		N				N
					6	Ef2 Em3		N		N		N				N
I	13/04/99	E	N. Wales	32	7	Ef1 Gm1		N	N		N	N	N	N	N	N
		G	Mediterr		8	Gf1 Gm1		N	N		N	N	N	N	N	N
II	25/05/99	E	Canada	32	9	Ef1 Em1	E/Tf Em	N			N	N	N	N	N	N
		T	Canada		10	Ef1 Em2	E/Tf Em									N
					11	Ef1 Tm1	E/Tf E/Tm	N			N	N	N	N	N	A
					12	Tf1 Em1	Tf Em	N			N	N	N	N	N	
					13	Tf1 Tm1	Tf E/Tm	N			N	N	N	N	N	
					14	Tf1 Tm2										N
III	14/06/99	E	Scotland	32	15	Ef1 Em1		N			N	N	N	N	N	A
		G	Mediterr		16	Ef1 Gm1		N			N	N	N	N	N	
					17	Gf1 Em1		N			N	N	N	N	N	N
					18	Gf1 Gm1		N			N	N	N	N		
					19	Gf1 Gm2										
IV	23/07/99	E	Scotland	25	20	Ef1 Em1		N				N	N	N	N	N
		G	Mediterr		21	Ef1 Tm1	Ef E/Tm	N				N	N	N	N	N
		T	Baltic		22	Tf1 Em1	Ef Em	N				N	N	N	N	A
					23	Tf1 Tm1	Ef E/Tm	N				N	N	N	N	A
					24	Tf1 Gm1	Ef Gm	N				N	N	N	N	
					25	Gf1 Tm1	Gf E/Tm	N				N	N			
					26	Gf1 Gm1		N				N	N	N	N	N

Table 2.1 continued

Trial	Date	Species	Location	Salinity (ppt)	Family no.	Parents	Me15/16 genotype	Samples of larvae obtained (N=normal, A=abnormal) 3h	6h	8h	15h	18h	21h	24h	48h	72h
V	17/08/99	E G T	Scotland Mediterr Baltic	25		No viable gametes obtained										
VI	24/08/99	E T	Scotland Baltic	25		No viable gametes obtained										
VII	28/09/99	E	Canada	32	27	Ef1 Tm1		N				N		N	N	N
		T	Canada		28	Tf1 Tm1		N				N		N	N	N
					29	Tf1 Tm2		N				N		N	N	N
VIII	19/06/00	E	Scotland	32	30	Ef1 Em1		N				N		N	N	N
		G	Mediterr		31	Ef1 Em2		N				N		N	N	N
					32	Ef2 Em1		N				N		N	N	N
					33	Ef2 Em2		N				N		N	N	N
					34	Ef1 Gm1		N				N		N	N	N
					35	Ef1 Gm2		N				N		N	N	N
					36	Ef2 Gm1		N				N		N	N	N
					37	Ef2 Gm2		N				N		N	N	N
					38	Gf1 Em1		N				N		N	N	N
					39	Gf1 Em2		N				N		N	N	N
					40	Gf2 Em1		N				N		N	N	N
					41	Gf2 Em2		N				N		N	N	N
					42	Gf1 Gm1		N				N		N	N	N
					43	Gf1 Gm2		N				N		N	N	N
					44	Gf2 Gm1		N				N		N	N	N
					45	Gf2 Gm2		N				N		N	N	N
IX	15/08/00	E G	Scotland Mediterr	32		As for Trial VIII										
X	29/08/00	E G	Scotland Mediterr	32		As for Trial VIII										

2.3 Sampling of Mussel Tissues

For DNA analysis, samples of adductor muscle and gill tissue were taken from each individual and preserved in 70% ethanol / 30% seawater in 1.5 ml microtubes at 4°C. Replicate samples were frozen at -20°C. For allozyme analysis, samples of muscle/gill and digestive gland/foot were stored in separate 1.5 ml microtubes at -70°C.

2.4 Sampling of Gametes, Embryos and Larvae

Gametes were sampled from each parent, and embryos and larvae from each cross. Eggs, embryos and larvae were sampled by taking 1-2 ml from the relevant container using a disposable pipette and releasing this into a 20 μm mesh sieve tube over a beaker for the seawater to drain into. The sieve, retaining the samples, was then placed into a beaker of 70% ethanol / 30% seawater and the same pipette was used to draw up the samples from the bottom of the sieve, in 1 ml of the ethanol solution. This 1 ml sample was then transferred to a pre-labelled 1.5 ml microtube.

For spermatozoa, which might pass through a 20 μm sieve mesh, a different method was used. A 1 ml sample was taken from the glass jar using a disposable pipette and released into a 1.5 ml microtube. This was centrifuged for approximately 5 seconds (up to 10 000 rpm) to concentrate the sperm in the bottom of the tube. The supernatant (seawater) was then carefully removed by pipette and replaced with 70% ethanol / 30% seawater.

2.5 DNA Analysis

DNA markers were used for species identification and detection of the F and M mitotypes in adult mussels and larvae.

2.5.1 DNA Extraction

For each adult mussel, 1-2 mm^3 of adductor muscle tissue was dissected and added to 300 µl CTAB buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB) in a 1.5 ml microtube. The dissecting instruments were cleaned thoroughly between each dissection to avoid contamination between samples. The tissue was macerated using a pestle with a pinch of sterilized sand and 0.15 mg proteinase K was added to give a final concentration of 0.5 mg ml^{-1}. The tube was centrifuged briefly, then incubated at 55°C in a water bath for 1-2 h.

Following removal from the water bath, the samples were left to cool for 1-2 minutes. Extractions of DNA followed the general phenol: chloroform method of Sambrook *et al.* (1989). All operations involving phenol or chloroform were carried out in a fume hood. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the sample was mixed gently by inversion for 1 minute, then centrifuged at 13 000 rpm for 5 minutes. The supernatant (aqueous phase) was removed taking care to avoid any solid material at the interface and transferred to a new microtube The sample was then mixed gently by inversion with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove proteins and centrifuged at 13 000 rpm for 5 minutes. Again, the supernatant was transferred to a new microtube and mixed gently by inversion with an equal volume of chloroform/isoamyl alcohol (24:1) to remove any remaining traces of phenol, then centrifuged at 13 000 rpm for 5 minutes. The supernatant was transferred to a new microtube, mixed with an equal volume of cold isopropanol to precipitate the DNA and left at -20°C for 20 minutes. The microtubes were then centrifuged (hinge outwards) at 13 000 rpm for 10 minutes, or until a pellet of DNA was visible near the base of the tube. The liquid was removed by pipette, taking care not to dislodge the DNA pellet. 1 ml of cold 70% ethanol was added and the microtubes were centrifuged (hinge inwards) at 13 000 rpm for 10 minutes, or until the pellet of DNA had transferred to the opposite (outer) tube wall. The liquid was carefully removed by pipette and the DNA pellet was dried at room temperature for 30-60 minutes.

The DNA pellet was then redissolved in 50 µl sterile water (0.2 µm filtered, deionised, UV treated, autoclaved and stored at -20°C) by leaving to stand at room temperature overnight.

2.5.2 Preparation of larvae for PCR

To release DNA from individual mussel larvae a lysis protocol was employed (slightly modified from that in Sutherland *et al.* 1998). Each individual larva was washed and isolated in 1 µl 0.2 µm filtered/UV treated seawater, using a 1-10 µl pipette, then transferred to a 0.5 ml microtube containing 15 µl 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). The samples were incubated in a thermocycler at 37°C for 1.5 h followed by 99°C for 10 minutes.

2.5.3 PCR Amplification

PCR mixes were made up immediately prior to the reaction for the number of samples to be amplified. For each reaction 2 mixes were made and aliquotted separately. Mix A contained the PCR buffer, $MgCl_2$, dNTPs and sterile water. Mix B contained the Taq DNA polymerase, forward and reverse primers and sterile water. Once both mixes had been aliquotted into labelled, autoclaved, 0.5 ml microtubes, 1 µl of extracted DNA or the relevant volume of lysate, was added inside the lid of each tube. In all cases the negative control for PCR was prepared by adding sterile water in place of DNA/lysate. The thermocyler block was held at 94°C while the tubes were centrifuged briefly (to transfer the DNA/lysate to the bottom of the tube), loaded into the block and then the temperature cycle was started. The thermocycler used was a Techne Genius model unless otherwise stated. Reaction conditions are given for each set of primers used in the relevant chapters.

2.5.4 PCR Product Electrophoresis and Detection Protocols

PCR products were electrophoresed and visualised by one of two methods: an agarose gel stained with ethidium bromide or a polyacrylamide gel stained with silver nitrate. Bromophenol blue loading dye (2 µl per gel lane) was added to all PCR products prior to electrophoresis.

2.5.4.1 Agarose gel electrophoresis

Gels were prepared by boiling (in a microwave oven) the relevant amount of agarose in 1 x TBE buffer (see Appendix C). Gels were cast in the horizontal submarine perspex electrophoresis rig in which they were run (Figure 2.1). A mould was created using a glass or perspex plate as the base and perspex end plates. The mould joints were sealed with a small amount of hot agarose solution and a comb was fitted into the mould to create sample wells. Once the agarose solution had cooled to approximately 60°C, ethidium bromide (0.5 µg ml^{-1} agarose) was added and the gel was mixed thoroughly, before pouring into the mould. Gels were left to set for approximately 30 minutes, prior to electrophoresis. Once the gel was set, the comb and end plates were removed and the gels was submerged in 1x TBE buffer. Samples were loaded into the wells and electrophoresed at 60-80 ma until the bromophenol blue loading dye reached the end of the gel. For high percentage agarose gels (eg. 4%), ethidium bromide was not added to the gel prior to casting. Instead, the gel was stained for 15-30 minutes in ethidium bromide solution (350 µg l^{-1}) following electrophoresis. PCR products were visualized under ultraviolet light and gels were photographed with a Polaroid camera.

2.5.4.2 Polyacrylamide gel electrophoresis

Gels were cast between two vertical glass plates, separated by a plastic spacer on each side and held together by bulldog clips. The gap at the bottom of the plates was sealed by pipetting a small amount of hot 1% agarose solution between the plates. The agarose was allowed to set and the plate edges were

sealed with masking tape. The gel mixture (see Appendix C) was prepared and poured immediately into the gap at the top of the plates. A perspex comb was then inserted between the top of the plates and the gel was allowed to polymerize for 2-24 hours, usually overnight. Prior to loading samples, the masking tape, agarose strip and comb were removed and the wells were washed with 1x TBE buffer to remove any loose pieces of polyacrylamide. The gel plates were then clamped onto a vertical electrophoresis rig (Figure 2.2) with an aluminium cooling plate and the buffer chambers were filled with 1x TBE. After ensuring that no air bubbles remained in the wells, the samples were loaded using gel loading pipette tips. Electrophoresis was at 28 ma for 2-3 h, until the bromophenol blue loading dye reached the bottom of the gel. Following electrophoresis, PCR products were visualised by silver staining, using a method slightly modified from that in Skibinski *et al.* (1994) (See Appendix B) and photographed on a white light box.

Figure 2.1. A perspex horizontal submarine electrophoresis rig, showing the gel casting apparatus.

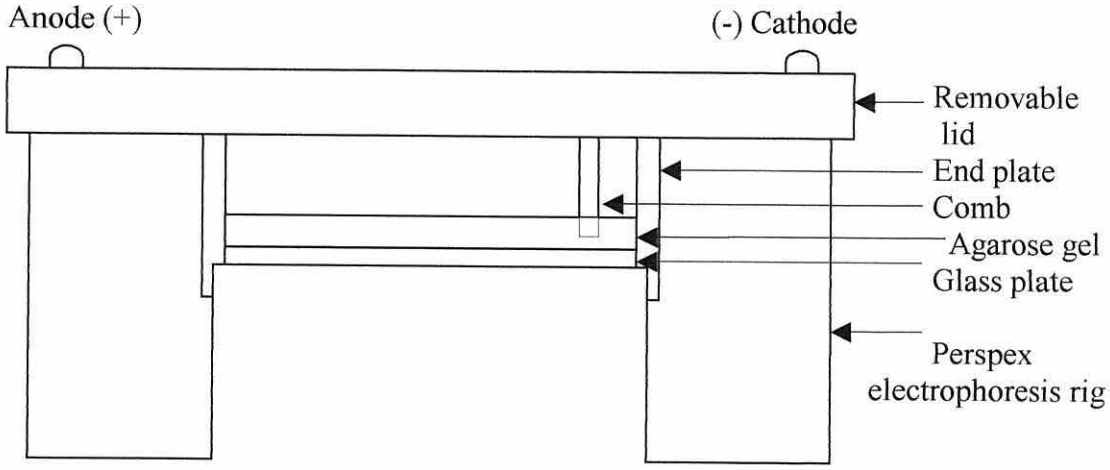
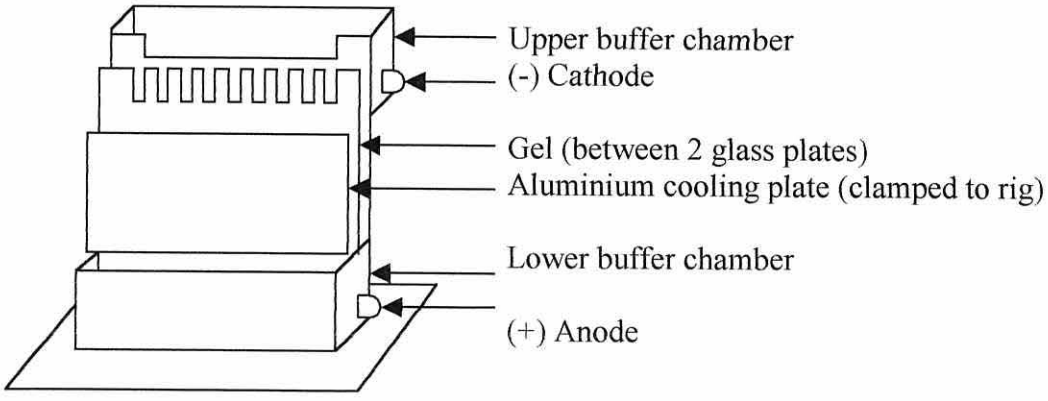


Figure 2.2. A vertical polyacrylamide gel electrophoresis rig.



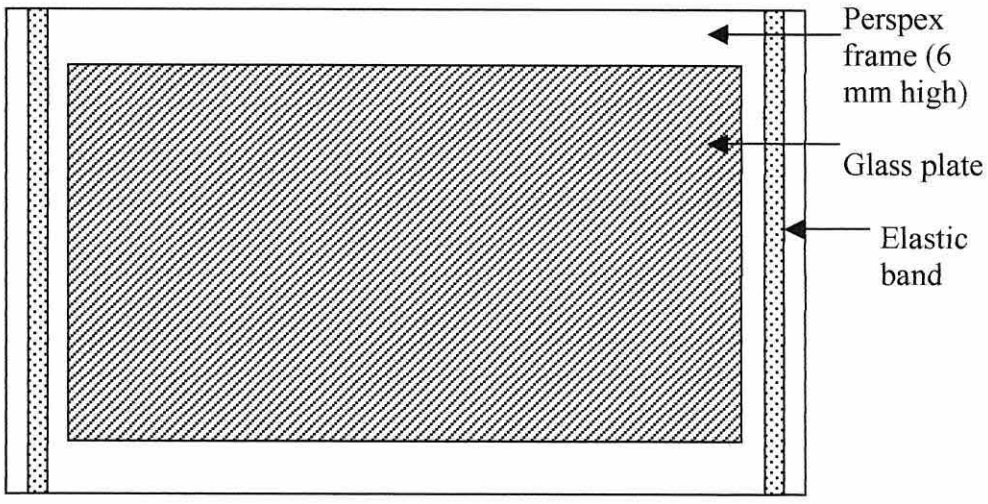
2.6 Allozyme Electrophoresis

Allozyme loci were employed for comparison with nuclear DNA markers in species identification of adult mussels.

2.6.1 Preparation of starch gels

A gel mould was prepared by securing a 6 mm high perspex frame onto a glass plate, using elastic bands (Figure 2.3) and this was placed on a second glass plate on a level surface. 12% starch gels were made with a 1 in 10 dilution of the electrode buffer (see Appendix D), in a 1 l heavy duty conical flask. The ingredients were mixed by swirling the flask and then heated in a microwave on full power (650W) for approximately 8 minutes, until the gel mixture started to boil. During this time the flask was removed from the microwave and swirled every 15-30 seconds to ensure that the gel mixture was heated evenly. Degassing of the gel was carried out under vacuum, to produce a bubble-free, semi-transparent viscous liquid. The gel was poured into the prepared mould to a level slightly higher than the frame and left to set at room temperature for 10-15 minutes and then at 4°C for 2-3 h.

Figure 2.3. Mould for pouring starch gels. Gel dimensions: 180 x 150 x 6 mm.



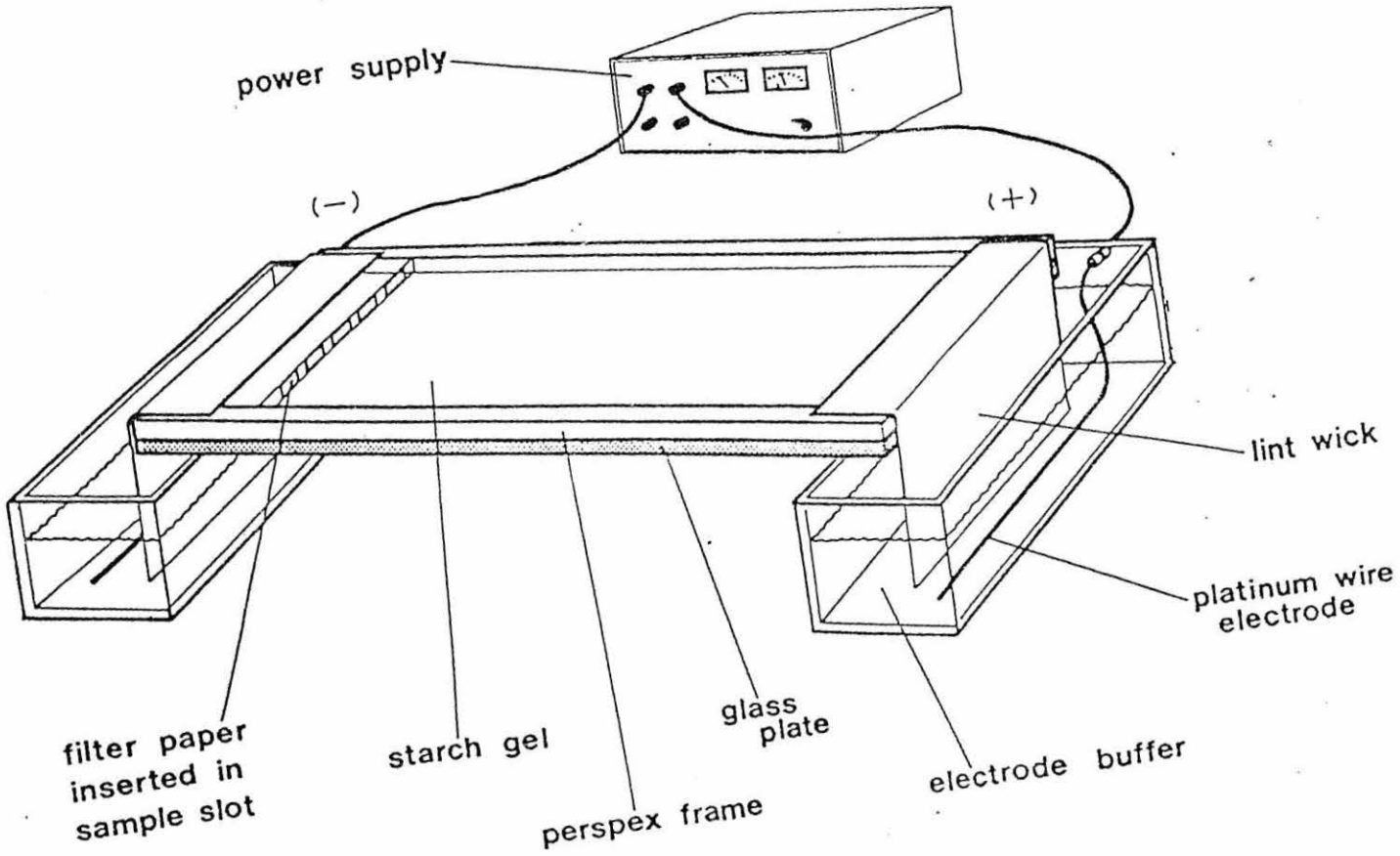
2.6.2 Sample preparation

Approximately 3 mm^3 of adductor muscle tissue was placed into a 1.5 ml microtube with 0.5 ml cold electrode buffer. A pinch of sterile sand was added and the sample was macerated using a steel rod, then centrifuged at 10 000 rpm for 5 minutes. The microtubes were held on ice following each stage of the procedure.

2.6.3 Gel loading and electrophoresis

The elastic bands were removed from the gel mould and the top of the gel was sliced off using cheese wire, to the level of the perspex frame. A slit was then made across the gel, approximately 3 cm from one end. The perspex frame was removed and the slit was opened, separating the two parts of the gel. A pre-prepared strip of filter paper (approximately 3 x 5 mm) was held at one end using forceps and the other end was placed briefly into the supernatant from a sample. The paper was then placed onto the vertical cut face of the larger part of the gel. Other samples were loaded sequentially across the gel, leaving a small gap between each filter paper. One piece of filter paper was used to absorb a small amount of horse spleen ferritin (a brown coloured protein) and loaded on the gel to act as a visual marker for the progress of electrophoresis. Finally, the slit between the two parts of the gel was carefully closed and the perspex frame was replaced around the gel. The gel was covered with a thin plastic sheet to avoid evaporation and electrophoresis was carried out in a refrigerator at 4°C, at 80 V per gel, for approximately 22 h. The electrophoresis apparatus is shown in Figure 2.4.

Figure 2.4 Starch gel electrophoresis apparatus.



2.6.4 Staining of allozyme loci

Following electrophoresis, the filter paper inserts were removed from the gel using forceps and the 6 mm perspex frame was replaced with a 3 mm frame. The gel was sliced in half horizontally using cheese wire and a plastic plate on top of the gel to keep the gel flat. A second glass plate was placed alongside the gel and covered with a small amount of distilled water to prevent the gel sticking to the plate. The upper slice of the gel was removed and placed, cut face up, onto the second glass plate. A 6 mm perspex frame was placed around each gel slice and labelled with the slice type (upper or lower) and the stain to be applied.

The stains were applied to the surface of the gel in a solution of warm 1% agar. The perspex frame contained the solution until the agar set. The gel was then transferred to a 37°C incubator to speed the development of the enzyme reaction. Stain recipes are given in Appendix D.

Chapter 3. Species Identification in the *Mytilus edulis* Complex

3.1 Introduction

The *Mytilus edulis* complex comprises three closely related marine mussel species (*M. edulis*, *M. galloprovincialis*, *M. trossulus*) whose taxonomic status has long been a topic of debate. Despite widespread hybridisation where the species distributions overlap the three taxa maintain morphological and genetic differences (McDonald *et al.* 1991). To date there is no consensus on whether these three taxa merit full specific status or should instead be considered as semi-species or subspecies, but to avoid further confusion they are referred to as *M. edulis*, *M. galloprovincialis* and *M. trossulus* and collectively as the *M. edulis* complex (Gosling 1992). *Mytilus* taxa were first defined using shell morphometrical characters but these are now known to be heavily influenced by various environmental factors (Seed 1968). Morphometrical characteristics can occasionally be used to identify mussel species on a local scale, but their reliability over larger spatial scales is questionable (Rawson *et al.* 1996). The taxa also show differentiation in chromosomal markers (Insua *et al.* 1994, Martinez-Lage *et al.* 1995), sperm morphology (Hodgson and Bernard 1986), immunology (Brock 1985) and physiology (Hilbish *et al.* 1994). Allozyme electrophoresis has proved useful in clarifying taxonomy in the *M. edulis* complex and several allozyme loci have been extensively used for distinguishing *Mytilus* species. However, no single locus is completely diagnostic between the three species (McDonald *et al.* 1991).

More recent studies have focussed on the use of DNA methodologies to characterize taxonomic relationships in the *M. edulis* complex. Both mitochondrial DNA (e.g. Skibinski 1985; Edwards and Skibinski 1987; Rawson and Hilbish 1995a) and nuclear DNA (e.g. Corte-Real *et al.* 1994; Heath *et al.* 1995; Inoue *et al.* 1995; Beynon and Skibinski 1996; Heath *et al.* 1996; Rawson *et al.* 1996; Ohresser *et al.* 1997; Suchanek *et al.* 1997; Borsa *et al.* 1999; Daguin and Borsa 1999; Rawson *et al.* 1999) markers have been widely used in the study of *Mytilus* populations. However, the use of mitochondrial DNA

markers is complicated by the unusual mode of mitochondrial DNA inheritance found in *Mytilus*, termed Doubly Uniparental Inheritance. This involves two highly diverged mitotypes with separate transmission routes down female and male lineages. Widespread introgression of mitochondrial DNA through hybridisation and at least one occurrence of transfer of a mitotype between the female and male lineages (Hoeh *et al.* 1997) limit the use of mitochondrial DNA markers in distinguishing species.

Several existing nuclear DNA (nDNA) markers appear to be of some use in distinguishing *Mytilus* species (Corte-Real *et al.* 1994; Heath *et al.* 1995; Inoue *et al.* 1995; Rawson *et al.* 1996; Ohresser *et al.* 1997). Indeed, two of these markers are claimed to distinguish mussels of all three species in the *M. edulis* complex (Inoue *et al.* 1995; Rawson *et al.* 1996). Both markers are located within the nuclear gene encoding a polyphenolic adhesive protein, important in attachment of mussels to the substrate. The marker reported by Rawson *et al.* (1996), Glu-5', produced species-specific bands from a total of 208 mussels from 6 populations, but additional bands were also present. For Inoue *et al.* (1995)'s marker, Me 15/16, single diagnostic bands were produced from each species. Band sizes were 180 bp for 16 *M. edulis* from Lewes (Delaware, USA), Tromsö (Norway) and Brittany (France), 168 bp for 16 *M. trossulus* from Juneau (Alaska, USA) and 126 bp for 36 *M. galloprovincialis* from 2 locations in Japan and from Sete (France). A later study (Inoue *et al.* 1997) found mussels from other Japanese populations that were heterozygous for the bands typical of *M. galloprovincialis* and *M. trossulus* and hybridisation was given as a possible explanation. A third adhesive protein gene marker, Glu-3' (Rawson *et al.* 1996) produced species-specific PCR products from *M. edulis* (from Lewes, Delaware and Cape Anne, Massachusetts, USA) and *M. galloprovincialis* (from San Diego, California, USA and Sete, France) which differed in size by 6 bp and could be more easily distinguished by restriction digestion with *Dde*I. Rawson *et al.* (1996) found that the Glu-3' primers did not amplify any PCR product from *M. trossulus* (from Newport and Port Orford, Oregon, USA).

Heath *et al.* (1995) developed two PCR-based RFLP markers which distinguished *M. trossulus* (from Newport, Oregon, USA) from both *M. edulis* (from Lewes, Delaware, USA) and *M. galloprovincialis* (from San Diego, California, USA). The ITS marker was based on the internal transcribed spacer regions between the 18S and 28S nuclear rDNA coding regions (Hillis and Dixon 1991) and the PLIIa marker amplified part of the coding region of the protamine-like sperm packaging protein PLII*, sequenced by Carlos *et al.* (1993).

Two further nDNA markers, mac-1 (Ohresser *et al.* 1997) and CaM-1 (Corte-Real *et al.* 1994), are based on intron-length polymorphisms and were used in limited studies of *M. edulis* and *M. galloprovincialis* but neither study included *M. trossulus*. Ohresser *et al.* (1997) identified an intron-length polymorphism at the actin gene locus mac-1. PCR primers amplified 3 alleles, 2 of which were found in both *M. edulis* (from Boyard and Aiguillan, France) and *M. galloprovincialis* (from Sete, France and Polzeath, England). The third mac-1 allele was found only in *M. galloprovincialis*. Corte-Real *et al.* (1994) found 2 length-variants at the calmodulin gene intron 3 locus (CaM-1) in *M. edulis* (various British populations) and *M. galloprovincialis* (from Venice lagoon, Italy). However, the allele frequencies showed little variation between the 2 species. A more recent study in this laboratory (Pers. comm., C. Sherman) found a third CaM-1 length variant in *M. trossulus* (Baltic Sea and Nova Scotia) which was not detected in *M. edulis* (Menai Strait and Nova Scotia) or *M. galloprovincialis* (Montpellier, France).

Several studies have used one or more of the above nDNA markers to identify the species composition of *Mytilus* populations in various locations (e.g. Rawson, Secor and Hilbish 1996; Suchanek *et al.* 1997; Borsa *et al.* 1999; Comesana *et al.* 1999; Rawson *et al.* 1999; Daguin *et al.* 2001; Rawson *et al.* 2001). However, given that the original studies used relatively low numbers of individuals from only a few populations, it seems inappropriate to assume that markers which appeared to be diagnostic for these three species in the original studies will still be so when applied to greater numbers of individuals from

different populations. The present study aims to evaluate the usefulness of the seven published nDNA markers described above in identifying *M. edulis*, *M. galloprovincialis* and *M. trossulus*. Six natural *Mytilus* populations are surveyed and identification of species using the nDNA markers is compared with that based on allozyme loci for two of these populations.

3.2 Materials and Methods

The samples used in this study were from the *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* populations described in Chapter 2.1.

3.2.1 DNA Analysis

In an initial survey approximately 50 ng extracted DNA from each of 5 Menai Strait *M. edulis*, 2 Montpellier *M. galloprovincialis* and 3 Gulf of Gdansk *M. trossulus* individuals was amplified by PCR for seven nuclear DNA (nDNA) markers which were published in the literature as being useful in distinguishing these 3 species. Further individuals from all 6 populations described in Chapter 2.1 were genotyped for the 3 nDNA markers which appeared most useful from the initial survey.

3.2.1.1 PCR protocols

The nDNA markers used and PCR protocols are described below:

1. Me 15/16: a fragment of the nonrepetitive region of the adhesive protein gene (primers Me 15/16 of Inoue *et al.* 1995). The 25 µl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 4 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, 90 s at 70°C and a final extension period of 4 min at 70°C.

2. and 3. Glu-5' and Glu-3': fragments of the repetitive region of the adhesive protein gene (primers JH-5/54 (Glu-5') and JH-4/PR-8 (Glu-3') of Rawson *et al.* 1996). For Glu-5' the 25 µl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM of each

dNTP, 0.5 μM of each primer, 0.1% TritonX®-100 and 0.25 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 3 min at 94°C followed by 30 cycles of 40 s at 94°C, 40 s at 55°C, 60 s at 72°C and a final extension period of 10 min at 72°C (Pers. comm., Smita Apte). This protocol produced more successful amplifications than that given in the original publication. For Glu-3' the 12.5 μl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 0.5 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 3 min at 94°C followed by 30 cycles of 20 s at 94°C, 20 s at 53°C and 45 s at 72°C.

4. mac-1: part of the actin gene (Ohresser *et al.* 1997). The 12.5 μl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 0.5 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 3 min at 96°C followed by 30 cycles of 90 s at 94°C, 60 s at 45°C and 30 s at 72°C.

5. ITS: part of the internal transcribed spacer (ITS) region between the 18S and 28S nuclear rDNA coding regions (Heath *et al.* 1995). The 25 μl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 3 min at 94°C followed by 30 cycles of 20 s at 94°C, 20 s at 55°C and 45 s at 72°C.

6. PLIIa: part of the coding region of a protamine-like sperm packaging protein, PLII* (Heath *et al.* 1995). The 25 μl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each

dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 3 min at 94°C followed by 30 cycles of 20 s at 94°C, 20 s at 50°C and 45 s at 72°C.

7. CaM-1: the intron 3 of the calmodulin gene (primers CAD 6/7 of Corte-Real *et al.* 1994). The 20 μl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega). The temperature cycle consisted of an initial denaturation period of 2 min at 94°C followed by 35 cycles of 45 s at 94°C, 60 s at 61°C, 90 s at 72°C and a final extension period of 5 min at 72°C.

For the Glu-3', ITS and PLIIa markers, PCR products were digested with *Dde*I, *Hha*I or *Hinf*I respectively, as in the original publications (10 μl PCR product and 3 units restriction enzyme at 37°C for 2.5 h). PCR and restriction digest products were electrophoresed on agarose gels of a suitable concentration and stained with ethidium bromide. Improved resolution of Me 15/16 alleles was achieved by electrophoresis on 8% polyacrylamide gels and silver staining (see Appendix B).

3.2.1.2 Results of initial survey of nDNA markers

Expected results for the seven markers are shown in Table 3.1. PCR amplification was successful to varying degrees for the different markers.

1. Inoue *et al.*(1995)'s Me 15/16 marker amplified successfully from 10 out of 10 individuals and produced the three expected alleles.

2. Glu-5' PCR products were detected in 6 out of 10 individuals. Four of the five expected alleles were detected.

3. Glu-3' PCR and *Dde*I digestion products of the expected sizes were detected in Menai Strait *M. edulis* and Montpellier *M. galloprovincialis*. Gulf of Gdansk *M trossulus* gave PCR and digestion products of approximately the same size as *M. edulis*. PCR and digestion products were detected in 9 out of 10 individuals.

4. mac-1 PCR products were detected for 2 out of 10 individuals. Two Menai Strait *M. edulis* gave a band at approximately 324 bp and one of these also had a band at approximately 360 bp. No PCR products were detected from *M. galloprovincialis* or *M. trossulus*.

5. The ITS primers produced PCR products in 9 out of 10 individuals examined. All bands were approximately 950 bp. No bands were detected at the expected 1250 bp. Digestion of PCR products with *Hha*I produced results from 8 individuals. The expected bands of 480 bp and 180 bp were detected in addition to an extra band at approximately 380 bp. The *M. trossulus* associated band at 280 bp (Heath *et al.*, 1995) was not detected in this study.

6. The PLIIa primers produced weak PCR products at the expected 475 bp in 6 out of 10 individuals. No bands were detected following digestion with *Hinf*I.

7. CaM-1 PCR products were detected in 9 out of 10 individuals. In addition to the 2 alleles detected in *M. edulis* by Corte-Real *et al.* (1994) (525 bp and 460 bp), 2 other bands were detected at approximately 500 bp and 600bp. The ~ 500 bp band was found in 2 of the 3 *M. trossulus* individuals. The 600 bp band was detected only in one *M. galloprovincialis* individual.

From the results of the initial survey, the Me 15/16, Glu-5' and CaM-1 markers were selected as being potentially useful for distinguishing *M. edulis*, *M. galloprovincialis* and *M. trossulus*.

Table 3.1. Expected results according to original publications for the 7 markers used in the initial survey. E, *Mytilus edulis*. G, *M. galloprovincialis*. T, *M. trossulus*. +/ = either or both bands present.

Marker	Species distinguished	Expected PCR product sizes (bp). Figures in parentheses are restriction enzyme digest products.		
		E	G	T
Me 15/16	E from G from T	180	126	168
Glu-5'	E from G from T	350 +/ 380	300 +/ 500	240
Glu-3'	E from G	220 (*Dde*I, 2 bands)	214 (*Dde*I, 1 band)	Unknown
mac-1	Possibly E from G	324 +/ 360	324 +/ 360 +/ 388	Unknown
ITS	T from E/G	1250 (*Hha*I, 450 + 180)	1250 (*Hha*I, 450 + 180)	1250 (*Hha*I, 280 + 180 + <100)
PLIIa	T from E/G	475 (*Hinf*I, 200 + <100)	475 (*Hinf*I, 200 + <100)	475 (*Hinf*I, 475 + 200 + <100)
CaM-1	Possibly E from G from T	525 +/ 460	525	525 / 480

3.2.2 Allozyme analysis

Starch gel electrophoresis was carried out on adductor muscle samples from 5 *M. edulis* and 15 *M. trossulus* from the Bay of Lunenburg, Nova Scotia following the methods outlined in Section 2.6. Staining was performed for the phosphoglucomutase (PGM, EC 2.7.5.1), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9.) and mannose-6-phosphate isomerase (MPI, EC 5.3.1.8) loci. Stain recipes are given in Appendix D. These loci were selected from those used to distinguish between *M. edulis* and *M. trossulus* on the Atlantic coast of Canada in previous studies (e.g. McDonald *et al.* 1991; Saavedra *et al.* 1996; Penney and Hart 1999).

3.2.3 Data Analysis

Data analyses were carried out using the programs GENEPOP version 3.2 (Raymond and Rousset 2000), FSTAT version 2.9.3 (Goudet 1995, 2001), POPGENE version 1.32 (Yeh *et al.* 1999) and PHYLIP version 3.5c (Felsenstein 1993). Where appropriate, *P*-values were corrected for multiple testing with the sequential Bonferroni technique (Holm 1979; Rice 1989).

3.2.3.1 Conformance of populations to Hardy-Weinberg expectations

Genotype frequencies observed in each of the 6 populations at the 3 nDNA loci and 3 allozyme loci (2 populations only) were tested for conformation to Hardy-Weinberg expectations using the GENEPOP program. Weir and Cockerham (1984)'s estimator (f) of Wright (1951)'s F_{IS} was calculated and a Markov chain method (Guo and Thompson 1992) was used to estimate the exact *P*-values. Whenever possible, the standard errors associated with the *P*-values were kept < 0.01 by adjusting the Markov chain parameters (Raymond and Rousset 2000).

3.2.3.2 Genotypic disequilibrium

The null hypothesis that genotypes at one locus were independent from genotypes at another locus was tested using the GENEPOP program. Fisher exact tests were performed, using a Markov chain method, for each locus pair within each population and across all populations.

3.2.3.3 Population differentiation

Weir and Cockerham (1984)'s estimator (θ) of Wright (1951)'s F_{ST} was calcuated for each nDNA locus (between 6 populations) and each allozyme locus (between 2 populations) using the FSTAT program (Goudet 1995, 2000). This estimator takes account of differing sample sizes by weighting allele frequencies accordingly. The null hypothesis that $\theta = 0$ (100% gene flow) was tested as described by Waples (1987).

3.2.3.4 Homogeneity of population allele frequency distributions

The null hypothesis of homogeneity of allele frequencies between populations was tested using the GENEPOP program. Unbiased estimates of *P*-values of Fisher exact tests were obtained using a Markov chain method.

Initially, all pairs of samples within species were tested for heterogeneity in allele frequency distributions at each nDNA locus. Where there was no indication of heterogeneity, samples were pooled within species and the pooled groups were those used in further analyses. All between species group pairs were tested for heterogeneity in allele frequency distributions at each nDNA locus in order to compare the extent of heterogeneity detected by each of the 3 markers.

3.2.3.5 Genetic distance

Nei's genetic distance (Nei 1972) was calculated from allele frequencies for all population pairs and between species groups for each nDNA locus and across the three nDNA loci. These values were graphically represented as UPGMA dendrograms (unweighted pair group method with arithmetic means) using the POPGENE and PHYLIP programs.

3.3 Results

3.3.1 PCR Amplifications

Inoue *et al.* (1995)'s Me 15/16 marker amplified successfully and consistently from 180 of 181 (99%) samples and produced the three expected alleles (Figure 3.1). The Menai Strait and Loch Etive *M. edulis* samples contained only the 180 bp (Me) allele. However, the remaining four populations each contained more than one allele.

Glu-5' PCR products were detected in 26 of 64 (41%) samples. The five expected alleles were detected in addition to several extra bands which occurred too infrequently to be of use in distinguishing species (Figure 3.2). Rawson *et al.* (1996) also reported the detection of extra bands, which they attributed to non-specific amplification.

CaM-1 PCR products were detected for 67 of 71 (94%) samples (Figure 3.3). In addition to the 2 alleles detected in *M. edulis* by Corte-Real *et al.* (1994) (525 bp and 460 bp), 3 other relatively rare bands were detected at approximately 500 bp, 600 bp and 850 bp. The ~ 500 bp band was found only in *M. trossulus* and Bay of Lunenburg *M. edulis* and probably corresponds to the 480 bp band found previously (Pers. comm., C. Sherman). The 505 bp band detected previously in Bay of Lunenburg *M. edulis* (Pers. comm., C. Sherman) was not detected in this study, possibly due to a lack of resolution in the lower percentage agarose gel used. The 600 bp band was detected in individuals of all 3 species, but only in combination with the 525 bp band. A single Gulf of Gdansk *M. trossulus* individual was homozygous for the 850 bp band.

Figure 3.1 Me 15/16 PCR products obtained from mussels of the genus *Mytilus*, visualised on a silver stained 8% polyacrylamide gel. Lane 1 = *M. galloprovincialis*, Montpellier (126/126); lane 2 = *M. edulis*, Menai Strait (180/180); lanes 3,4 = *M. trossulus*, Gulf of Gdansk (180/168); L = pBR322 *Msp*I size marker.

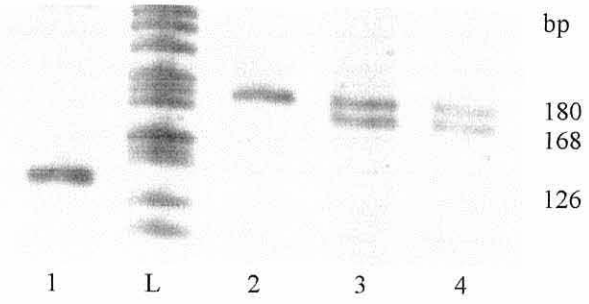


Figure 3.2 Glu-5' PCR products obtained from mussels of the genus *Mytilus*, visualised on an ethidium bromide stained 1% agarose gel. Lane 1 = *M. trossulus*, Gulf of Gdansk (240/240); lanes 2, 3 = *M. trossulus*, Gulf of Gdansk (380/240); lanes 4,5 = *M. galloprovincialis*, Montpellier (300/500); lanes 6, 7 = *M. edulis*, Menai Strait (380/380); L = 100 bp DNA ladder.

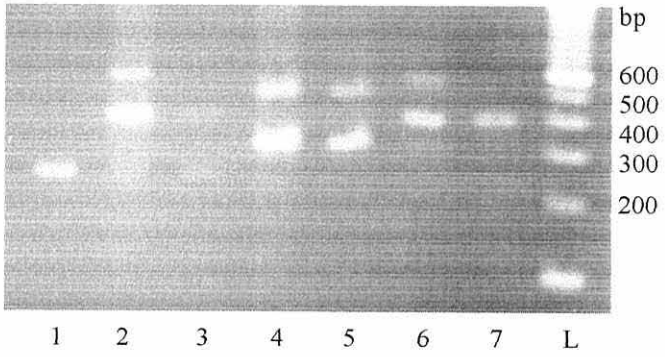
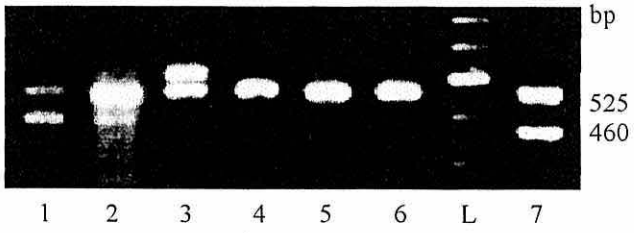


Figure 3.3 CaM-1 PCR products obtained from mussels of the genus *Mytilus*, visualised by ethidium bromide staining of a 1% agarose gel. Lanes 1, 2 = *M. trossulus*, Nova Scotia (525/500); lane 3 = *M. galloprovincialis*, Montpellier (600/525); lane 4 = *M. galloprovincialis*, Montpellier (525/525); lanes 5-6 = *M. edulis*, Loch Etive (525/525); lane 7 = *M. edulis*, Loch Etive (525/460); L = 100 bp DNA ladder.



3.3.2 Allozyme Analysis

The PGM and GPI loci gave results for all 20 mussels analysed. Three of the five *M. edulis* and five of the fifteen *M. trossulus* failed to give results for the MPI locus. Six alleles were observed at the PGM locus, eight at the GPI locus and 3 at the MPI locus. The alleles observed generally corresponded to those previously found in, and used to identify, *M. edulis* and *M. trossulus* on the Atlantic coast of Canada (e.g. McDonald *et al.* 1991; Saavedra *et al.* 1996; Penney and Hart 1999).

3.3.3 Conformance of populations to Hardy-Weinberg expectations

Allele frequencies, expected and observed heterozygosity, as well as probabilities for conformity of genotype frequencies to Hardy-Weinberg expectations are given in Table 3.2 for the nDNA and allozyme loci. General agreement to Hardy-Weinberg expectations was obtained for all loci, with three exceptions. In the Gulf of Gdansk *M. trossulus* sample, CaM-1 showed a heterozygote deficiency ($f = +0.616$) which was statistically significant at the 0.05 level. In the Nova Scotia *M. trossulus* sample, PGM and GPI showed heterozygote deficiencies significant at the 0.05 (PGM, $f = +0.550$) and 0.01 (GPI, $f = +0.826$) levels.

Table 3.2. Genotype and allele frequencies for each of the 6 *Mytilus* samples, sample sizes (N), expected and observed number of heterozygotes (He and Ho respectively), and probabilities of conformance of genotype frequencies to Hardy-Weinberg expectations (P) for the nDNA and allozyme loci. * indicates significance at the 0.05 level and ** at the 0.01 level, following the sequential Bonferroni correction for multiple testing with k = 15 (Holm 1979; Rice 1989). *M. edulis* samples are EMS (Menai Strait), ELE (Loch Etive), ENS (Nova Scotia). *M. trossulus* samples are TGD (Gulf of Gdansk), TNS (Nova Scotia). GMP is *M. galloprovincialis* from Montpellier.

nDNA locus		Population					
		EMS	ELE	ENS	TGD	TNS	GMP
Me 15/16							
	180/180	6	10	28	18	2	0
	126/126	0	0	0	0	0	28
	168/168	0	0	2	6	39	0
	180/126	0	0	0	0	0	1
	180/168	0	0	3	18	18	0
	168/126	0	0	0	1	0	0
	180	1.000	1.000	0.894	0.628	0.186	0.017
	126	0.000	0.000	0.000	0.012	0.000	0.983
	168	0.000	0.000	0.106	0.360	0.814	0.000
	N	6	10	33	43	59	29
	He	-	-	6.354	20.694	18.051	1.000
	Ho	-	-	3.000	19.000	18.000	1.000
	P	-	-	0.0240	0.3881	1.0000	-
Glu-5'							
	380/380	2	0	1	5	1	0
	350/350	1	3	0	0	0	0
	300/300	1	0	0	0	0	8
	380/240	0	0	0	1	1	0
	500/300	0	0	0	0	0	2
	240	0.000	0.000	0.000	0.083	0.250	0.000
	300	0.250	0.000	0.000	0.000	0.000	0.900
	350	0.250	1.000	0.000	0.000	0.000	0.000
	380	0.500	0.000	1.000	0.917	0.750	0.000
	500	0.000	0.000	0.000	0.000	0.000	0.100
	N	4	3	1	6	2	10
	He	2.857	-	-	1.000	1.000	1.895
	Ho	0.000	-	-	1.000	1.000	2.000
	P	0.0331	-	-	-	-	1.0000
CaM-1							
	850/850	0	0	0	1	0	0
	525/525	0	4	1	17	5	15
	500/500	0	0	0	2	0	0
	600/525	0	0	1	0	1	2
	525/500	0	0	3	3	6	0
	525/460	4	1	0	0	0	1
	460	0.500	0.100	0.000	0.000	0.000	0.028
	500	0.000	0.000	0.300	0.152	0.250	0.000
	525	0.500	0.900	0.600	0.804	0.708	0.917
	600	0.000	0.000	0.100	0.000	0.042	0.056
	850	0.000	0.000	0.000	0.043	0.000	0.000
	N	4	5	5	23	12	18
	He	2.286	1.000	3.000	7.711	5.435	2.886
	Ho	4.000	1.000	4.000	3.000	7.000	3.000
	P	0.3152	-	1.0000	0.0030*	0.6307	1.0000

Table 3.2 (continued)

Allozyme locus		Population	
		ENS	TNS
PGM			
	126/126	0	3
	116/116	0	5
	109/109	0	1
	100/100	3	1
	136/126	0	1
	126/109	0	3
	126/100	1	1
	109/88	1	0
	88	0.100	0.000
	100	0.700	0.100
	109	0.100	0.167
	116	0.000	0.267
	126	0.100	0.433
	136	0.000	0.033
	N	5	15
	He	2.667	10.897
	Ho	2.000	5.000
	P	0.3132	0.0012*
GPI			
	105/105	0	1
	100/100	2	0
	97/97	0	1
	94/94	0	1
	91/91	0	7
	85/85	0	2
	81/81	0	1
	110/91	1	0
	105/94	0	1
	105/85	1	1
	100/91	1	0
	81	0.000	0.067
	85	0.100	0.167
	91	0.200	0.467
	94	0.000	0.100
	97	0.000	0.067
	100	0.500	0.000
	105	0.100	0.133
	110	0.100	0.000
	N	5	15
	He	3.778	11.138
	Ho	3.000	2.000
	P	0.2404	<0.0001**
MPI			
	110/110	1	0
	100/100	1	0
	84/84	0	10
	84	0.000	1.000
	100	0.500	0.000
	110	0.500	0.000
	N	2	10
	He	1.333	-
	Ho	0.000	-
	P	0.3340	-

3.3.4 Genotypic disequilibrium

Tests for genotypic disequilibrium between pairs of loci within each population and across all populations showed no significant deviation from the null hypothesis of independence of loci ($P > 0.23$ in all cases).

3.3.5 Population differentiation

θ –values indicate that all loci show highly significant ($P<0.001$) levels of population / species differentiation (Table 3.3). The θ –values for the MPI, Glu-5' and Me 15/16 loci were considerably higher (0.644 – 0.878) than those for the other loci (0.102 – 0.230).

Table 3.3. Estimates of genetic structure for nDNA loci (between 6 populations from 3 *Mytilus* spp.) and allozyme loci (between 2 populations, ENS & TNS). θ is Weir and Cockerham (1984)'s estimator of Wright (1951)'s F_{ST}. The null hypothesis that $\theta = 0$ (100% gene flow) was tested using the equation given by Waples (1987). *** indicates significance at the 0.001 level.

Locus	θ
Me 15/16	0.644***
Glu-5'	0.664***
CaM-1	0.102***
PGM	0.230***
GPI	0.106***
MPI	0.878***

3.3.6 Homogeneity of population allele frequency distributions

All pairs of samples within species were tested for heterogeneity in allele frequency distributions at each nDNA locus (Table 3.4). Within *M. edulis*, the 3 samples showed no statistically significant evidence for heterogeneity. On this basis, data from the Menai Strait and Loch Etive samples were pooled to form a UK *M. edulis* group (EUK) for further analyses. The Nova Scotia *M. edulis* sample was retained as a separate group, in view of the distant geographical location from the UK *M. edulis* samples. The Gulf of Gdansk and Nova Scotia *M. trossulus* samples showed significant heterogeneity at the Me 15/16 locus and were retained as separate groups, although there was no statistically significant evidence for heterogeneity at the Glu-5' and CaM-1 loci. The UK *M. edulis* group was tested for deviation from Hardy-Weinberg equilibrium and was not significantly different from the expected values at the CaM-1 locus but showed a significant heterozygote deficiency ($f = +1.000$, $P = 0.0022$) at the Glu-5' locus.

All between species pairs of the five groups were tested for heterogeneity of allele frequency distributions at each locus (Table 3.4), in order to compare the effectiveness of each locus at distinguishing the three species. All tests for the Me 15/16 locus showed highly significant heterogeneity between samples. The Glu-5' locus showed significant heterogeneity between the Montpellier *M. galloprovincialis* sample and each of the other four samples, however, in the *M. edulis* vs. *M. trossulus* comparisons only the EUK vs. TGD test was significant. For the CaM-1 locus, significant heterogeneity was observed in the same five sample pairs as for Glu-5', but the consistently higher *P*-values for CaM-1 indicate less power in distinguishing samples for CaM-1 than for Glu-5'. The CaM-1 locus also showed significant heterogeneity in the EUK vs. TNS comparison.

Table 3.4. Within species and between species pairwise P-values for heterogeneity of allele frequency distributions. * indicates significance at the 0.05 level, ** at the 0.01 level, *** at the 0.001 level and ns indicates nonsignificant results following the sequential Bonferroni correction for multiple testing with k = 9 for within *M. edulis* tests, k = 3 for within *M. trossulus* tests, k = 6 for *M. edulis* vs. *M. galloprovincialis* tests, k = 12 for *M. edulis* vs. *M. trossulus* tests and k = 6 for *M. galloprovincialis* vs. *M. trossulus* tests (Holm 1979; Rice 1989). Sample abbreviations are as in Table 3.2, EUK = pooled data from EMS & ELE.

Samples	Locus		
	Me 15/16	Glu-5'	CaM-1
Within species			
EMS vs ELE	1.00000 ns	0.02524 ns	0.11711 ns
EMS vs ENS	0.58452 ns	1.00000 ns	0.02713 ns
ELE vs ENS	0.19366 ns	0.03713 ns	0.14457 ns
TGD vs TNS	<0.00001 ***	0.44941 ns	0.30505 ns
Between species			
EUK vs GMP	<0.00001 ***	<0.00001 ***	0.01922 *
ENS vs GMP	<0.00001 ***	0.00821 *	0.01025 *
EUK vs TGD	<0.00001 ***	0.00075 **	0.00111 **
EUK vs TNS	<0.00001 ***	0.04267 ns	0.00217 *
ENS vs TGD	0.00037 **	1.00000 ns	0.14262 ns
ENS vs TNS	<0.00001 ***	1.00000 ns	0.56810 ns
GMP vs TGD	<0.00001 ***	<0.00001 ***	0.00444 **
GMP vs TNS	<0.00001 ***	0.00009 ***	0.00409 **

3.3.7 Genetic distance

Nei's genetic distances between the five groups for each nDNA locus and for the three nDNA loci combined are shown in Table 3.5 and graphically represented in Figure 3.4. Genetic distance values are greater with the Me15/16 locus than with the Glu-5' and CaM-1 loci for seven of the eight possible between species comparisons, the exception being the EUK vs TGD comparison for which the Glu-5' locus gives the highest value. Particularly high genetic distances (3.46 to 5.54) are produced between the GMP sample and all other samples with the Me 15/16 locus. Within *M. edulis*, the Me 15/16 locus produces the lowest genetic distance and the Glu-5' locus the highest. Within *M. trossulus*, the CaM-1 locus produces the lowest genetic distance and the Me 15/16 locus the highest. Figure 3.4 indicates that the Me 15/16 locus provides the clearest separation of species groups, with *M. edulis* and *M. trossulus* more closely related to each other than to *M. galloprovincialis*. In contrast, the dendrograms produced from Glu-5' and CaM-1 distance estimates show more confused patterns of relationships between species groups. Statistical support for the dendrograms for individual loci cannot be calculated using a bootstrap procedure, as the data come from just one locus for each dendrogram. However, these dendrograms facilitate comparison of the effectiveness of the three markers at separating the species. The bootstrapping resampling procedure was applied over the three nDNA loci and the consensus tree for one hundred replicates is shown in Figure 3.4 with the support for each branch.

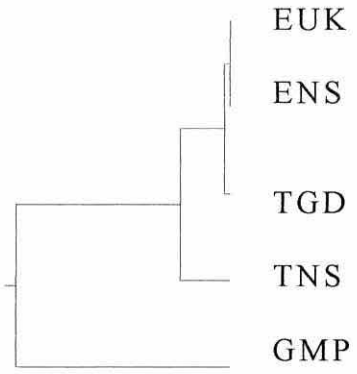
Comparisons with previously reported evolutionary relationships for the three species using allozymes and mitochondrial DNA (mtDNA) are shown in Table 3.6. Evolutionary relationships deduced from the nDNA markers in the present study resemble those estimated from mtDNA, but the distances are much greater with nDNA. Both nDNA and mtDNA show *M. edulis* and *M. trossulus* as the most closely related pair and *M. galloprovincialis* and *M. trossulus* as the most distant. This is in contrast to the evolutionary relationships deduced from allozymes which show the greatest distance between *M. edulis* and *M. trossulus*.

Table 3.5. Pairwise genetic distance (Nei 1972) estimates between *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* populations calculated for the Me 15/16, Glu-5' and CaM-1 loci separately and across the 3 nDNA loci (in the column "All loci"). Sample abbreviations are as in Table 3.2, EUK = pooled data from samples EMS & ELE.

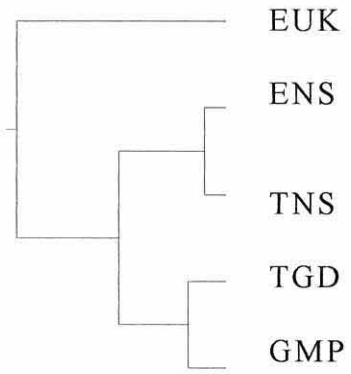
Samples	Locus			All loci
	Me 15/16	Glu-5'	CaM-1	
Within species				
EUK vs ENS	0.0070	0.8291	0.1915	0.2852
TGD vs TNS	0.3872	0.0269	0.0146	0.1289
Between species				
EUK vs GMP	4.0432	1.5284	0.0597	1.0408
ENS vs GMP	4.0502	0.0000	0.1148	1.4539
EUK vs TGD	0.1426	0.8332	0.0880	0.3250
EUK vs TNS	1.4989	0.8818	0.1292	0.7628
ENS vs TGD	0.0837	0.0041	0.0512	0.0522
ENS vs TNS	1.0887	0.0527	0.0120	0.3174
GMP vs TGD	3.4652	0.0000	0.0213	1.1159
GMP vs TNS	5.5421	0.0000	0.0590	1.2246

Figure 3.4 UPGMA dendrograms constructed from Nei (1972)'s genetic distance estimates, showing relationships between the five *Mytilus* species groups as determined from the Me 15/16, Glu-5' and CaM-1 loci separately and together. Statistical support is shown for the "All loci" dendrogram which is the consensus tree from one hundred bootstrapping replicates. *M. edulis* samples are EUK (Menai Strait & Loch Etive), ENS (Nova Scotia). *M. trossulus* samples are TGD (Gulf of Gdansk), TNS (Nova Scotia). GMP is *M. galloprovincialis* from Montpellier.

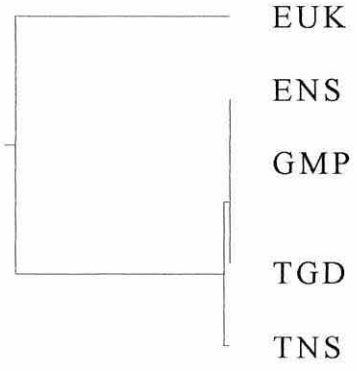
Me 15/16



CaM-1



Glu-5'



All loci

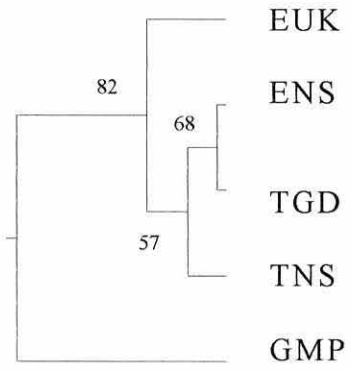


Table 3.6. Pairwise genetic distance (Nei 1972) estimates between *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* calculated from allozymes (Väinölä and Hvilsom 1991), mtDNA (Wenne and Skibinski 1995) and from the nDNA loci in this study (Me 15/16, Glu-5' and CaM-1). E = *M. edulis*, G = *M. galloprovincialis*, T = *M. trossulus*.

	E vs G	E vs T	G vs T
Allozymes	0.21	0.25	0.21
mtDNA	0.63	0.10	0.76
nDNA	1.05	0.39	1.14

3.3.8 Comparison of nDNA markers and allozymes for species identification

Table 3.7 shows a comparison of species identification methods for 12 *Mytilus* spp. individuals from Nova Scotia. These mussels were initially assigned to either *M. edulis* or *M. trossulus* by their location and by shell morphometry (Pers. comm., A. Mallet). The MPI genotype was then used independently to assign each mussel to one of the two species. All were identified as homozygotes for MPI alleles typical of the species to which they had been morphometrically assigned (MPI^{100} or MPI^{110} for *M. edulis* and MPI^{84} for *M. trossulus*). However, with the Me 15/16 marker, seven of these mussels (one *M. edulis* and six *M. trossulus*) were heterozygotes for the 180 bp and 168 bp alleles. Glu-5' genotypes were only obtained for 2 of these 12 individuals, both identified as *M. trossulus* by morphometry and MPI genotype. One of these mussels was identified as *M. trossulus* by Me 15/16 and as a *M. edulis/M. trossulus* hybrid by Glu-5'. The other was identified as a *M. edulis/M. trossulus* hybrid by Me 15/16 and as *M.edulis* by Glu-5'.

Table 3.7. Comparison of identification methods for 12 *Mytilus* spp. from Nova Scotia. Mussels were identified as either *M. edulis*, *M. trossulus* or hybrid using shell morphometry, MPI genotype ($MPI^{100/100}$ or $MPI^{110/110}$ for *M. edulis* and $MPI^{84/84}$ for *M. trossulus*) and Me 15/16 genotype. E = *M. edulis*, T = *M. trossulus*, E/T = hybrid.

Species identification			Frequency
Morphometry	MPI genotype	Me 15/16 genotype	
E	E	E	1
E	E	E/T	1
T	T	T	4
T	T	E/T	6

3.4 Discussion

According to the conclusions of Inoue *et al.* (1995) and Rawson *et al.* (1996), pure species populations would be expected to be fixed for the species-associated Me 15/16 alleles: *M. edulis* for the 180 bp allele, *M. galloprovincialis* for 126 bp and *M. trossulus* for 168 bp; and Glu-5' alleles: *M. edulis* for the 350 and/or 380 bp alleles, *M. galloprovincialis* for 300 and/or 500 bp and *M. trossulus* for 240 bp. For the CaM-1 marker, previous work in this laboratory (Pers. comm. C. Sherman) suggests that the 525 bp allele would be present in all three species but that the 460 bp allele might be restricted to *M. edulis* and the 500 bp allele to *M. trossulus*. The highly significant θ-values, the significant heterogeneity of genotype frequencies in some or all between-species comparisons, and the high genetic distance values indicate that the Me 15/16, Glu-5' and CaM-1 loci all reveal high levels of differentiation between the *Mytilus* populations studied. At the CaM-1 locus, the 525 bp allele was the most common allele in all six populations, with a frequency ranging from 0.500 to 0.917. Although the 500 bp allele was restricted to the two *M. trossulus* and the Nova Scotia *M. edulis* populations, its frequency was too low for the CaM-1 marker to be useful for distinguishing species independently of other markers. The following discussion compares the results of the Me 15/16 and Glu-5' markers in the six populations from the present study with those from previous studies of species distributions in the *M. edulis* complex.

Of the six populations analysed in this study, the Loch Etive *M. edulis* sample is the only one in which the expected Me 15/16 and Glu-5' alleles were detected. The remaining five populations all contained alleles typical of more than one species at one or more loci. In the Montpellier *M. galloprovincialis* population the unexpected allele was present only at low frequency (0.017), but the Menai Strait and Nova Scotia *M. edulis* populations and both *M. trossulus* populations contained higher frequencies of unexpected alleles (0.106 – 0.917). There are two possible explanations for this, firstly that the populations may not be "pure species" but have acquired introgressed alleles through hybridisation, and

secondly that the markers may not be completely diagnostic in identifying these three species.

One Menai Strait *M. edulis* individual was a homozygote for the Glu-5'- 300 bp allele which is typical of *M. galloprovincialis*. All other mussels analysed from this population had *M. edulis* –associated Glu-5' genotypes and at the Me 15/16 locus only the expected 180 bp allele was detected. No previous studies of the Glu-5' or Me 15/16 loci have included *M. edulis* from North Wales. However, Silva (1998) detected the Me 15/16 – 126 bp allele at very low levels in two out of four South Wales populations analysed which were thought to be pure *M. edulis*. An earlier study of allozyme loci in several North Wales populations by Skibinski *et al.* (1983) found no evidence of *M. galloprovincialis* alleles. Recent introgression of alleles from *M. galloprovincialis* does not seem a likely explanation, given that the nearest *M. galloprovincialis* populations to North Wales are in south-west England and on the southern coast of Ireland.

In the Mediterranean Sea *M. galloprovincialis* population (GMP), the results of the present study are similar to those of Silva (1998). The Me 15/16- 180 bp allele was detected in one out of twenty-nine *M. galloprovincialis* in the present study and in three out of forty-three individuals from Peniscola (Spain, Mediterranean Sea) by Silva (1998). Introgression from Atlantic *M. edulis* is possible but unlikely due to the oceanographic barrier to gene flow marked by the Almeria – Oran front. Quesada, Beynon and Skibinski (1995) found evidence for limited mitochondrial DNA gene flow across this feature between Atlantic and Mediterranean *M. galloprovincialis* populations, but this gene flow appeared to be restricted to the populations adjacent to the front. In the present study, Glu-5' genotypes for ten *M. galloprovincialis* were all as expected. However, Borsa *et al.* (1999) found *M. edulis* –associated Glu-5' alleles in one out of thirty-nine *M. galloprovincialis* from Sète (Mediterranean Sea) and suggested introduction of *M. edulis* spat via imported juvenile oysters, *Crassostrea gigas*, as a possible source from which introgression could have occurred.

The Gulf of Gdansk *M. trossulus* population showed a higher frequency of the Me 15/16 – 180 bp allele than the expected Me 15/16 – 168 bp allele and one individual was heterozygous for the Me 15/16 – 168 bp and 126 bp alleles. A previous study of Me 15/16 genotypes in seven *M. trossulus* from Sopot, near Gdansk, also found the 180 bp allele to be at a higher frequency than the 168 bp allele (Silva 1998). At the Glu-5' locus, all individuals except one were homozygous for *M. edulis*-typical alleles, the one exception was a heterozygote for *M. edulis*- and *M. trossulus*- typical alleles. Hybridisation between *M. edulis* and *M. trossulus* is known to be widespread in the Øresund region at the entrance to the Baltic Sea but, on the basis of allozyme studies (Väinölä and Hvilsom 1991; Wenne and Skibinski 1995), the Gulf of Gdansk is thought to contain only *M. trossulus*. Previous studies have found evidence of *M. edulis* alleles in Gdansk *M. trossulus* at the Glu-5' locus (Borsa *et al.* 1999) and also evidence of introgression of *M. edulis* mtDNA into Baltic Sea *M. trossulus* (Quesada, Wenne and Skibinski 1995, 1999; Rawson and Hilbish 1998), however there is no previous record of *M. galloprovincialis* alleles in Baltic Sea *M. trossulus*. The Me 15/16 and Glu-5' nDNA markers reveal similarities between Baltic Sea *M. trossulus* and Atlantic *M. edulis* which are not apparent from allozyme studies.

The putative *M. edulis* and *M. trossulus* obtained from Nova Scotia were selected on the basis of shell morphometry from an area containing both species (Pers. comm. A. Mallet). This method of species identification has previously shown a high level of concordance with allozyme results in this area (Mallet and Carver 1995). However, these two samples both contained homozygotes and heterozygotes for the Me 15/16 – 180 bp and Me 15/16 – 168 bp alleles, an observation which is best explained by hybridisation between *M. edulis* and *M. trossulus*. Previous studies have suggested that hybridisation between *M. edulis* and *M. trossulus* does occur in Atlantic Canada (McDonald *et al.* 1991; Mallet and Carver 1995; Saavedra *et al.* 1996) but that it is relatively rare compared to the levels observed between *M. edulis* and *M. galloprovincialis* in Europe (Comesana *et al.* 1999). Recent research by Comesana *et al.* (1999) shows that certain nDNA markers (ITS and Glu-5') reveal higher levels of hybridisation

than the allozyme loci mannose-6-phosphate isomerase (MPI) and esterase-D (EST-D) which have been regarded as diagnostic and almost diagnostic, respectively, for identifying *M. edulis* and *M. trossulus* in Atlantic Canada (McDonald *et al.* 1991; Mallet and Carver 1995; Saavedra *et al.* 1996; Penney and Hart 1999). Comesana *et al.* (1999) found that 18% of mussels classified as pure *M. trossulus* using MPI and EST-D were reclassified, mainly as *M. trossulus*-biased backcross individuals, when the nDNA markers were used in addition to the allozyme loci. Allozyme electrophoresis results for the MPI locus were obtained for ten Nova Scotia *M. trossulus* and two Nova Scotia *M. edulis* from the present study. All were identified as homozygotes for MPI alleles typical of the species to which they had been morphometrically assigned (MPI^{100} for *M. edulis* and MPI^{84} for *M. trossulus*). However, with the Me 15/16 marker, seven of these mussels (one *M. edulis* and six *M. trossulus*) were heterozygotes for the Me 15/16 – 180 bp and Me 15/16 – 168 bp alleles. Glu-5' genotypes were obtained for two of the mussels identified as *M. trossulus* by morphometry and MPI genotype and, for both of these mussels, the Glu-5' locus gave different species identification results than the Me 15/16 and MPI loci. These results highlight the discrepancy between nDNA markers and allozymes in this area and also the need for use of a range of markers when classifying mussels as pure species or hybrids.

A comparison of evolutionary relationships within the *M. edulis* complex deduced from nDNA markers and allozymes (Table 3.6) also suggests disagreement between the two marker types. Allozyme data show *M. edulis* and *M. trossulus* as the most distantly related pair, although the genetic distance value (0.25) is not much greater than that for the other two species pairs (0.21). In contrast, the nDNA data from the present study show *M. edulis* and *M. trossulus* as the most closely related pair and *M. galloprovincialis* as being more closely related to *M. edulis* than *M. trossulus*. This pattern for nDNA markers closely matches that for mtDNA. This is interesting because a widely used explanation for the discordance between allozyme and mtDNA variation is differential introgression of mtDNA and nDNA (e.g. Quesada, Wenne and Skibinski 1995). If this were the case in the *M. edulis* complex then

disagreement between nDNA and mtDNA would be expected. However, it is important to bear in mind that evolutionary relationships deduced from just one or a few markers, as in the present study, are likely to be more strongly influenced by genetic drift than those deduced from many markers (Nei 1987) and thus the observed agreement or disagreement between marker types may be strongly influenced by interlocus variability and may not reflect real similarities or differences. Examination of the pairwise genetic distances (Table 3.5) shows that the relationships deduced in the present study are heavily influenced by the high frequency of the *M. edulis* –associated Me 15/16 allele (180 bp) in the Gulf of Gdansk *M. trossulus* population. This results in a higher genetic distance between the two *M. trossulus* population than between the Baltic Sea *M. trossulus* and the two *M. edulis* populations for Me 15/16, which is not so for Glu-5' and CaM-1. Additionally, the possibility remains that selection may influence allele frequencies at allozyme loci and at the Me 15/16 and Glu-5' loci. The neutrality of allozyme loci is a controversial issue (Karl and Avise 1992) and the adhesive protein gene is important in attachment of mussels to the substrate and thus might be subject to selective effects. Indeed, Rawson *et al.* (1996) found evidence of selection at the Glu-5' locus in a hybrid *Mytilus* population, where allele frequencies were associated with shell length. More data from a larger number of loci would be necessary in order to draw any conclusions about the evolutionary forces influencing the different marker types and the true evolutionary relationships between the three species.

Given the extensive hybridisation and introgression known to exist in the *M. edulis* complex, it is possible that perfectly diagnostic markers for identifying species might not exist. Nevertheless, the nDNA markers studied here revealed high levels of heterogeneity between populations and species and are therefore useful for population genetic studies. The Me 15/16 marker consistently showed higher levels of heterogeneity and higher genetic distance estimates than the Glu-5' and CaM-1 loci. Taking account of the extremely high amplification success rate (99%) and the ability to distinguish all three species by simple PCR product length variation, the Me 15/16 locus is clearly the most effective nDNA marker available for species identification in the six *M. edulis* complex

populations studied. The results of this and previous studies (Inoue *et al.* 1995, Silva 1998) indicate that the Me 15/16 marker is diagnostic or almost diagnostic of *M. edulis* and *M. galloprovincialis* in Europe and Japan and of *M. trossulus* in Alaska but its value in distinguishing *M. trossulus* in eastern Canada or the Baltic Sea is in doubt. Either these populations, identified as *M. trossulus* by allozymes and morphometry, have acquired introgressed alleles through hybridisation with *M. edulis*, or the Me 15/16 marker is polymorphic in *M. trossulus*. Further studies are required to equate the allozymic and the DNA detection of *Mytilus* species, particularly *M. trossulus*, from different regions of the world.

Chapter 4. Species Identification in *Mytilus* Larvae and Inheritance of the Me 15/16 marker

4.1 Introduction

The *Mytilus edulis* complex includes three marine mussel species (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) with a worldwide distribution. Hybridisation occurs to varying degrees where the species distributions overlap, however the taxa maintain morphological and genetic differences (McDonald *et al.* 1991). *Mytilus* species have a prolonged larval stage enabling dispersal over great distances, but little is known about how the hybrid zones are maintained. The availability of a rapid method for identifying larvae of these three species and their hybrids would facilitate studies of hybridisation and would aid the study of larval dispersal for other purposes.

Mytilus taxa were first defined using morphological characters but shell shape is now known to be heavily influenced by various environmental factors (Seed 1968). More recently, several allozyme loci have been extensively used for identifying *Mytilus* species, although no single locus is diagnostic between the three closely related species (McDonald *et al.* 1991). However, scoring allozymes in larvae, though possible (Hu *et al.* 1992), is difficult.

Bivalve larvae are notoriously difficult to distinguish morphologically (Loosanoff *et al.* 1966; Chanley and Andrews 1971; Le Pennec 1980) and no diagnostic criteria exist at the species level for early stage *Mytilus* larvae. Immunological identification techniques exist for some marine invertebrate larvae (Miller *et al.* 1991; Demers *et al.* 1993) but there can be variation in the reliability of these techniques between life history stages (Medeiros-Bergen *et al.* 1995). PCR-based DNA methodology offers potential for rapid processing of large samples. It requires very small amounts of template DNA and has already been applied to various marine invertebrate larvae (e.g. Medeiros-Bergen *et al.* 1995; Andre *et al.* 1999; Hare *et al.* 2000) including mussel larvae

(Corte-Real *et al.* 1994; Claxton and Boulding 1998; Sutherland *et al.* 1998; Toro 1998; Martel *et al.* 2000). However, no previous study has distinguished the larvae of *M. edulis*, *M. galloprovincialis* and *M. trossulus*.

Recently, two PCR-based DNA markers have been developed which claim to distinguish adult mussels of all three species in the *M. edulis* species complex (Inoue *et al.* 1995; Rawson *et al.* 1996). Both markers are located within the nuclear gene encoding a polyphenolic adhesive protein, important in attachment of mussels to the substrate. The marker reported by Rawson *et al.* (1996), Glu-5', produced species specific bands, but additional bands were also present and evidence was found for intragenic recombination and association of allele frequencies with mussel size in a hybrid population. Inoue *et al.* (1995)'s marker, *Me 15/16*, produced single diagnostic bands for individuals from each of the three species. Band sizes were 180 bp for *M. edulis* from Lewes (Delaware, USA), Tromsö (Norway) and Brittany (France), 168 bp for *M. trossulus* from Juneau (Alaska, USA) and 126 bp for *M. galloprovincialis* from two locations in Japan and from the Mediterranean coast of France. A later study (Inoue *et al.* 1997) found mussels from other Japanese populations that were heterozygous for the bands typical of *M. galloprovincialis* and *M. trossulus* and hybridisation was given as a possible explanation.

During initial PCR amplification trials using DNA extracted from adult mussels the results obtained with Me 15/16 were more consistent and reproducible than those from Glu-5' (see Chapter 3). The Me 15/16 marker has several other advantages with regard to PCR amplification from larvae. Firstly, the short sequence should amplify efficiently from a small amount of template DNA and secondly, the diagnostic length differences avoid the need for further manipulation of PCR products.

In population genetic studies an important criterion for any nuclear marker is to confirm its Mendelian inheritance. Many factors can complicate the inheritance of alleles at particular loci, for example linkage to a sex-determining gene could

cause sex-biased inheritance. This study investigates the detection and inheritance of the Me 15/16 marker in individual pure species and hybrid larvae from laboratory crosses between *M. edulis*, *M. galloprovincialis* and *M. trossulus*.

4.2 Materials and Methods

Intra- and inter-specific crosses were performed between *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* and samples of parents and larvae were taken as described in Chapter 2. For this study larvae from trials II, III, IV and VIII were examined at 3 h and 72 h or 2 weeks after fertilisation. The primers Me 15 and Me 16 (Inoue *et al.* 1995) were used for PCR amplification from 1 µl (approx. 50 ng) extracted DNA from each parent or 3 µl lysate from each larva. PCR conditions and temperature cycle were as described in Chapter 3. PCR products were electrophoresed on 8% non-denaturing polyacrylamide gels and bands were visualised by a silver staining method (see Appendix B).

4.3 Results

Data were obtained for all possible pure species crosses. In spite of repeated efforts it was not possible to obtain data for 3 of the 6 possible reciprocal hybrid crosses (Me/Me female x Mt/Mt male, Mt/Mt female x Mg/Mg male and Mg/Mg female x Mt/Mt male), mainly because there was a low frequency of parents with the Mt/Mt genotype.

Me 15/16 PCR products of the three expected sizes (180 bp, 168 bp and 126 bp for *M. edulis*, *M. trossulus* and *M. galloprovincialis* respectively (Figure 4.1)) were obtained for 247 larvae from thirteen families. A total of 315 larvae were analyzed, 64 at 3 h, 224 at 72 h and 27 at 2 weeks after fertilisation. Amplification success rates varied with age of larvae: 38% in 3 h old larvae, 90% in 72 h old larvae and 78% in 2 week old larvae (chi-square 81.612, d.f. 2, $P<0.001$). Presumably PCR products without a detectable band were due to insufficient DNA for successful amplification or the presence of PCR inhibitors.

Me 15/16 genotypes for parents and larvae are shown in Table 4.1. Allele nomenclature follows that of Inoue *et al.* (1995). Unexpected genotypes were detected in two of the thirteen families. In family 37 one larva was scored as a homozygote for the Mg allele when the expected genotype was Me/Mg. One larva from family 12 (expected genotype Me/Mt) scored three bands of equal intensity of the approximate sizes of the Me, Mg and Mt alleles. In a total of nine 72 h old larvae from families 31 (8) and 32 (1), a very faint band was detected at the approximate position of the Mg allele, in addition to the expected Me band. This faint band had a consistently lower intensity than the Me band and was not observed in the parents of these families. In other families where heterozygote larvae were observed, both bands were of equal intensity (Figure 4.2).

Figure 4.1 Me 15/16 PCR products obtained from mussels of the genus *Mytilus*, visualised on a silver stained 8% polyacrylamide gel. Lane 1 = *M. galloprovincialis*, Montpellier (126/126); lane 2 = *M. edulis*, Menai Strait (180/180); lanes 3,4 = *M. trossulus*, Gulf of Gdansk (180/168); L = pBR322 *Msp*I size marker.

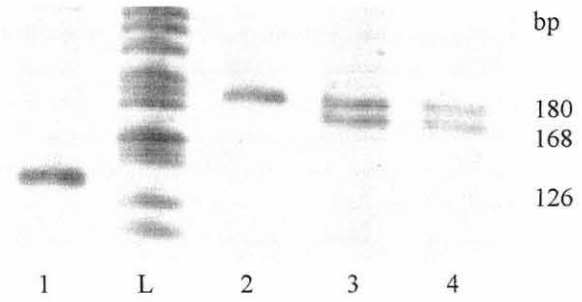
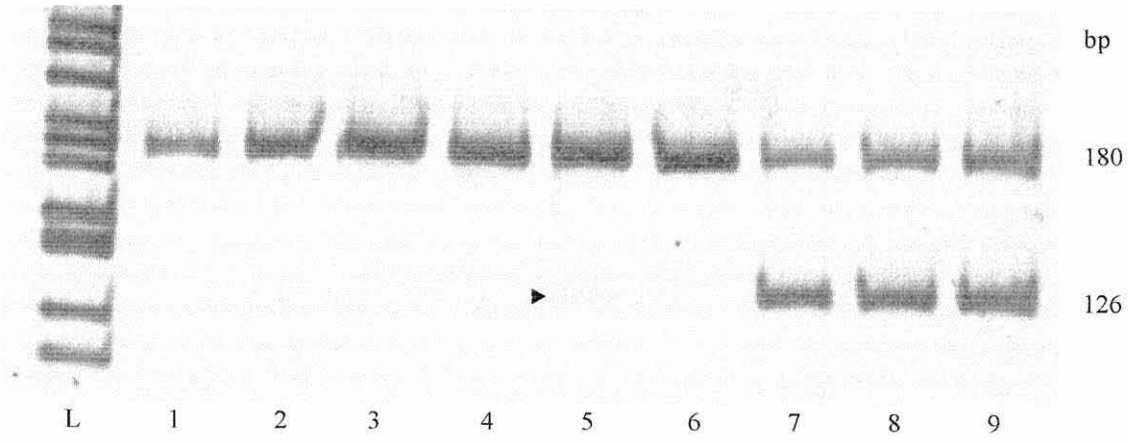


Table 4.1. Me 15/16 genotype for parents and larvae from 13 controlled laboratory crosses, representing 8 different genotype combinations. #, family number. [C], crosses involving *M. trossulus* from Canada. [B], crosses involving *M. trossulus* from the Baltic Sea. Age of larvae is the time after fertilisation at which larvae were sampled. h, hours. wk, weeks. Allele nomenclature: Me, 180 bp; Mt, 168 bp; Mg, 126 bp.

Trial / #	Parent genotypes		Age of larvae	No. of larvae		Genotype numbers						
	Female	Male		Unscorable	Scorable	Me/Me	Me/Mt	Mt/Mt	Mt/Mg	Mg/Mg	Me/Mg	Me/Mt/Mg
IV/**20**	Me/Me	Me/Me	72 h	2	13	13	0	0	0	0	0	0
VIII/**31**	Me/Me	Me/Me	3 h	10	6	6	0	0	0	0	0	0
			72 h	3	31	31	0	0	0	0	0	0
VIII/**32**	Me/Me	Me/Me	72 h	0	10	10	0	0	0	0	0	0
VIII/**44**	Mg/Mg	Mg/Mg	3 h	0	16	0	0	0	0	16	0	0
			72 h	5	39	0	0	0	0	39	0	0
VIII/**43**	Mg/Mg	Mg/Mg	72 h	0	10	0	0	0	0	10	0	0
II/**14**[C]	Mt/Mt	Mt/Mt	72 h	8	7	0	0	7	0	0	0	0
VIII/**37**	Me/Me	Mg/Mg	3 h	16	0	0	0	0	0	0	0	0
			72 h	0	10	0	0	0	0	1	9	0
VIII/**41**	Mg/Mg	Me/Me	3 h	14	2	0	0	0	0	0	2	0
			72 h	2	31	0	0	0	0	0	31	0
VIII/**38**	Mg/Mg	Me/Me	72 h	2	8	0	0	0	0	0	8	0
III/**17**	Mg/Mg	Me/Me	72 h	0	13	0	0	0	0	0	13	0
II/**12**[C]	Mt/Mt	Me/Me	2 wk	3	11	0	10	0	0	0	0	1
II/**9**[C]	Me/Mt	Me/Me	72 h	0	15	6	9	0	0	0	0	0
IV/**25**[B]	Mg/Mg	Me/Mt	72 h	0	15	0	0	0	9	0	6	0
			2 wk	3	10	0	0	0	6	0	4	0

Figure 4.2 Me 15/16 PCR products obtained from larvae of the genus *Mytilus*, visualised on a silver stained 8% polyacrylamide gel. Lanes 1-6 = Me/Me, arrow indicates faint band at approximately 126 bp in lane 5; lanes 7-9 = Me/Mg, showing both bands at equal intensity; L = pBR322 MspI size marker.



Genotype frequencies from families 9 and 25, which each had one heterozygous and one homozygous parent, were tested for agreement with Mendelian inheritance of alleles using a goodness of fit chi-square test. The data from 72 h and 2 wk old larvae from family 25 were tested both separately and pooled together. In family 9, equal proportions of Me homozygotes and Me/Mt heterozygotes were expected. In family 25 equal proportions of Me/Mg and Me/Mt heterozygotes were expected. No significant deviation from the expected genotype proportions was detected (Table 4.2).

Table 4.2. Results from goodness of fit chi-square test for agreement of observed genotype frequencies with those expected according to Mendelian inheritance.

Family	Age of larvae	Observed and expected (in parentheses) genotype frequencies				Chi-square	df	Exact significance
		Me/Me	Me/Mt	Me/Mg	Mt/Mg			
9	72 h	6 (7.5)	9 (7.5)	-	-	0.600	1	0.607
25	72 h	-	-	6 (7.5)	9 (7.5)	0.600	1	0.607
25	2 wk	-	-	4 (5.0)	6 (5.0)	0.400	1	0.754
25	pooled	-	-	10 (12.5)	15 (12.5)	1.000	1	0.424

4.4 Discussion

Me 15/16 alleles were detected in 78% of the larvae analysed. This success rate varied with the age of the larvae, being lowest (38%) in 3 h old larvae and highest (90%) in 72 h old larvae. The low amplification success rate in 3 h old larvae may be a result of the relatively low template copy number compared to older larvae. However there was considerable variation between families, with the success rate ranging from zero in family 37 to 100% in family 44. Significant heterogeneity in success rate in 3 h old larvae from families 31, 37, 41 and 44 was detected using the Monte Carlo simulation method of Roff and Bentzen (1989) in the Monte program from REAP (McElroy *et al.* 1992) (chi-square 40.53, d.f. 3, $P<0.001$). Variation in DNA quality or in efficiency of the lysis procedure are possible explanations.

Each cross produced larvae of all the expected genotypes suggesting that there are no genotype-specific barriers to fertilisation among the crosses from which larvae were analysed. The crosses analysed demonstrate that each allele can be inherited from either the female or male parent. However, two of the thirteen families contained a small proportion of larvae with unexpected genotypes. Possible explanations for these observations might include: (1) external input of gametes or larvae via seawater supplies, (2) contamination of larvae analysed with adhered sperm, (3) contamination of gametes or larvae between families, (4) PCR contamination, (5) chromosomal loss (aneuploidy), (6) failure of PCR to detect an allele (allele dropout), (7) PCR artefacts. The first explanation is very unlikely as all seawater was passed through a 0.2 μm filter prior to use, which should eliminate any gametes or larvae. Precautions were taken to prevent contamination of gametes or larvae between families, to remove any adhered sperm by washing larvae and to minimise the possibility of PCR contamination, however these three explanations cannot be ruled out completely.

In family 37 one larva was scored as a homozygote for the Mg allele when the expected genotype was Me/Mg. This could be explained by allele dropout

(failure of the PCR to detect the Me allele in this larva), by aneuploidy, for which there are precedents in *M. edulis* (Dixon 1982) or by contamination between families.

One larva from family 12 (expected genotype Me/Mt) scored three bands of equal intensity at the approximate sizes of the Me, Mt and Mg alleles. No *M. galloprovincialis* individuals were present when this cross was performed, so contamination with *M. galloprovincialis* sperm can be ruled out. PCR contamination or non-specific amplification appear to be the only plausible explanations for this observation.

The faint band at approximately 126 bp observed in some larvae from families 31 and 32 is not regarded as a Me 15/16 allele for two reasons. First, because it exhibited a much lower staining intensity than the Me band, and second, because it was not detected in any of the parents of these families. The possibility of contamination with larvae from other families or from adhered *M. galloprovincialis* sperm was discounted for some of the larvae by detection of mitochondrial DNA markers which distinguished the parents of families 31 and 32 from those of the other families (data not shown). It is likely that this faint band at approximately 126 bp, present only in larvae with parents from the Loch Etive site yet not detected in the parents themselves, is a PCR artifact produced by non-specific amplification. The only non-specific band previously recorded with the Me 15/16 marker was a minor band at approximately 300 bp found in Japanese *Mytilus* populations by Inoue *et al.* (1997). The occurrence of these minor bands highlights the value of inheritance studies and the need for care when PCR bands of different size at a locus are sometimes observed at different intensity. The identity of these unexpected bands might be determined by cloning and sequencing the PCR products.

The data from families 9 and 25 showed segregation of alleles which did not differ significantly from Mendelian expectations. In the remaining families, in which larvae were expected to be either homozygotes or heterozygotes of one

type only, just 2 out of 207 scorable larvae (<1%) had unexpected genotypes. This further supports a conclusion of Mendelian inheritance for this locus.

In conclusion, the Me 15/16 marker is readily detected in individual mussel larvae, appears to be inherited in a Mendelian manner and will be of potential use in the study of larvae in the *Mytilus edulis* species complex.

Chapter 5. Method Development for mtDNA Genome Detection

5.1 Introduction

Previous studies of mtDNA in *Mytilus* have developed PCR primers that amplify specifically either the F or the M mitotype (eg. Skibinski *et al.* 1994; Stewart *et al.* 1995; Rawson, Secor and Hilbish 1996; Sutherland *et al.* 1998). Other studies have employed universal primers (Rawson and Hilbish 1995) or *Mytilus* specific primers that amplify from both the F and M mitotypes, combined with restriction site assays which distinguish between the F and M mitotypes (eg. Zouros *et al.* 1994; Stewart *et al.* 1995).

In order to investigate inheritance of the F and M mitotypes in both pure species and hybrid larvae from *M. edulis*, *M. galloprovincialis* and *M. trossulus*, it is necessary to develop a methodology for distinguishing the F and M mitotypes from the three species, or at least from the mother and father of a particular family. The high level of sequence divergence between the F and M mitotypes, estimated as between 9 and 31% (Fisher and Skibinski 1990; Hoeh *et al.* 1991; Skibinski *et al.* 1994; Quesada *et al.* 1996; Beagley *et al.* 1997), allows relatively easy distinction. However, where possible the methodology also needs to distinguish between the F mitotype from the mother and that from the father of each cross, and between the M mitotypes in different males. Skibinski *et al.* (1994) estimated within mitotype sequence divergence to be 0 – 1.3% for the F mitotype and 0 – 2.3% for the M mitotype. A further complication is that in *M. trossulus* from the Baltic Sea, no M mitotype has been detected using either M specific or universal primers. Instead, some male Baltic Sea *M. trossulus* are heteroplasmic for two length variants of the F mitotype, one of which appears to behave as an M mitotype (Wenne and Skibinski 1995).

Although there is a large body of DNA sequence data for *Mytilus* and some mtDNA primers are already available, the task of distinguishing at least six mtDNA variants remains a complex one. The use of mitotype specific primers and/or species specific primers would be time efficient and would ultimately

allow a higher level of differentiation. In an ideal situation, primers would be used which were both mitotype and species specific. Following amplification, restriction enzyme digests could be used to distinguish between individuals, for example to distinguish the F mitotype PCR products from the mother and father of a cross. In the case of primers which amplified from both F and M mitotypes, restriction enzyme digests would be necessary to distinguish these mitotypes and it might be over-complicated to attempt to also distinguish between individuals using restriction enzymes.

In addition, the methodology needs to be suitable for analysis of individual larvae, in which the copy number of both mitotypes, but particularly the M mitotype, will be very low. *Mytilus* eggs have been estimated to contain approximately 200000 mtDNA molecules (Skibinski *et al.* 1994) and sperm contain five mitochondria (Longo and Dornfeld 1967) which would probably have only a few copies of mtDNA each, although the precise copy number is not known. PCR amplification is a technique which can produce detectable amounts of DNA from very small quantities of template DNA. PCR-based DNA analysis has already been applied to various marine invertebrate larvae (eg. Medeiros-Bergen *et al.* 1995; Andre *et al.* 1999; Hare *et al.* 2000) including mussel larvae (Corte-Real *et al.* 1994; Claxton and Boulding 1998; Sutherland *et al.* 1998; Toro 1998; Martel *et al.* 2000). Indeed, Sutherland *et al.* (1998) investigated inheritance of the F and M mitotypes in 18-48 h old *M. edulis* larvae using mitotype specific primers.

The extraction of DNA that is suitable for PCR amplification from small larvae is complicated by the small quantity of tissue present. Methods used for preparation of embryos and larvae for PCR range from total DNA extraction using phenol/chloroform (Toro 1998) or Chelex (Andre *et al.* 1999), to simply adding a larva directly to the PCR mixture prior to thermocycling (Medeiros-Bergen *et al.* 1995). Phenol/chloroform and Chelex extractions produce high quality pure DNA but such extractions using toxic reagents and / or involving several handling steps are difficult when very small amounts of tissue are involved and are relatively time consuming. On the other hand, the quicker

method of adding the larva directly to a PCR tube will only allow one PCR amplification from each larva (unless PCRs with different primers can be multiplexed). It is also doubtful whether this method would be successful with shelled mussel larvae, which would require some method of breaking down the shell tissue in order to make DNA available for PCR. An alternative which has been successfully used for mussel larvae (Corte-Real *et al.* 1994; Sutherland *et al.* 1998) and marine snail embryos (Simpson *et al.* 1999) is to use a lysis method to release DNA into solution prior to PCR. This type of method is relatively simple and rapid and has yielded sufficient template DNA from a single embryo (0.4 – 0.7 mm diameter) for up to four separate PCRs (Simpson *et al.* 1999). In the present study, multiple PCRs would be necessary in order to determine the presence or absence of the F and M mitotypes and to check species identity in each *Mytilus* larva.

To confirm that the DUI system does occur in mussels of the genus *Mytilus*, it is necessary to study the inheritance of the F and M mitotypes in single family crosses. The mitotypes present in each parent must be identified and distinguished and their presence or absence in the offspring confirmed. Zouros *et al.* (1992) provided direct evidence for the DUI system by demonstrating that the F mitotype present in adult mussels is that of the mother and not the father, in addition to showing that the M mitotype is present in both father and sons but not in mother and daughters. The possibility was raised that the M mitotype could be present in females at undetectable levels and be inherited maternally. To test for this Zouros *et al.* (1992) examined mtDNA inheritance in four pairs of families with the same mother but different fathers. Each of these pairs of families consisted of an intraspecific cross and an interspecific cross involving *M. edulis* and *M. trossulus*. Offspring of the crosses were reared for 12-15 months before DNA analysis. Total DNA from parents and offspring was digested with restriction enzymes and then probed with cloned mtDNA fragments. The assay produced different patterns for the two male parents of each pair of families. They found that some offspring had the father's pattern in addition to the mother's pattern, which was present in all offspring. In all pairs, the correct father's pattern was reflected in the offspring of each cross.

However, the DUI system has not yet been directly demonstrated in pure species families. Furthermore, Garrido-Ramos *et al.* (1998) have detected low levels of the M mitotype in some somatic tissues, and very rarely in the gonad, of some female mussels using a sensitive DIG/PCR assay. Therefore the possibility that the M mitotype might be inherited maternally cannot be ruled out.

The present study evaluates the usefulness of existing and new mitotype specific PCR primers for detection and discrimination of the F and M mitotypes in adults and larvae of the *M. edulis* complex. DNA extraction methods, PCR amplifications and DNA detection methods are optimized for use on 3 to 72 h old larvae. *M. edulis* larvae from single parent crosses are analysed with F and M mitotype specific primers and RFLP assays in an attempt to confirm the DUI system.

5.2 Method Development

5.2.1 Survey of existing PCR primers for *Mytilus* mtDNA

Two sets of published F-specific mtDNA primers (Skibinski *et al.* 1994; Sutherland *et al.* 1998) and three sets of M-specific mtDNA primers (Skibinski *et al.* 1994; Rawson and Hilbish 1995; Sutherland *et al.* 1998) were selected for preliminary trials using extracted DNA from adult mussels. PCR amplifications were attempted with each set of primers on samples from all of the *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus* populations described in Chapter 2. For each set of primers, a minimum of 10 female and 10 male mussels were analysed from each species. The PCR conditions and temperature cycles were those given in the original publications. Where the results obtained were unsatisfactory, reoptimization of PCR conditions was attempted.

5.2.1.1 F1/2 primers

The F-specific primers used by Sutherland *et al.* (1998) were renamed F1 (forward primer) and F2 (reverse primer). The primer sequences were as follows:

F1 5'-TATGTACCAGGTCCAAGTCCGTG-3'
F2 5'-CACATACACTAAGCACCACAATG-3'

These primers amplify a 753 bp fragment of the COIII gene (Sutherland *et al.* 1998). The reaction mixture contained approximately 50 ng total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM NH_4Cl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 1.25 units Taq DNA polymerase (Promega) in a 25 μl reaction volume. The temperature cycle consisted of an initial denaturation period of 4 min at 94°C followed by 40 cycles of 30 s at 94°C, 90 s at 59°C, 30 s at 72°C, followed by a final extension period of 4 min at 72°C. Initially PCR products were electrophoresed on 5% polyacrylamide gels and visualized by silver staining. Once successful amplification had been established

a 1% agarose gel stained with ethidium bromide proved a sufficiently sensitive detection method.

The F1/2 primers produced a single, strong band of the expected size when amplifying from male and female mussels from all 3 species (Figure 5.1).

5.2.1.2 F3/4 primers

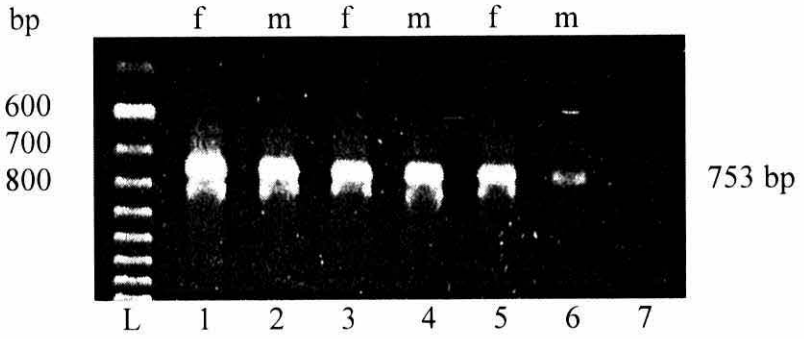
The F-specific primers from Skibinski *et al.* (1994) were named F3 (forward primer) and F4 (reverse primer). The primer sequences were as follows:

F3 5'-TCTTGGTACAACTGCGGGAA-3'
F4 5'-ACCAAGAAACGGAGGCATC-3'

These primers amplify a 1300 bp fragment which spans the join between the COIII and ND2 genes (Skibinski *et al.* 1994). The reaction mixture contained approximately 50 ng total DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μm of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega) in a reaction volume of 25 μl. The temperature cycle consisted of an initial denaturation period of 3 min at 94°C followed by 33 cycles of 60 s at 94°C, 30 s at 53°C, 90 s at 72°C, followed by a final extension period of 5 min at 72°C. PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide.

A band of the expected size was obtained when amplifying DNA from male and female *M. edulis* and *M. galloprovincialis* but no detectable PCR product was obtained from male or female *M. trossulus*. These primers have previously been used to amplify the expected product from Gulf of Gdansk *M. trossulus* by Quesada *et al.* (1999). These authors used purified mtDNA as the template for PCR which may have resulted in more efficient amplification than the total genomic DNA template used in the present study.

Figure 5.1 F1/2 PCR products obtained from male and female mussels of the genus *Mytilus*, visualised on an ethidium bromide stained 1% agarose gel. Lanes 1, 2 = *M. edulis* (Menai Strait); lanes 3, 4 = *M. galloprovincialis* (Montpellier); lanes 5, 6 = *M. trossulus* (Gulf of Gdansk); lane 7 = negative control; f = female; m = male; L = 100 bp DNA ladder.



5.2.1.3 M1/2 primers

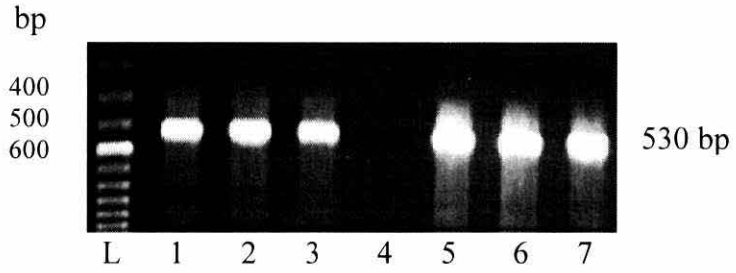
The M-specific primers used by Sutherland *et al.* (1998) were renamed M1 (forward primer) and M2 (reverse primer). The primer sequences were as follows:

M1 5'-TGGAGTCGCTTTATTTATTTTATCTGA-3'
M2 5'-ATACTACAAACCACAGCCTCACTCATA-3'

These primers were designed by Sutherland *et al.* (1998) to be M-specific and to amplify a 530 bp fragment of the COIII gene from *M. edulis* but not *M. trossulus*. The reaction mixture contained approximately 50 ng total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM NH_4Cl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 1.25 units Taq DNA polymerase (Promega) in a 25 μl reaction volume. The temperature cycle consisted of an initial denaturation period of 4 min at 94°C followed by 40 cycles of 30 s at 94°C, 90 s at 63°C, 30 s at 72°C, followed by a final extension period of 4 min at 72°C. PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide.

PCR products of approximately 530 bp were obtained from male, but not female, UK and Canadian *M. edulis* samples (Figure 5.2) and no detectable PCR products were obtained from male or female *M. galloprovincialis* or *M. trossulus*. Therefore these primers appear to be both M mitotype and *M. edulis* – specific.

Figure 5.2 M1/2 PCR products obtained from DNA extracted from adult *Mytilus edulis* samples from male mussels, visualised on an ethidium bromide stained 1% agarose gel. Lanes 1-3 = *M. edulis* (Menai Strait); lane 4 = negative control; lanes 5-7 = *M. edulis* (Loch Etive), L = 100 bp DNA ladder.



5.2.1.4 M3/4 primers

The M-specific primers from Skibinski *et al.* (1994) were named M3 (forward primer) and M4 (reverse primer). The primer sequences were as follows:

M3 5'-TCTTGGTACAACTGCGGGAA-3'
M4 5'-ACCAAGAAACGGAGGCATC-3'

These primers amplify a 1500 bp fragment which spans the join between the COIII and ND2 genes (Skibinski *et al.* 1994). The reaction mixture, temperature cycle and electrophoresis conditions were as given for the F3/4 primers.

No bands were obtained from any individual from any of the three species. PCR products were then electrophoresed on 5% polyacrylamide gels and silver stained, but no bands of the expected size were detected (some small bands <300 bp were present). Cloned M fragments (clones 25 and 27 from Skibinski *et al.* 1994) were used as positive controls and the expected band of 1500 bp was amplified. To test for the presence of PCR inhibitors in the DNA samples, some amplifications were performed using combined cloned DNA and mussel DNA as a template. Any inhibitors in the DNA sample might prevent amplification of the cloned DNA. However the amplification was successful, hence there is no evidence that the failure to amplify mussel DNA was due to the presence of PCR inhibitors.

Attempts were made to optimize these primers in various ways:

- Lowering the annealing temperature from 53°C to 52°C, then 50°C and finally to 45°C.
- A range of magnesium chloride concentrations (1-5 mM), with an annealing temperature of 50°C.
- A 50 µl reaction volume was used, as in Skibinski *et al.* (1994).
- Doubling the amount of Taq DNA polymerase.
- A range of template DNA concentrations (ten-fold and one hundred-fold dilutions and two-fold and ten-fold increases).

- Extra annealing time (1 minute instead of 30 s).
- 40 cycles instead of 33.

However the amplifications were unsuccessful.

The successful amplification from the cloned M mitotype fragments suggests that the problem in amplifying from extracted mussel DNA may be with the template quality rather than the PCR conditions. It may be that the purified mtDNA template used by Skibinski *et al.* (1994) facilitated more efficient PCR amplification than the total genomic DNA template used in the present study.

5.2.1.5 PR17/18 primers

These primers were designed by Rawson and Hilbish (1995a) to amplify a 440 bp fragment of the mitochondrial 16S rRNA gene, from the M genome but not the F genome, in *M. edulis*, *M. galloprovincialis* and *M. trossulus*. A restriction enzyme digest with *Dde*I was used to distinguish PCR products from the three species. Three distinct RFLP patterns were detected, the first was found only in *M. trossulus* and the second only in *M. galloprovincialis* but the third pattern was found in *M. edulis* and in a small proportion of *M. galloprovincialis* individuals. The primer sequences were as follows:

PR17 5'-GCTTCTACACCTCTAGGACAC-3'

PR18 5'-CTGCCC(A/T)[§](G/A) TGCAACTAAATTAAC-3'

[§] Parentheses indicate mixed base sites, where approximately 50% of primer molecules include one of the bases given and other primer molecules include the other base given.

The reaction mixture contained approximately 50 ng total DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 1 unit Taq DNA polymerase (Promega) in a reaction volume of 25 μl. The temperature cycle consisted of an initial denaturation period of 3 min at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 58°C, 45 s at 72°C, followed by a final extension period of 3 min at 72°C.

PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide.

A strong band of the expected size was amplified from *M. galloprovincialis* and Canadian *M. trossulus* males (Figure 5.3). A weak band of the same size was also obtained from Loch Etive and Canadian *M. edulis* males and from some Canadian *M. trossulus* females. No bands were obtained from *M. trossulus* from the Baltic Sea or from female *M. edulis* or *M. galloprovincialis*.

Following digestion of PCR products with *Dde*I, three RFLP patterns were observed. One pattern was found only in *M. galloprovincialis* males and the second pattern only in *M. trossulus* males. The third pattern was found in both Canadian *M. edulis* males and Canadian *M. trossulus* females (Figure 5.4). Hence the *Dde*I digest appears to enable identification of the M mitotype in *M. galloprovincialis* and Canadian *M. trossulus* males. The unreliable and weaker amplification from *M. edulis* males and the shared RFLP pattern between *M. edulis* males and some *M. trossulus* females indicate that these primers cannot be reliably used to identify the *M. edulis* M mitotype.

Figure 5.3 PR 17/18 PCR products obtained from male mussels of the genus *Mytilus*, visualised on an ethidium bromide stained 1% agarose gel. Lanes 1-3 = *M. galloprovincialis* (Montpellier); lanes 4, 5 = *M. trossulus* (Nova Scotia); L = 100 bp DNA ladder.

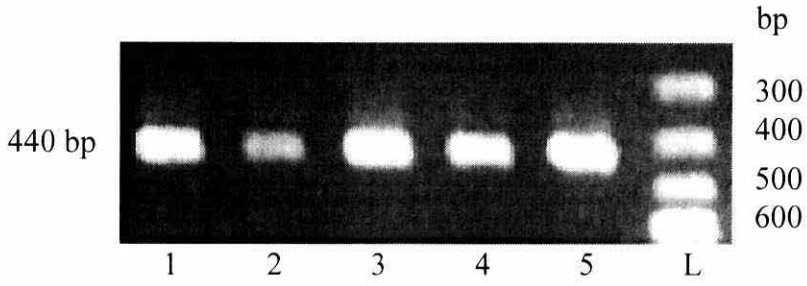
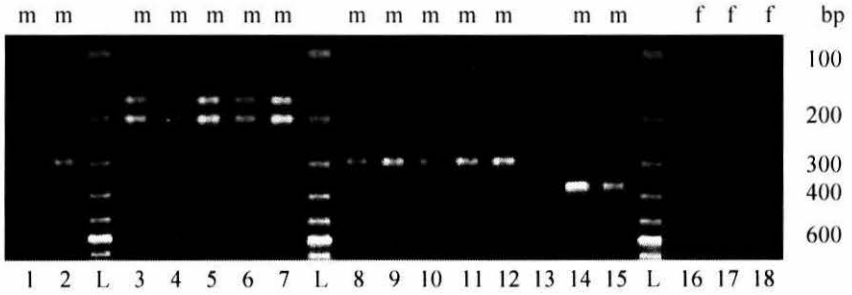


Figure 5.4 PR 17/18 PCR products obtained from male and female mussels of the genus *Mytilus* and digested with *Dde*I. Lanes 1, 3-7 = *M. galloprovincialis* (Montpellier); lanes 2, 8-12, 16-18 = *M. trossulus* (Nova Scotia); lane 13 = negative control; lane 14= *M. edulis* (Loch Etive); lane 15 = *M. edulis* (Nova Scotia); L = 100 bp DNA ladder; f = female; m = male.



5.2.2 Development of new primers

The literature and the GenBank (Benson *et al.* 2000) database were searched to find all available mtDNA sequence data for the F and M mitotypes for *M. edulis*, *M. galloprovincialis* and *M. trossulus*. Sequence data were available for 2 regions for individuals from all 3 species for both F and M mitotypes: approximately 450 bp of the 16S rRNA gene (Rawson and Hilbish 1995b) and 296 bp of the COIII gene (Quesada, Warren and Skibinski 1998; Stewart *et al.* 1995). All available sequences for these regions were aligned using the ClustalW computer program (Higgins *et al.* 1996). The 16S rRNA gene sequence data contained very little variation between mitotypes / species (15% of sites were polymorphic) and were not considered to be useful, however the COIII gene sequences were far more variable (47% of sites were polymorphic) and could potentially be of use for designing species / mitotype-specific PCR primers. The available COIII gene sequences (see Table 5.1) were divided into 6 groups according to species (*M. edulis*, *M. galloprovincialis* or *M. trossulus*) and mitotype (F or M) and the sequences within each group were aligned using ClustalW. From these alignments a typical sequence was chosen from each group. The *M. edulis* and *M. galloprovincialis* groups contained sequences from individuals from different locations, the typical sequences chosen were from British Isles *M. edulis*, an Atlantic *M. galloprovincialis* female and a Mediterranean *M. galloprovincialis* male.

The internet based computer program Primer3 (Rozen and Skaletsky 1996, 1997) was used to design 18-27 bp PCR primers to amplify 100-300 bp long fragments from the 6 typical sequences. For each mitotype the *M. edulis* and *M. galloprovincialis* sequences were almost identical and the few differences that did occur were not in positions that made it possible to design effective primers to distinguish between these 2 species. Four primer pairs were selected to amplify from the following groups: *M. edulis* / *M. galloprovincialis* F mitotype (F(ed/ga)), *M. edulis* / *M. galloprovincialis* M mitotype (M(ed/ga)), *M. trossulus* F mitotype (F(tr)) and *M. trossulus* M mitotype (M(tr)).

Table 5.1. Sources of COIII gene sequence data used. NS = Nova Scotia, Canada.

Species	Mitotype	Number of Sequences	Source	Reference
M. edulis	F	9	British Isles	Quesada *et al.* (1998)
		3	Bay of Lunenburg, NS	Stewart *et al.* (1995)
	M	10	British Isles	Quesada *et al.* (1998)
		2	Bay of Lunenburg, NS	Stewart *et al.* (1995)
M. galloprovincialis	F	9	Mediterranean Sea	Quesada *et al.* (1998)
		6	NW Spain (Atlantic)	Quesada *et al.* (1998)
	M	10	Mediterranean Sea	Quesada *et al.* (1998)
		6	NW Spain (Atlantic)	Quesada *et al.* (1998)
M. trossulus	F	4	Bay of Lunenburg, NS	Stewart *et al.* (1995)
	M	3	Bay of Lunenburg, NS	Stewart *et al.* (1995)

Primers were selected based on the following criteria (see Table 5.2):

1. The non-complementarity score (defined as the number of bases which differed between the primer and a typical sequence). Primers with a non-complementarity score of less than 2 for any sequence from a different group were discarded.
2. Low 3'end complementarity (as calculated by Primer3) for individual primers and primer pairs. Primers with scores greater than 2 were discarded.
3. Low 3' end non-specificity score (defined as the number of conserved bases, within the 6 typical sequences, in the five 3' end bases). Primers with a score of 5 were discarded.
4. PCR product length. The aim was to amplify products of different lengths, separable by agarose gel electrophoresis, from each group.
5. Tm (as calculated by Primer3). Primer pairs with similar Tm values were selected.
6. DNA duplex stability (delta G). Scores were calculated according to the method of Rychlik (1993), for the five 3' end bases and the five 5' end bases. Where possible, primers with an unstable 3' end (-6 to -9 kcal mol^{-1}) and a relatively stable (more negative delta G) 5' end were selected.

Table 5.2. Data for primer selection criteria for the 4 pairs of primers selected. F = F mitotype; M = M mitotype; ed = *M. edulis*; ga = *M. galloprovincialis*; tr = *M. trossulus*; L = forward primer; R = reverse primer; bp = base pairs. † = as calculated by Primer 3. § = the number of conserved bases, within the 6 typical sequences, in the five 3' end bases. ¶ = the number of bases which differed between the primer and a typical sequence. Delta G values were calculated according to the method of Rychlik (1993).

Primer	F(ed/ga)L	F(ed/ga)R	F(tr)L	F(tr)R	M(ed/ga)L	M(ed/ga)R	M(tr)L	M(tr)R
Primer length (bases)	24	23	20	22	22	21	22	20
Product length (bp)	288		202		201		228	
Tm (°C)†	59.8	59.9	60.2	59.9	59.9	59.9	59.5	59.9
3' end complementarity†	2	1	1	0	1	1	0	2
Pair 3' end complementarity†	0		2		2		0	
3' end non-specificity§	4	4	3	4	3	4	3	4
3' end delta G (kcal mol^{-1})	-7.3	-6.9	-6.1	-8.2	-6.7	-6.4	-6.7	-7.5
5' end delta G (kcal mol^{-1})	-12.9	-8.1	-8.1	-8.1	-8.2	-7.0	-6.7	-7.3
Non-complementarity score¶								
M. edulis F	0	0	4	5	4	9	7	4
M. galloprovincialis F	0	0	4	5	4	9	7	4
M. trossulus F	5	5	0	0	4	10	3	3
M. edulis M	3	3	5	2	0	0	6	2
M. galloprovincialis M	3	3	5	2	0	0	6	2
M. trossulus M	5	3	4	4	6	9	0	0

The sequences of the group specific primers selected were as follows (L indicates the forward primer and R the reverse):

F(ed/ga)L 5'-GCGGGAATATTATTGAAACTCCTA-3'
F(ed/ga)R 5'-CCATCCCATATCTTAAACCATCA-3'

F(tr)L 5'-CACGTAGTCGTGGGGACTCT-3'
F(tr)R 5'-CGTCTCATATTTTGAACCACCA-3'

M(ed/ga)L 5'-GCAGACAGTGTTTATGGCAGAG-3'
M(ed/ga)R 5'-CACAAATCACAGCCTCACTCA-3'

M(tr)L 5'-TCAGATAGCGTGTACGGAAGAG-3'
M(tr)R 5'-TAACCAACCCCCAAACCATA-3'

PCR amplifications with the 4 selected primer pairs were attempted on extracted DNA from samples from the *M. edulis*, *M. galloprovincialis* and *M. trossulus* populations described in Section 2.1. The reaction mixture contained approximately 50 ng total DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.1% TritonX®-100 and 1 unit of Taq DNA polymerase (Promega) in a reaction volume of 25 μl. $MgCl_2$ concentration was optimized (1-5 mM) for each primer pair. The temperature cycle consisted of an initial denaturation period of 4 min at 94°C followed by 30 cycles of 30 s at 94°C, 60 s at 55°C, 60 s at 72°C, followed by a final extension period of 4 min at 72°C. PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. Where initial results were unsatisfactory, optimization of the annealing temperature was attempted.

In the initial trial the F(ed/ga) primers gave the expected band of 288 bp in *M. edulis* and *M. galloprovincialis* females and males. The strongest bands were produced with 4 mM $MgCl_2$. No bands were detected in *M. trossulus*. Further amplifications under the same conditions and also with increased annealing

temperatures (up to 57°C) produced inconsistent results, with bands detected at approximately 200 bp and not at 288 bp.

The F(tr) primers produced the expected band of 202 bp in Canadian and Baltic Sea *M. trossulus* females and males. 2 mM $MgCl_2$ gave the strongest bands. No bands were detected in *M. edulis* or in *M. galloprovincialis* males, but a faint band at approximately 200 bp was detected in some *M. galloprovincialis* females, which was not consistently eliminated by increasing the annealing temperature (up to 66°C).

The M(ed/ga) primers gave the expected band of 201 bp in *M. edulis* and *M. galloprovincialis* males and no band was produced in females. In *M. edulis* the band was strongest at 2 mM $MgCl_2$, but in *M. galloprovincialis* 4 mM $MgCl_2$ gave the strongest band. A faint band at approximately 200 bp was detected in some Canadian and Baltic Sea *M. trossulus* males and females, which was not consistently eliminated by increasing the annealing temperature (up to 58°C).

Under the initial PCR conditions the M(tr) primers produced the expected band of 228 bp in females and males of all 3 species. Increasing the annealing temperature increased the specificity of these primers. An annealing temperature of 67°C and 3 mM $MgCl_2$ consistently gave the expected band in Canadian, but not Baltic Sea, *M. trossulus* males and no bands were detected in *M. trossulus* females, *M. edulis* or *M. galloprovincialis*.

No further optimization was attempted with the F(ed/ga), F(tr) and M(ed/ga) primers due to the non-specific amplification detected. As most of the non-specific amplification products were of a size similar to that expected from the target sequence, the non-specificity of the primers was probably due to insufficient sequence differences between the species / mitotypes at the primer annealing sites. However it may be that non-specific amplification resulted from primer(s) annealing at other sites within the genome. The M(tr) primers appeared to be specific and so PCR amplification from lysate from larvae was attempted with these primers. The larvae used were 72 h old pure species

Canadian *M. trossulus* larvae from family 14 (trial II) and the PCR conditions were as described above, with 3 mM $MgCl_2$ and an annealing temperature of 67°C. No bands were detected using 2% agarose gel electrophoresis and ethidium bromide staining or 5% polyacrylamide gel electrophoresis and silver staining. It is possible that these primers would, with further optimization, amplify from larvae, however this was not attempted as no full reciprocal crosses involving Canadian *M. trossulus* had been successfully achieved.

5.2.3 Selection of primers for detection of F and M mitotypes in *Mytilus* larvae

The F1/2 primers were selected as F-specific primers for use on all three species. For *M. trossulus* from the Baltic Sea, where no M mitotype has been detected, restriction digests of the F1/2 PCR products might detect mussels heteroplasmic for more than one variant of the F mitotype. The M1/2 primers were selected as *M. edulis* M-specific primers and the PR17/18 primers were selected to detect the M mitotype in *M. galloprovincialis* and Canadian *M. trossulus*. Attempts were made using all these selected primers to amplify lysate from mussel larvae from the crosses described in Chapter 2.

5.2.4 Optimization of amount of lysate used in PCR

In order to determine the presence or absence of both mitotypes in each larva and to confirm the species identity, it was necessary to do 3 PCRs for each larva. The lysis protocol used (see Section 2.6) produces 15 µl lysate for each larva. For each set of mitochondrial gene PCR primers selected, the optimal amount of lysate to be used as a PCR template was determined, to ensure the accuracy of results and the feasibility of the study.

Three and 72 h old *Mytilus edulis* (for F1/2 and M1/2 PCRs) and *M. galloprovincialis* (for PR17/18 PCRs) larvae were used for these experiments. PCR conditions were as given above (Section 5.2.1). PCR products were electrophoresed on 5% polyacrylamide gels and visualized by silver staining.

For F1/2 and M1/2 primers, no PCR products were detected using less than 1µl lysate as the PCR template. Amplifications using 2 to 5 µl lysate gave a band of the expected size. For M1/2 primers stronger bands were obtained with greater volumes of lysate, hence it was decided to use 3 µl lysate as a template for F1/2 and M1/2 PCRs.

For the PR17/18 primers no PCR products were detected using 3 µl lysate. Varying the $MgCl_2$ concentration, lowering the annealing temperature and increasing the number of cycles did not produce detectable PCR products. Using 6 µl lysate produced a faint band of the expected size and 9 µl lysate gave a stronger band. It would not be feasible to use more than 9 µl lysate for these primers in addition to the 3 µl required for each of the other 2 PCRs, so an additional experiment was performed in order to confirm that this protocol was capable of detecting all occurrences of the M mitotype. The lysis and PR 17/18 PCR protocols were repeated on 18 h old *M. galloprovincialis* larvae which, according to the results of Sutherland *et al.* (1998), should all contain the M mitotype. The expected band was amplified from 9 out of 10 larvae and it was decided that 9 µl lysate was a sufficient template volume for the PR 17/18 primers.

We would expect a larva to contain fewer copies of the M mitotype than the F mitotype because the sperm's contribution to the mtDNA pool of the zygote would be minimal compared to that of the egg. Therefore the copy number in a given volume of lysate would be less for the M mitotype than for the F mitotype and so we would expect that a greater volume of lysate would be required to provide sufficient template for PCR for the M mitotype than for the F mitotype. The higher volume of lysate required to produce a detectable PR 17/18 PCR product suggests that the PR 17/18 primers were less efficient than the F1/2 and M1/2 primers.

5.2.5 Comparison of lysis methods to release DNA from larvae

Two previous studies had performed PCR on mussel larvae (Sutherland *et al.* 1998; Corte-Real *et al.* 1994). Corte-Real *et al.* (1994) used distilled water and alternating periods of freezing on dry ice and heating to 95°C to release DNA from individual larvae into solution. Sutherland *et al.* (1998) used a lysis buffer solution and proteinase K treatment (see Section 2.6). These two methods were compared for 18 and 72 h old *Mytilus galloprovincialis* larvae using F1/2 and PR17/18 primers. The methods used were those described in the original papers except that for Corte-Real *et al.* (1994)'s method the volume of sterile water used was 15 µl instead of 3 µl to make the volume equal to that of Sutherland *et al.* (1998)'s method, thereby eliminating any effect of DNA template concentration. PCR products were electrophoresed on 5% polyacrylamide gels and visualized by silver staining.

The results were compared by counting the number of bands present and by assessing the band strength on a scale of strong, medium or weak (Table 5.3). For the F1/2 PCR, the results for Sutherland *et al.* (1998)'s method were better than those for Corte-Real *et al.* (1994)'s method. The composition of Sutherland *et al.* (1998)'s lysis buffer was similar to that of the F1/2 PCR buffer whereas Corte-Real *et al.* (1994)'s method used distilled water. The contribution from the lysis buffer to the overall concentration of components of the PCR reaction mixture (e.g. $MgCl_2$) might explain why Sutherland *et al.* (1998)'s method gave better F1/2 PCR results than Corte-Real *et al.* (1994)'s method. For the PR17/18 PCR the results were similar for both methods. To enable detection of both mitotypes in a single larva it is necessary to use one lysis method for all primers. It was decided that Sutherland *et al.* (1998)'s method gave the best overall results and this was the method used for all future experiments.

Table 5.3. Results of comparison of lysis methods. h = hours.

Primers	Age of larvae	Bands obtained / no. of larvae analysed, band strength	
		Sutherland *et al.* (1998) method	Corte-Real *et al.* (1994) method
PR17/18	18 h	9 / 10, strong	9 / 10, strong + non-specific bands
PR17/18	72 h	4 / 10, strong	6 / 10, strong + non-specific bands
F1/2	18 h	10 / 10, strong	6 / 10, weak
F1/2	72 h	10 / 10, strong	7 / 10, weak

5.2.6 Comparison of DNA Detection Methods

In order to maximise detection of PCR products and time efficiency, four electrophoresis and DNA detection methods were compared for their effectiveness in detecting F1/2 and M1/2 PCR products from *Mytilus edulis* larvae (protocols are given in Section 2.8 and Appendix B):

1. agarose gel electrophoresis with ethidium bromide staining.
2. agarose gel electrophoresis with SYBR Gold (Molecular Probes) staining.
3. polyacrylamide gel electrophoresis with silver staining.
4. polyacrylamide gel electrophoresis with autoradiography following internal labelling of PCR products with α-^{33}PdCTP.

5.2.6.1 Comparison of methods 1, 2 and 3

SYBR Gold staining was found to be a more sensitive detection method than ethidium bromide staining, but not as sensitive as polyacrylamide gel electrophoresis with silver staining.

5.2.6.2 Comparison of methods 1 and 3

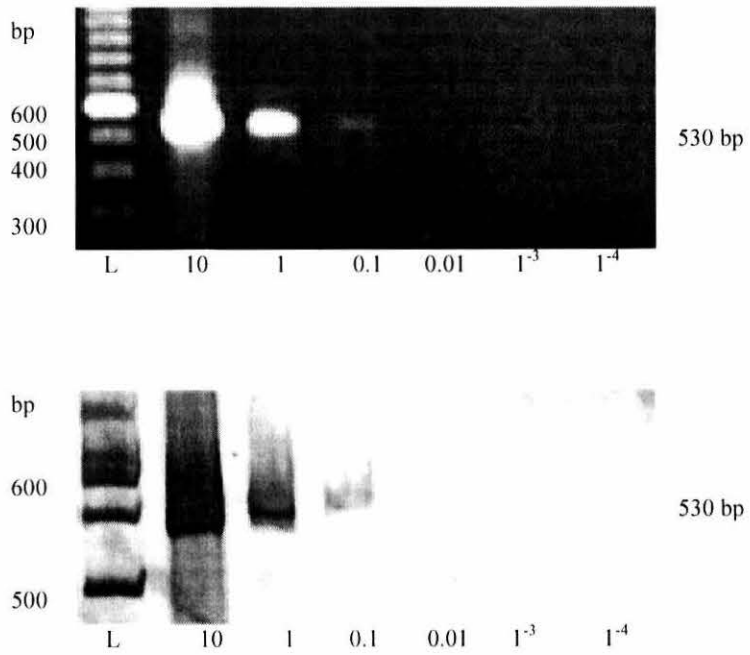
Dilutions of M1/2 PCR product amplified from extracted DNA from an adult mussel were electrophoresed and visualized using both methods. The results were compared by recording the presence or absence of a band for each dilution, and by assessing the band strength on a scale of very strong to very weak. Results are shown in Table 5.4 and Figure 5.5.

The results suggest that polyacrylamide gel electrophoresis with silver staining is approximately 100 times more sensitive than agarose gel electrophoresis with ethidium bromide staining.

Table 5.4. Detection of M1/2 PCR product from adult *M. edulis* extracted DNA.

Amount of PCR product present (μl)	Strength of band observed	
	Agarose / EtBr	PAGE / Silver
10	Strong	Very strong
1	Medium	Strong
0.1	Weak	Medium
0.01	-	Weak
0.001	-	Very weak
0.0001	-	-
0.00001	-	-
0.000001	-	-

Figure 5.5 Dilutions of M1/2 PCR product amplified from extracted DNA from an adult male *M. edulis*. PCR product dilutions were electrophoresed on a 1% agarose gel stained with ethidium bromide (top) and on a 5% polyacrylamide gel stained with silver nitrate (bottom). Amounts of PCR product present in each lane are given below each gel, in µl. L = 100 bp DNA ladder. The expected band of 530 bp was detected for 0.1 µl but not for 0.01 µl with the agarose gel and ethidium bromide staining method. The corresponding values for the polyacrylamide gel and silver staining method were 0.001 (1^{-3}) and 0.0001 (1^{-4}) µl.



To investigate how this applied to the PCR primers to be used with larvae, F1/2 and M1/2 PCRs were performed on lysate from 56 *M. edulis* larvae using the protocols given in Section 5.2.1. PCR products were then electrophoresed and visualized using both methods. The results are shown in Table 5.5.

For F1/2 PCR products, the results for both methods were very similar. The F mitotype was detected in 91% of larvae by the agarose gel / ethidium bromide staining method and 93% by the polyacrylamide gel / silver staining method. However, M1/2 PCR products had a much higher detection rate with the polyacrylamide gel / silver staining method (41% of larvae) than with the agarose gel / ethidium bromide staining method (2%). This suggests that agarose gel electrophoresis with ethidium bromide staining is sufficiently sensitive to detect F1/2 but not M1/2 PCR products. This implies that the amount of PCR product produced from larvae by M1/2 primers is less than that produced by F1/2 primers. This could happen if the M1/2 PCR was less efficient than the F1/2 PCR but this is unlikely to be the reason as both M1/2 and F1/2 PCR products from DNA extracted from adult mussels were readily detected by the agarose gel / ethidium bromide staining method (Section 5.2.1). A more likely explanation is that the M1/2 PCR starts with a lower template copy number than the F1/2 PCR, because a larva contains fewer copies of M than F.

Table 5.5. Detection of F1/2 and M1/2 PCR products from *M. edulis* larvae.

Age of larvae (h)	No. of larvae	No. of F1/2 PCR products detected		No. of M1/2 PCR products detected	
		Agarose/EtBr	PAGE/Silver	Agarose/EtBr	PAGE/Silver
3	10	10	10	0	4
15	10	8	9	1	5
18	10	8	8	0	4
21	8	7	7	0	4
24	8	8	8	0	4
48	10	10	10	0	2
Total:	56	51	52	1	23

5.2.6.3 Comparison of methods 3 and 4

Finally, in order to determine if silver staining (method 3) was a sufficiently sensitive method to detect all M mitotype PCR products, a comparison was made with a ^{33}P internal labelling method (4). M1/2 PCRs were performed on lysate from 34 3 h old *M. edulis* larvae, incorporating α-^{33}PdCTP into the PCR mix. PCRs were run on an Eppendorf Mastercycler Gradient thermocycler. It was not possible to use the Techne Genius thermocycler used for non-radioactive PCRs due to radiation safety regulations. Each PCR product was then electrophoresed on two 5% polyacrylamide gels, alongside a α-^{33}PdCTP 3'-end labelled GibcoBRL 100bp DNA ladder. Following electrophoresis, one gel was silver stained and the other gel was dried onto Whatman paper in a gel dryer and the PCR products were visualized by autoradiography.

The expected M1/2 PCR product was detected in all 34 larvae by both methods and bands were of similar intensity. This suggests that the silver staining method is sufficiently sensitive to detect M1/2 PCR products and any negative results can be interpreted as being due to either absence of the M mitotype or failure of the PCR.

5.2.6.4 Final methodology for detection of PCR products

The electrophoresis and DNA detection methods selected for use were as follows:

F1/2 primers: 1% agarose gel stained with 0.5 μg ethidium bromide per ml agarose. Any negative results were checked by running PCR products on a 5% polyacrylamide gel and silver staining.

M1/2 primers: 5% polyacrylamide gel and silver staining.

PR 17/18 primers: 5% polyacrylamide gel and silver staining.

5.2.7 Confirmation of DUI by PCR / RFLP

DUI has previously been demonstrated using pairs of intra- and inter-specific crosses involving *M. edulis* and *M. trossulus* (Zouros *et al.* 1992). In an attempt to confirm this for intraspecific *M. edulis* families, crosses were made between one female (Ef1) and 2 males (Em1 and Em2) and RFLPs of F1/2 and M1/2 PCR products were developed to distinguish the parents' mitotypes. These RFLP assays were then applied to 3 h old larvae from each family (families 1 and 2 from trial 0).

F1/2 and M1/2 PCR conditions and temperature cycles were as described in Section 5.2.1. PCR products from parents were electrophoresed on 1% agarose gels and stained with ethidium bromide. Bands of the expected sizes were obtained from all three parents with the F1/2 primers and from the two male parents, but not the female parent, with the M1/2 primers. However, when M1/2 PCR products were electrophoresed on 5% polyacrylamide and silver stained a faint band was detected in Ef1 at the same position as that detected in Em1 and Em2. 5 µl of each PCR product was incubated at 37°C for 2.5 h with 3 units of restriction enzyme for each of *Hae*III, *Hha*I, *Hinc*I, *Hinf*I, *Mbo*I and *Rsa*I. The resulting fragments were electrophoresed on 5% polyacrylamide gels and silver stained to determine which restriction enzymes were useful in distinguishing mitotypes from different parents. Suitable restriction enzymes were selected (*Hinf*I for F1/2 PCR products and *Hha*I for M1/2 PCR products) and PCR products from six 3 h old larvae from each family were digested (10 µl PCR product with 3 units restriction enzyme incubated at 37°C for 2.5 h). Digestion products from larvae were electrophoresed on 5% polyacrylamide gels and silver stained.

Digestion of F1/2 PCR products with *Hinf*I produced two different restriction patterns; one observed in the female parent and a second shared by the two male parents. None of the restriction enzymes tested distinguished the two male parents. All larvae examined from both families produced a pattern identical to that of the female parent and the pattern from the male parents was not observed

in any larvae (Figure 5.6). These results confirm that inheritance of the F mitotype is maternal and not paternal.

Digestion of M1/2 PCR products with *Hha*I produced a different restriction pattern for each of the three parents (Figure 5.7). The male parent Em2 was heteroplasmic for several different M types, including types found in Ef1 and Em1. However, each male parent had bands which were not found in Ef1 or in the other male parent and hence can be considered to be diagnostic. Em1 had two such bands and these bands were found in four of the six larvae from family 1 (Ef1 x Em1) but were not found in the larvae from family 2 (Ef1 x Em2). Em2 had three diagnostic bands, of which two were found in all six family 2 larvae but in none of the family 1 larvae. The third diagnostic band in Em2 was not found in any larvae from either family. These results demonstrate paternal inheritance of the M mitotype. The primary band detected in Ef1 (approx. 410 bp) was not observed in any larvae, however the three smaller minor bands in Ef1 were detected in larvae from both families. Two of these three bands were also present in Em1 and all three were present in Em2, so this does not necessarily demonstrate maternal inheritance of the M mitotype. However, the possibility that M is inherited both maternally and paternally cannot be ruled out from these results.

The restriction patterns observed for M1/2 PCR products were more complicated than those for F1/2 PCR products and it seems that at least some males are heteroplasmic for several variants of the M mitotype in addition to an F mitotype. It is conceivable that each sperm from such a male would not contain all of the M mitotype variants found in somatic tissues and that different sperm from the same male might contain different M variants. This would explain the observation that no single larva contained all the M1/2 PCR / *Hha*I digest bands detected in its male parent. The M1/2 PCR / *Hha*I bands for larvae were faint and at the limit of detection using this method. A more sensitive method would be necessary to confirm (or otherwise) exclusive paternal inheritance of the M mitotype.

Figure 5.6 F1/2 PCR / HinfI digest products from parents and 3 h old larvae from 2 *Mytilus edulis* families. Parents: lane 1 = Em1; lane 10 = Em2; lanes 2, 11 = Ef1. Lanes 3, 4, 6-9 = family 1 (Ef1 x Em1) larvae; lanes 12-17 = family 2 (Ef1 x Em2) larvae; lane 5 = negative control. L = 100 bp DNA ladder. All larvae show a pattern identical to that of the female parent (Ef1) and different to that of the male parents (Em1 & Em2).

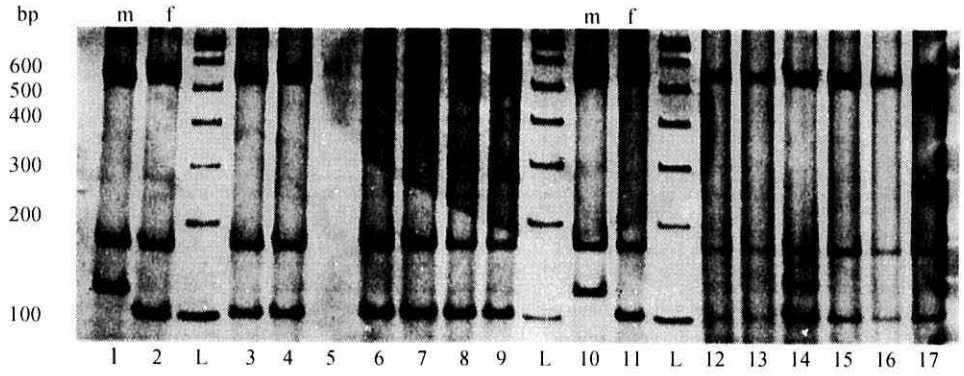
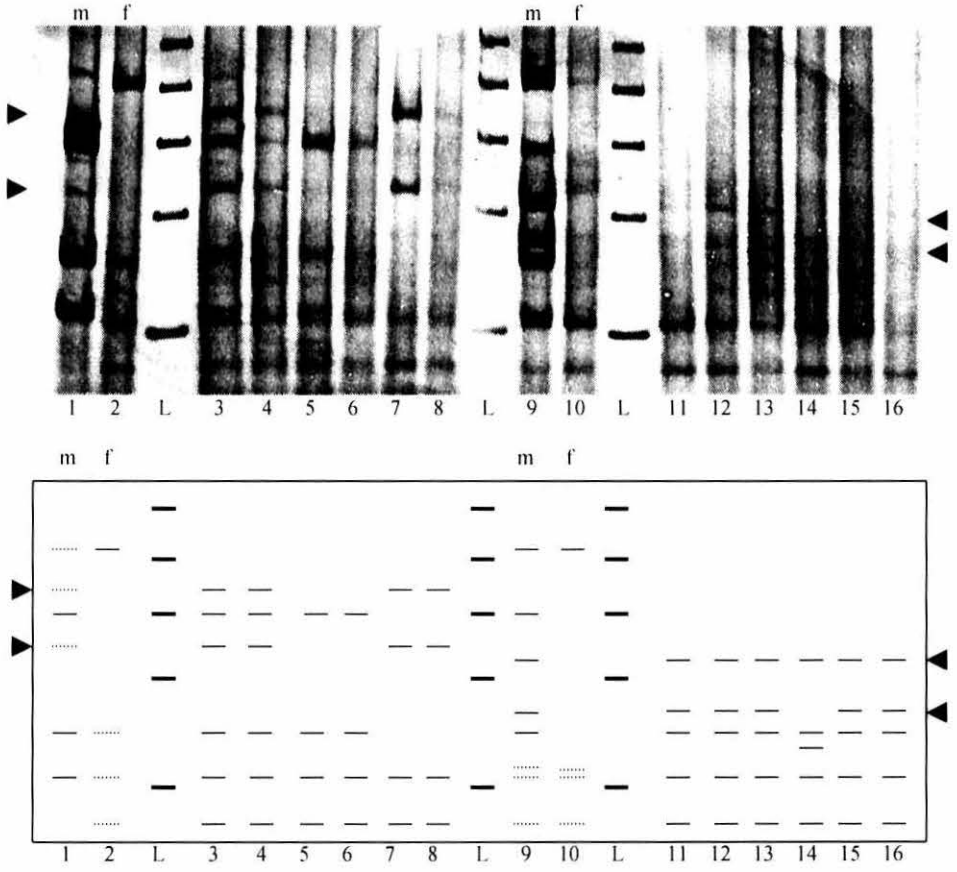


Figure 5.7 M1/2 PCR / *Hha*I digest products from parents and 3 h old larvae from two *Mytilus edulis* families. Parents: lane 1 = Em1 (m); lane 9 = Em2 (m); lanes 2, 10 = Ef1 (f). Lanes 3-8 = family 1 (Ef1 x Em1) larvae; lanes 11-16 = family 2 (Ef1 x Em2) larvae. L = 100 bp DNA ladder: bands are 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, in ascending order from the bottom of the gel. ► & ◄ indicate the position of bands found in larvae which were also found in the relevant male parent only. As the bands in larvae in the photo (top) are faint, the diagram (bottom) is included for clarity to show the bands as scored from the gel. In the parents, solid lines mark primary bands and broken lines mark minor bands.



Chapter 6. Time of Elimination of the M Mitotype in Female Mussels

6.1 Introduction

Mussels of the *Mytilus edulis* complex (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) exhibit an unusual type of mitochondrial DNA (mtDNA) inheritance, with two gender-associated mitotypes (Skibinski *et al.* 1994). The F mitotype is inherited maternally and the M mitotype is inherited paternally in a system termed Doubly Uniparental Inheritance (DUI) by Zouros *et al.* (1994). Female mussels are expected to contain only the F mitotype in their somatic tissues and eggs whereas male mussels are expected to contain both the F and M mitotypes in their somatic tissues but only the M mitotype in sperm. However, using very sensitive detection methods or with very high amounts of purified mitochondrial DNA as a template for PCR, low levels of the M mitotype have been detected in the somatic tissues, and very rarely in the gonads, of some female mussels (Quesada *et al.* 1996; Garrido-Ramos *et al.* 1998).

It appears that the inheritance of the F and M mitotypes is in some way linked to sex determination, although the occurrence of homoplasmic males (without M) and heteroplasmic females (with M) in interspecific (Zouros *et al.* 1994) and intraspecific (Saavedra *et al.* 1997) crosses suggests that mtDNA plays an indirect rather than a direct role. Although the population sex ratio in *Mytilus* has not been found to differ significantly from 1:1 (Sastry 1979; Brousseau 1983; Fisher and Skibinski 1990; Skibinski *et al.* 1994), sex ratio has been observed to vary extensively between different families produced in the laboratory (Zouros 1994; Saavedra *et al.* 1997). Furthermore, with few exceptions, families which shared the same mother but had different fathers had similar sex ratios, suggesting that sex ratio is predominantly under maternal control (Saavedra *et al.* 1997).

Longo and Dornfeld (1967) found that *M. edulis* sperm contained five mitochondria which were larger than those seen in somatic cells and Longo and

Anderson (1969) observed that the sperm is wholly incorporated into the egg during fertilisation. The sperm's contribution to the mtDNA pool of the zygote would be minimal compared to that of the egg (Avise 1991). Indeed Skibinski *et al.* (1994) estimated that there would be about 100000 times as many F mitotype molecules as M in a newly fertilised egg. However, in the somatic tissue of adult *M. edulis* males Skibinski *et al.* (1994) often found a predominance of M over F.

Three possible mechanisms have been proposed to explain why the M mitotype is readily detected in male but not female mussels (Skibinski *et al.* 1994; Zouros *et al.* 1994; Rawson, Secor and Hilbish 1996; Sutherland *et al.* 1998). (1) the sperm mitochondria (or M mitotype molecules) only enter eggs destined to become males and are excluded from those eggs destined to become females. (2) the sperm mitochondria (or M mitotype molecules) enter all eggs but are at some stage eliminated from those eggs destined to become females. (3) the M mitotype has a replicative advantage over the F mitotype in eggs destined to become males but this advantage is suppressed in eggs destined to become females. To investigate these possibilities, Sutherland *et al.* (1998) carried out laboratory crosses using *M. edulis* parents known to produce only female offspring and also parents known to produce offspring of both sexes. As it is not yet possible to determine the sex of mussel larvae or juveniles prior to reproductive maturity, this knowledge of the history of the parents was an important advantage. A series of families were produced from pair matings and offspring were sampled at 18, 24, 48 hours, 14 days, 3 and 6 months after fertilisation. Each individual was examined for the presence of the M mitotype. Almost all (98.5%) of the 18 hour old larvae showed presence of the M mitotype at a relatively high frequency, but a proportion of the 24 and 48 hour and 14 day, 3 and 6 month old larvae had no traces of the M mitotype. As some of these families were expected to contain only females, this is strong evidence that sperm mitochondria do enter the egg irrespective of which sex it will become, confirming the observations of Longo and Anderson (1969), and that the M mitotype may be eliminated in females between 18 and 24 hours after fertilisation (Sutherland *et al.* 1998).

The objective of the present study is to follow the inheritance and fate of the F and M mitotypes in developing *M. edulis* and *M. galloprovincialis* larvae from further laboratory crosses. In particular, the time at which the M mitotype is eliminated in larvae destined to become female mussels is investigated and the results are compared to those of Sutherland *et al.* (1998).

6.2 Materials and Methods

Intraspecific *M. edulis* and *M. galloprovincialis* crosses were performed and samples of parents and larvae were taken as described in Chapter 2. For this study, parents and samples of larvae taken between 3 and 72 h after fertilisation were analysed for the presence of F and M mitotypes. Analysis was performed between 1 and 21 months after sampling and samples were stored in 70% ethanol/seawater at 4°C during this time. The mitotype specific primers F1/2 (for the F mitotype in both species), M1/2 (for the M mitotype in *M. edulis*) and PR17/18 (for the M mitotype in *M. galloprovincialis*) were used for PCR amplification from 1 μl (approx. 50 ng) extracted DNA from each parent or 3 μl (9 μl for PR17/18 primers PCR) lysate from each larva. PCR conditions and temperature cycles were as described in Chapter 5. F1/2 PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. All M1/2 and PR17/18 PCR products and any F1/2 PCR products which gave negative results on agarose were electrophoresed on 5% non-denaturing polyacrylamide gels and bands were visualized by silver staining (see Appendix B).

6.3 Results

Parents and larvae from nine *M. edulis* families (1-6, 20, 31, 32) and three *M. galloprovincialis* families (8, 43, 44) were analysed for the presence of F and M mitotypes. PCR products of the expected sizes were obtained for at least one mitotype from all 19 parents, from 465 of 554 (84%) *M. edulis* larvae and from 143 of 150 (95%) *M. galloprovincialis* larvae. Where the expected band was detected for the F but not the M mitotype, or vice versa, this was interpreted as an absence of one mitotype in the individual concerned. Failure to detect either mitotype in some larvae could be due to poor DNA quality or presence of PCR inhibitors.

Data from 3 h old larvae, which are all expected to contain both F and M mitotypes, were examined for any relationship between storage time of larvae (after sampling and prior to analysis) and PCR amplification success using a G-test of independence (Sokal and Rohlf 1995). For *M. galloprovincialis* 3 h old larvae, data were obtained for storage times of 2 and 4 months and did not suggest any association between storage time and PCR amplification success for either F1/2 ($G = 1.71$, 1 df, $P>0.1$) or PR17/18 primers ($G = 3.23$, 1 df, $P>0.05$). For *M. edulis* 3 h old larvae, storage times ranged from 2 to 21 months. There was no significant association between storage time and amplification success rate with F1/2 primers ($G = 8.58$, 4 df, $P>0.05$) , but storage times greater than 9 months were associated with lower amplification success rates with M1/2 primers ($G = 74.95$, 4 df, $P<0.001$) (Table 6.1).

If the data with storage times of 20 and 21 months are removed, a test of the remaining data (for storage times of 2, 4 and 9 months) gives a non-significant result ($G = 0.77$, 2 df, $P>0.2$).

Table 6.1. Percentage of larvae for which the expected band was obtained for F1/2 and M1/2 or PR17/18 primers in 3 h old larvae analysed after different storage times. N, sample size.

Storage time (months)	*M. edulis*			*M. galloprovincialis*		
	N	F1/2 (%)	M1/2 (%)	N	F1/2 (%)	PR17/18 (%)
2	67	91	94	32	100	72
4	18	89	94	20	90	100
9	20	85	95	-	-	-
20	39	97	41	-	-	-
21	15	100	0	-	-	-

This was supported by data from three *M. edulis* families for which 3 h and 72 h old larvae were analysed firstly between 1 and 9 months after sampling and then again after 20-21 months. (Table 6.2). In larvae analysed after the shorter storage time the F mitotype detection rate was 89-100% and the M mitotype detection rate was 100% for 3 h old larvae and 30-76% for 72 h old larvae. For larvae analysed after the longer storage time the F mitotype was detected in all larvae but the M mitotype was not detected in any 3 h or 72 h old larvae.

The data suggest that storage of larvae in 70% ethanol/ 30% UV treated, 0.2 μm filtered seawater for longer than 9 months prior to analysis might adversely affect PCR amplification success for M1/2 primers, although not for F1/2 primers, hence data from samples of *M. edulis* larvae with storage times greater than 9 months were not included in further analyses. The reduction in PCR amplification success after storage times greater than 9 months for M1/2 primers but not F1/2 primers is most likely due to degradation of DNA, which would reduce the number of amplifiable copies of each mitotype. In early stage larvae the copy number of the M mitotype would be much smaller than that of the F mitotype and hence degradation of DNA over time would reduce the copy number below that detectable by PCR sooner for the M mitotype than for the F mitotype.

There was no significant variation in PCR amplification success with age of larvae for either *M. edulis* ($G = 3.27$, 5 df, $P>0.5$) or *M. galloprovincialis* ($G = 0.82$, 2 df, $P>0.5$) (Table 6.3).

Table 6.2. Percentage of larvae for which the expected band was obtained for F1/2 and M1/2 primers in 3 h and 72 h old *M. edulis* larvae analysed after different storage times. N, sample size. Heterogeneity between storage times was tested with a Fisher Exact test, ^{ns} = not significant, ** = significant at the 0.01 level and *** at the 0.001 level.

Family	Age of larvae (h)	Storage time (months)	N	F1/2 (%)	M1/2 (%)
1	72	1	35	89	31
		20	6	100^{ns}	0^{ns}
5	3	9	10	100	100
		20	10	100^{ns}	0***
	72	1	37	92	76
		20	5	100^{ns}	0**
6	72	7	10	100	30
		21	2	100^{ns}	0^{ns}

Table 6.3. PCR amplification success (percentage of larvae in which at least one mitotype was detected) in larvae sampled at different ages. h, hours.

Age of larvae (h)	*M. edulis*		*M. galloprovincialis*	
	N analysed	PCR amplification success (%)	N analysed	PCR amplification success (%)
3	109	96%	55	95%
18	10	100%	10	90%
21	10	100%	-	-
24	10	100%	-	-
48	10	80%	-	-
72	165	95%	85	96%

Once the data from larvae with storage times of 20 or 21 months have been removed, the remaining data come from six *M. edulis* families and three *M. galloprovincialis* families (Table 6.4). All families had different parents except for the *M. edulis* families 5 and 6 which shared the same female parent.

DNA extracted from the adductor muscle of all 17 parents was analysed for the presence of the F and M mitotypes. As expected, the F mitotype was detected in all parents. The M mitotype was detected in all nine male parents but not in any female parents by ethidium bromide staining. However, when PCR products were electrophoresed on polyacrylamide gels and detected by silver staining, the M mitotype was detected in one of the five *M. edulis* female parents (family 20) and in all three *M. galloprovincialis* female parents. The M mitotype band detected in female parents was consistently at a lower intensity than that detected in male parents. DNA was extracted from the eggs of these female parents and analysed for presence of the F and M mitotypes. In all cases, using polyacrylamide gel electrophoresis and silver staining, the F mitotype was detected in eggs but the M mitotype was not.

The F mitotype was detected in 67-100% of larvae from the nine families analysed. The M mitotype was detected in 90-100% of 3 h larvae with the exception of family 44 where the figure was 72%. In the 72 h samples the M mitotype was detected in 30-76% of larvae, varying between families. For family 20, data were available for 6 different ages from 3 to 72 h. From 3 to 24 h the M mitotype was detected in 90 - 100% of larvae but only in 63% of larvae at 48 h and 30% at 72 h. This suggests that elimination of the M mitotype occurred between 24 and 48 h after fertilisation in this family.

Table 6.4 Occurrence of the F and M mitotypes in larvae from 6 *M. edulis* families and 3 *M. galloprovincialis* families at 6 different ages. h = hours, N = sample size.

Family	Age (h)	N	F mitotype		M mitotype		F (%)	M (%)
			presence	absence	presence	absence		
M. edulis								
1	72	35	31	4	11	24	89	31
5	3	10	10	0	10	0	100	100
	72	37	34	3	28	9	92	76
6	72	10	10	0	3	7	100	30
20	3	10	7	3	9	1	70	90
	18	10	8	2	9	1	80	90
	21	10	5	5	9	1	50	90
	24	10	7	3	10	0	70	100
	48	8	8	0	5	3	100	63
	72	10	9	1	3	7	90	30
31	3	67	61	6	63	4	91	94
	72	34	32	2	20	14	94	59
32	3	18	16	2	17	1	89	94
	72	30	30	0	15	15	100	50
Totals	3	105	94	11	99	6	90	94
	72	156	146	10	80	76	94	51
M. galloprovincialis								
8	18	9	6	3	9	0	67	100
	72	19	17	2	10	9	89	53
43	3	20	19	1	20	0	95	100
	72	30	30	0	18	12	100	60
44	3	32	32	0	23	9	100	72
	72	33	32	1	21	12	97	64
Totals	3	52	51	1	43	9	98	83
	18	9	6	3	9	0	67	100
	72	82	79	3	49	33	96	60

For families where data were available for two or more ages of larvae, a Fisher Exact test was used to test for heterogeneity of frequency of larvae with the M mitotype between ages, both within families and for the combined data from all families within species for the 3 h and 72 h age samples (Table 6.5). For family 20 the data were grouped into two classes, 3 – 24 h and 48 – 72 h, and heterogeneity between classes was tested. In three of the four *M. edulis* families and two of the three *M. galloprovincialis* families the 72 h sample (48-72 h for family 20) had a significantly lower proportion of larvae with the M mitotype than the 3 h sample (3-24 h for family 20). The tests of heterogeneity of M mitotype frequency between the 3 h and 72 h samples for the combined data were also significant for both species. For comparison, the same tests were performed for the F mitotype data but showed no significant heterogeneity.

As families 5 and 6 share the same female parent they would be expected to have similar sex ratios (Saavedra *et al.*, 1997; Sutherland *et al.*, 1998). A Fisher Exact test showed significant heterogeneity of frequency of the M mitotype in 72 h larvae from families 5 and 6 ($P = 0.020$), suggesting that these families have different sex ratios.

Table 6.5 Results from Fisher Exact tests of heterogeneity of frequency of M mitotype between ages. * indicates significant heterogeneity at the 0.05 level and ** at the 0.01 level, following application of the sequential Bonferroni correction for multiple testing with k = 5 for *M. edulis* and k = 4 for *M. galloprovincialis*.

Family	Ages tested	*P* of homogeneity
M. edulis		
5	3 h, 72 h	0.091^{ns}
20	3–24 h, 48–72 h	<0.001**
31	3 h, 72 h	<0.001**
32	3 h, 72 h	0.001**
Combined data	3 h, 72 h	<0.001**
M. galloprovincialis		
8	18 h, 72 h	0.013*
43	3 h, 72 h	0.001**
44	3 h, 72 h	0.329^{ns}
Combined data	3 h, 72 h	0.004*

6.4 Discussion

Several mechanisms have been suggested to explain the observation that the M mitotype is detected in male *Mytilus* spp. but not in females. One possibility is that sperm mitochondria enter all eggs and then some factor ensures their elimination or suppresses their replicative advantage in females but not males. Another possibility is that sperm mitochondria only enter those eggs destined to become males and are somehow excluded from eggs destined to become females. Longo and Anderson (1969) observed that *M. edulis* sperm were wholly incorporated into the egg at fertilisation and the work of Sutherland *et al.* (1998) indicated that all *M. edulis* larvae do inherit the M mitotype. In the present study, the detection of the M mitotype in all or almost all 3 h old larvae from six *M. edulis* and three *M. galloprovincialis* families provides further evidence that the M mitotype enters all eggs at fertilisation in both these species.

Sutherland *et al.* (1998) observed elimination of the M mitotype in a proportion of *M. edulis* larvae between 18 and 24 h after fertilisation. In the present study, however, the M mitotype was detected in all 24 h old *M. edulis* larvae analysed (ten larvae from family 20) and the results indicate that elimination of the M mitotype occurred between 24 and 48 h after fertilisation. This difference in time of elimination of the M mitotype is relatively small and might be attributed to variation in rearing temperature between studies. Sutherland *et al.* (1998) used a rearing temperature of 20°C, at which larval development rate would be expected to be faster than that at the temperature of 14°C used in the present study (Bayne 1965). *M. galloprovincialis* larvae were analysed at 3, 18 and 72 h after fertilisation and elimination of the M mitotype in some larvae was detected between 18 and 72 h. Again the results support the conclusion that the pattern in *M. galloprovincialis* is not dissimilar to that in *M. edulis*. From the overall results of the present study, we can be sure that the elimination of the M mitotype begins at some point between 3 and 72 h after fertilisation in both *M. edulis* and *M. galloprovincialis*. This confirms that the mtDNA content of a mussel is determined at a very early stage in development, however the mechanism by which this occurs remains unknown.

The fact that the M mitotype is detectable in 3 h old larvae, and yet not in all 72 h larvae, lends support for the existence of some mechanism by which the M mitotype is eliminated or destroyed, rather than just the suppression of a replicative advantage. If the suppression of a replicative advantage of the M mitotype was solely responsible for the usual lack of detection of M in female mussels, we would expect the amount of M to increase throughout development in males but to remain constant in females. Thus if M is detected in all 3 h old larvae it should also be detected in all 72 h old larvae, it would only be the ratio of F to M that would change. If, in some larvae destined to become females, a few M mitotype molecules escaped elimination or destruction, the later replication of these molecules would explain the low levels of the M mitotype detected in some tissues of some adult female mussels (Garrido-Ramos *et al.* 1998).

Zouros *et al.* (1994) and Saavedra *et al.* (1997) found that sex ratio varied extensively among *Mytilus* families in laboratory crosses and their results suggested that sex ratio was predominantly under maternal control. Saavedra *et al.* (1997) carried out all possible crosses between five females and five males and determined the sex ratio of each family and also analysed a proportion of the offspring from each family for the presence of the M mitotype. For four of the five female parents, the sex ratio was consistent between families, irrespective of the male parent. For the fifth female parent, sex ratio did vary significantly among families with different male parents. Sutherland *et al.* (1998) also detected variation in the proportion of males from 53 – 76% among four families that shared the same mother. This suggests that sex ratio is predominantly under maternal control but that other factors are involved. In the present study, two of the families examined (5 and 6) shared the same mother and were therefore expected to have similar sex ratios. However, the proportion of 72 h old larvae with the M mitotype was significantly greater in family 5 than in family 6. Although the general pattern is that the frequency of offspring with the M mitotype would match the frequency of males in a family, exceptions to this rule do occur (see Saavedra *et al.* 1997; Sutherland *et al.* 1998). For

example, two of the families described by Saavedra *et al.* (1997) shared the same mother (F66) but had different fathers (M54 and M70). These families had similar sex ratios (approximately 1:1) but differed significantly in the proportion of offspring with the M mitotype due to the high frequency of M-negative males in one family (15 out of 16 males were M-negative). In the other family only one out of fifteen males was M-negative. All females in both families were M-negative as expected. These apparent differences from the expected pattern may be simply a result of sampling effects caused by the relatively low numbers of offspring analysed from the families concerned. The inability to determine the sex of a mussel prior to reproductive maturity, which usually takes a year or more (Sprung 1983; Seed and Suchanek 1992 and references therein; Zouros *et al.* 1992, 1994) to achieve and is therefore costly in terms of time and resources, makes it difficult to obtain both mtDNA content and gender data from large numbers of offspring. However, the data which are available suggests that in the vast majority of families the proportion of offspring which retain the M mitotype closely reflects the proportion of males.

Saavedra *et al.* (1997) proposed a model linking mtDNA inheritance and sex determination in *Mytilus* in which a paternally coded mitochondrial factor provides a replicative advantage and a maternally coded egg factor suppresses this advantage to varying degrees. The mechanism which ensures elimination of sperm mitochondria in other animals may simply be modified in mussels by a maternally encoded suppressor locus with two alleles: an active allele which suppresses the elimination of sperm mitochondria and an inactive allele which allows this elimination to proceed (Zouros 2000). Possibly a sperm-derived mitochondrial factor is necessary in early germ cells to induce production of a male gonad but continuous presence of this factor is not necessary to maintain maleness. The probability of the sperm mtDNA entering the germ line would be dependent on the mother's genotype and would be low in eggs from female biased mothers and high in those from male biased mothers. Saavedra *et al.* (1997) suggested that in female biased families the sperm mtDNA would only enter the germ line in a few eggs and would be vulnerable to elimination from these, giving rise to M-negative sons.

The results of the present study provide further evidence for the assumption of Saavedra *et al.* (1997)'s model that sperm mitochondria enter all eggs at fertilisation. Furthermore, the mtDNA content of a mussel and, most likely, its sex are determined very early on in development, between 3 and 72 h after fertilisation in *M. edulis* and *M. galloprovincialis*. However, the mechanism by which this occurs remains to be resolved.

Chapter 7. Disruption of Doubly Uniparental mtDNA Inheritance in *Mytilus edulis* x *M. galloprovincialis* Hybrids

7.1 Introduction

Mussels of the *Mytilus edulis* complex (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) are found at temperate latitudes throughout the Northern and Southern Hemispheres (McDonald *et al.* 1991). Hybridisation occurs to varying degrees where the species distributions overlap (Gosling 1992; McDonald *et al.* 1991) and yet the taxa seem to remain morphologically and genetically distinct in areas of allopatry. Where backcrossing of hybrids with parental taxa takes place, it can lead to introgression of nuclear and/or mitochondrial DNA from one taxon to the other (Harrison 1991; Arnold 1992), thus for the taxa to maintain their genetic integrity the level of introgression must be limited in some way.

The largest *Mytilus* hybrid zone is between *M. edulis* and *M. galloprovincialis*, extending over more than 1000 km from the north coast of Spain to the British Isles. More restricted hybrid zones occur between *M. edulis* and *M. trossulus* at the entrance to the Baltic Sea (approx. 100 km) and in eastern Canada, and between *M. galloprovincialis* and *M. trossulus* on the Pacific coast of the USA. It is generally thought that, in terrestrial species, selection against hybrids is more important than environmental factors in maintaining hybrid zones (Barton and Hewitt 1989). However, for *Mytilus*, there is a variety of evidence indicating that the fitness of hybrid mussels is not inferior to that of parental taxa (Gardner 1994). Little is known about how these hybrid zones are maintained in this group of taxa which have a prolonged planktonic larval stage facilitating dispersal over great distances. No evidence for gamete incompatibility between taxa has been found, which suggests that post-zygotic factors limit the degree of introgression where species distributions overlap.

The doubly uniparental mtDNA inheritance (DUI) in *Mytilus* and the tissue-specific distribution of the M and F mitotypes in males may impose greater

requirements for compatibility between nuclear and mitochondrial genomes than in species with standard maternal inheritance (Saavedra *et al.* 1996). For introgression to occur, compatibility of one species' nuclear DNA with the other species' mtDNA would be required in addition to compatibility of one species' F mitotype with the other species' M mitotype in hybrid males (Saavedra *et al.* 1996). If the system of DUI is disrupted by hybridisation, then the level of introgression among *Mytilus* taxa may be affected, which could be an important factor in the maintenance of genetic integrity for each taxon (Rawson, Secor and Hilbish 1996).

Several studies of DUI in *Mytilus* have found male mussels homoplasmic for the F mitotype and female mussels heteroplasmic for both F and M mitotypes, providing evidence for disruption of DUI. A low level of disruption has been observed in intraspecific *M. galloprovincialis* crosses (Saavedra *et al.* 1997). There is little evidence for disruption of DUI in *M. edulis* / *M. galloprovincialis* hybrids but disruption appears to be widespread in *M. galloprovincialis* / *M. trossulus* hybrids (Rawson, Secor and Hilbish 1996). Disruption of DUI has also been detected in laboratory produced hybrids between *M. edulis* and *M. trossulus* (Zouros *et al.* 1994), but not in a natural *M. edulis* / *M. trossulus* hybrid population (Saavedra *et al.* 1996). This previous work has concentrated largely on adult mussels from natural populations where the precise genetic and geographic background of a hybrid individual is uncertain. Laboratory-based hybridisations offer an opportunity to study DUI in pure species and hybrid larvae and eliminate some of these uncertainties.

We expect all normal *Mytilus* zygotes to receive both F and M mitotypes, so 3 hour old larvae should contain both F and M. In larvae destined to become females, the M mitotype is somehow eliminated and this normally occurs between 18 and 48 hours after fertilisation (Sutherland *et al.* 1998; Chapter 6 this study). Therefore we expect all 72 hour old larvae to contain the F mitotype and a proportion (those which are destined to become males) to also contain the M mitotype. Where there is disruption of DUI, homoplasmic males may arise because of a failure in either the inheritance or retention of the M mitotype.

Heteroplasmic females must presumably arise from a breakdown in the mechanism which would normally cause elimination of the M mitotype. In the majority of cases where it has been tested in the laboratory, half-sib families from the same mother have similar sex ratios. This has been demonstrated for pure species *M. galloprovincialis* families (Saavedra *et al.* 1997) and for comparisons between pure species *M. edulis* and hybrid *M. edulis* x *M. trossulus* families (Zouros *et al.* 1994). Furthermore, it is a feature of the DUI system that in normal families the proportion of larvae with the M mitotype should reflect the sex ratio (Zouros *et al.* 1994; Saavedra *et al.* 1997). If a pure species and a hybrid family of larvae share the same mother, disruption of DUI in the hybrid family might result in a difference in the proportion of larvae with the M mitotype from that observed in the pure species family. The objective of this study is to assess the resilience of DUI to hybridisation between *M. edulis* and *M. galloprovincialis* by comparing the inheritance and elimination of the M mitotype in pure species and hybrid larvae.

7.2 Materials and Methods

Intra- and inter-specific crosses were performed between *M. edulis* and *M. galloprovincialis* and samples of parents and larvae were taken as described in Chapter 2. For this study, parents and samples of larvae taken at 3 h and 72 h after fertilisation from trial VIII (families 31, 32, 34, 37, 38, 41, 43 and 44) were analysed for the presence of F and M mitotypes. These 8 families form 2 groups, each comprising the 4 possible crosses between one male and one female from each of the 2 species. This enables comparison of pure species and hybrid families which share either a female or male parent.

DNA analysis was carried out between 1 and 5 months after sampling of larvae. The mitotype specific primers F1/2 (for all families), M1/2 (for families with a *M. edulis* male parent) and PR17/18 (for families with a *M. galloprovincialis* male parent) were used for PCR amplification from 1 µl (approx. 50 ng) extracted DNA from each parent or 3 µl (9 µl for PR17/18 primers) lysate from each larva. PCR conditions and temperature cycles were as described in Chapter 5. F1/2 PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. All M1/2 and PR17/18 PCR products and any F1/2 PCR products which gave negative results on agarose were electrophoresed on 5% non-denaturing polyacrylamide gels and bands were visualized by silver staining (see Appendix B).

The species identity of parents and larvae was confirmed by amplifying 1 µl (approx. 50 ng) DNA or 3 µl lysate using the Me15/16 primers of Inoue *et al.* (1995), with PCR conditions and temperature cycle as described in Chapter 3. Me15/16 PCR products were electrophoresed on 8% non-denaturing polyacrylamide gels and bands were visualized by silver staining. Identity of the female parent of each family was confirmed using a Restriction Fragment Length Polymorphism (RFLP) which distinguished F1/2 PCR products from the different parents. For each parent 5 µl of F1/2 PCR product was incubated at 37°C for 2.5 h with 3 units of restriction enzyme for each of *Dde*I, *Hae*III, *Hha*I, *HincII*, *Hinf*I and *Rsa*I. The resulting fragments were electrophoresed on 2%

agarose gels and stained with ethidium bromide to determine which restriction enzymes were useful in distinguishing mitotypes from different parents. Digestion with *Dde*I or *Hinf*I produced 3 digest patterns: one in Ef1, a second in Ef2 and a third shared by Gf1 and Gf2. F1/2 PCR products from a sub-sample of 72 h old larvae from each family were digested with *Dde*I (10 μl PCR product and 3 units *Dde*I) at 37°C for 2.5 h. Digestion products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Matching of the RFLP pattern in the larvae with that of the appropriate female parent, together with the Me 15/16 genotype, confirmed the family identity of each sample of larvae.

7.3 Results

Parents and larvae from 8 families were analysed for the presence of the F and M mitotypes. As expected, the F mitotype was detected in all 8 parents. Using ethidium bromide staining the M mitotype was detected in all 4 male parents but not in any female parents. However, when PCR products were electrophoresed on polyacrylamide gels and detected by silver staining, the M mitotype was detected at a weak band intensity in the 2 *M. galloprovincialis* female parents. DNA was extracted from the eggs of these 2 female parents and analysed for the presence of the F and M mitotypes. In both cases, using polyacrylamide gel electrophoresis and silver staining, the F mitotype was detected but the M mitotype was not.

Table 7.1 shows the numbers of larvae in which the F and M mitotypes were detected for the 8 families. For both the 3 h and 72 h samples, the F mitotype was detected in almost all larvae (89-100%), as expected. At 3 h, the M mitotype is detected in most larvae, although the percentage is lower for family 44 (72% for family 44, 88 – 100% for the other families). As family 44 is a pure species *M. galloprovincialis* family, this difference is not a result of hybridisation affecting the inheritance of the M mitotype. At 72 h, the M mitotype is detected in 50 – 64% of larvae from the pure species families. However, in the hybrid families the proportion of larvae with the M mitotype appears to differ, ranging from 9 – 87%.

Table 7.1 Occurrence of the F and M mitotypes in pure species and hybrid larvae from eight *Mytilus* families at 3 h and 72 h post-fertilization. # = family number, N = sample size, E = *M. edulis*, G = *M. galloprovincialis*, f = female, m = male.

#	Parents	Family type (pure species or hybrid)	N	% of larvae with F mitotype	% of larvae with M mitotype
3 h old larvae					
31	Ef1xEm2	Pure	33	94	88
34	Ef1xGm1	Hybrid	35	100	97
44	Gf2xGm1	Pure	32	100	72
41	Gf2xEm2	Hybrid	33	97	91
32	Ef2xEm1	Pure	18	89	94
37	Ef2xGm2	Hybrid	19	100	89
43	Gf1xGm2	Pure	20	95	100
38	Gf1xEm1	Hybrid	18	100	94
72 h old larvae					
31	Ef1xEm2	Pure	34	94	59
34	Ef1xGm1	Hybrid	34	100	82
44	Gf2xGm1	Pure	33	97	64
41	Gf2xEm2	Hybrid	32	100	9
32	Ef2xEm1	Pure	30	100	50
37	Ef2xGm2	Hybrid	30	97	87
43	Gf1xGm2	Pure	30	100	60
38	Gf1xEm1	Hybrid	29	100	14

To test for any association between family type and proportion of larvae with the M mitotype, a Fisher Exact test was used to compare results for the 2 pure species families in each group and for each pair of pure species and hybrid families in each group for the 3 h and 72 h samples (Table 7.2). Families which share the same female parent would be expected to have the same sex ratio. If sex ratio is assumed to be equal to the proportion of pure species larvae with the M mitotype then these comparisons should reveal any disruption of either inheritance or elimination of the M mitotype in hybrid larvae.

Table 7.2 *P*-values from Fisher Exact test for independence of family and proportion of larvae with the M mitotype. E = *M. edulis*, G = *M. galloprovincialis*, f = female, m = male, ^{ns} indicates nonsignificant results, * indicates significance at the 0.05 level and ** at the 0.01 level after correction for multiple testing using the sequential Bonferroni method with k = 8 for pure species vs. hybrid family comparisons (Holm 1979; Rice 1989).

Families compared (family numbers in parentheses)	3 h samples	72 h samples
Ef1xEm2 (31) vs. Gf2xGm1 (44)	0.130^{ns}	0.803^{ns}
Ef1xEm2 (31) vs. Ef1xGm1 (34)	0.191^{ns}	0.061^{ns}
Ef1xEm2 (31) vs. Gf2xEm2 (41)	1.000^{ns}	<0.001**
Gf2xGm1 (44) vs. Ef1xGm1 (34)	0.005*	0.104^{ns}
Gf2xGm1 (44) vs. Gf2xEm2 (41)	0.061^{ns}	<0.001**
Ef2xEm1 (32) vs. Gf1xGm2 (43)	0.474^{ns}	0.435^{ns}
Ef2xEm1 (32) vs. Ef2xGm2 (37)	1.000^{ns}	0.005*
Ef2xEm1 (32) vs. Gf1xEm1 (38)	1.000^{ns}	0.005*
Gf1xGm2 (43) vs. Ef2xGm2 (37)	0.231^{ns}	0.039^{ns}
Gf1xGm2 (43) vs. Gf1xEm1 (38)	0.474^{ns}	<0.001**

There was no significant difference in the proportion of larvae with the M mitotype between pure *M. edulis* and pure *M. galloprovincialis* families at either 3 h or 72 h. For the 3 h samples there were no significant differences in the proportion of larvae with the M mitotype between pure species and hybrid families, with the exception of the Gf2xGm1 vs. Ef1xGm1 comparison (family 44 vs. family 34). The proportion of larvae with the M mitotype in family 44 was significantly less than that in family 34 at 3 h. It is the pure *M. galloprovincialis* family (44), rather than the hybrid family (34), which differs from the biological expectation, so this significant result does not indicate any effect of hybridisation on the inheritance of the M mitotype. The lower proportion of 3 h larvae with the M mitotype in family 44 might suggest that elimination of the M mitotype had commenced before 3 h, but is perhaps more likely the result of a higher frequency of PCR failure in this family.

At 72 h, however, 5 of the 8 pure species vs. hybrid family comparisons showed significant differences. These pure species vs. hybrid family comparisons can be grouped according to whether the two families being compared share the same female or male parent. The two comparisons involving *M. galloprovincialis* females crossed with males of both species (families 44 vs. 41 and 43 vs. 38) both show significant differences. The proportion of larvae with the M mitotype in the hybrid families is significantly lower than that in the pure species families. This indicates that when the female parent is *M. galloprovincialis*, the *M. edulis* M mitotype is eliminated more frequently than the *M. galloprovincialis* M mitotype. Two further comparisons involve *M. edulis* females crossed with males of both species (31 vs. 34 and 32 vs. 37). The data suggest that the proportion of larvae with the M mitotype in the hybrid families is greater than that in the pure species families. The comparison between families 32 and 37 shows a significant difference, and that between families 31 and 34 is approaching significance. These two comparisons were tested for overall significance using the "combining probabilities from tests of significance" technique (Sokal and Rohlf 1995). This technique combines the probabilities from a series of separate significance tests of the same hypothesis on different data sets. The *P*-values are transformed to give a quantity "$-2\Sigma \ln P$"

which has a chi-square distribution with 2k degrees of freedom, where k is the number of separate *P*-values used. For the 2 comparisons in this case, $-2\Sigma \ln P = 16.190$ with 4 degrees of freedom, which is significant at the 0.005 level. This shows that when the female is *M. edulis*, the *M. galloprovincialis* M mitotype is eliminated significantly less frequently than the *M. edulis* M mitotype. Where a *M. edulis* male is crossed with females of both species, both comparisons (31 vs. 41 and 32 vs. 38) show that the proportion of larvae with the M mitotype is significantly lower in the hybrid family than in the pure species family. This suggests that the *M. edulis* M mitotype is eliminated more frequently by *M. galloprovincialis* females than by *M. edulis* females. Where a *M. galloprovincialis* male is crossed with females of both species, the data from both comparisons (44 vs. 34 and 43 vs. 37) suggest that the proportion of larvae with the M mitotype in the hybrid family is greater than in the pure species family, but the comparisons are not individually significant. If the two comparisons are tested for overall significance as described above, $-2\Sigma \ln P = 11.015$ with 4 degrees of freedom, which is significant at the 0.05 level. This suggests that the *M. galloprovincialis* M mitotype is eliminated less frequently by *M. edulis* females than by *M. galloprovincialis* females. Overall the results show that, in hybrid families, the *M. edulis* M mitotype is eliminated more frequently, and the *M. galloprovincialis* M mitotype is eliminated less frequently, than in pure species families.

7.4 Discussion

The results of the present study confirm that hybridisation between *M. edulis* and *M. galloprovincialis* successfully produces larvae which survive until at least 72 h after fertilisation, some of which have F and M mitotypes from different species. This is in line with the widespread hybridisation observed between these species in the wild (Skibinski *et al.* 1983; McDonald *et al.* 1991).

As the M mitotype was detected in nearly all 3 h old larvae examined from the four hybrid families in this study, there is no evidence that hybridisation disrupts the inheritance of the M mitotype. However, the results do suggest that hybridisation between *M. edulis* and *M. galloprovincialis* may disrupt the normal regulation of elimination of the M mitotype. The differences in the proportion of 72 h old larvae with the M mitotype between pure species and hybrid families might be due to variation in sex ratio among families but, given that families from the same mother usually have similar sex ratios (Zouros *et al.* 1994; Saavedra *et al.* 1997), it is more likely that they reflect the presence of M-negative males or M-positive females in the hybrid families. Two features of the results of the present study are striking. First, the apparent frequency of breakdowns observed in the present study appears to be far greater than the low levels detected in *M. edulis* x *M. galloprovincialis* hybrid populations in the wild (Rawson, Secor and Hilbish 1996). Second, the nature of the breakdowns observed in the present study varies with the type of cross. In comparison to the pure species families, the proportion of 72 h old larvae with the M mitotype is higher in the *M. edulis* female x *M. galloprovincialis* male families, which suggests the presence of M-positive females in these families. In contrast, the proportion of 72 h old larvae with the M mitotype is much lower in the *M. galloprovincialis* female x *M. edulis* male families, indicating that these families contain M-negative males.

To assess the extent of the breakdowns of DUI in the hybrid families it is necessary to make two assumptions, firstly that the sex ratio is identical in each pair of pure species and hybrid families which have the same mother and

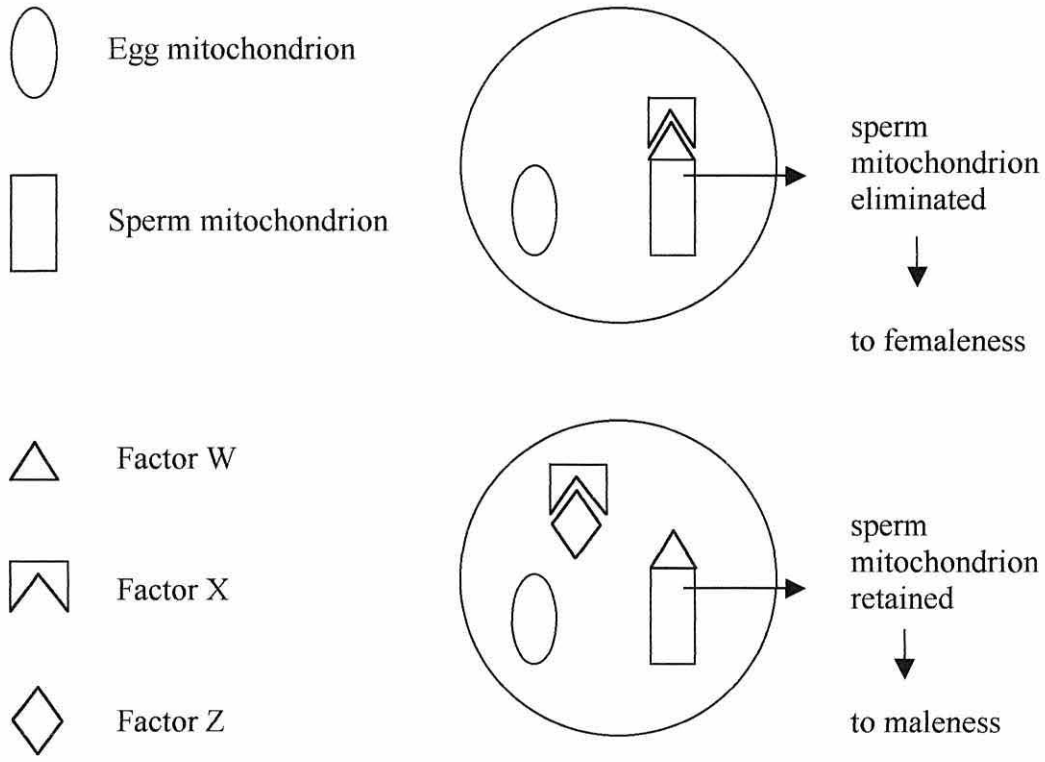
secondly that the proportion of pure species larvae with the M mitotype is equal to the proportion of males. The expected number of each sex can then be calculated for the hybrid families and hence the number of putative M-positive females and M-negative males can be deduced. From the four hybrid families in the present study it appears that 36% of hybrid larvae expected to be females were M-positive and that 42% of those expected to be males were M-negative (calculated as an average for all larvae in the four hybrid families). It may be that both M-negative males and M-positive females are present in one or more of these families but this cannot be confirmed because the sex of a larva cannot be determined. The presence of both forms of DUI breakdown in equal numbers in a family would cancel out any effect on the proportion of larvae with the M mitotype, therefore the assumption that any differences in this proportion are produced by the presence of only one form of breakdown will give minimal estimates of the frequency of DUI breakdown.

High levels of DUI disruption have also been found in natural hybrids between *M. galloprovincialis* and *M. trossulus* from San Francisco Bay, California, where Rawson, Secor and Hilbish (1996) found that 35% of males were M-negative and 43% of females were M-positive. In the same study Rawson, Secor and Hilbish (1996) found much lower levels of disruption in *M. edulis* x *M. galloprovincialis* hybrids from Whitsand Bay, UK. They examined 25 female and 36 male *M. edulis* x *M. galloprovincialis* adult hybrids, of which 54% had putative F_1 genotypes, and found only one M-positive female (4% of females) and four M-negative males (11% of males). Even if it is assumed that all of the observed breakdowns occurred in putative F_1 mussels, the percentage of adult F_1 individuals showing breakdown of DUI is only 15%, compared to 40% estimated from the F_1 hybrid 72 h old larvae in the present study. This difference may indicate that disruption of DUI is associated with increased mortality of later larval stages or juveniles, or alternatively disruption to DUI might be increased by some factor(s) associated with laboratory crosses. Studies of adults from pure species *M. edulis* and *M. galloprovincialis* laboratory crosses have detected only very low levels of disruptions to DUI (Saavedra *et al.* 1997) or no disruption at all (Zouros *et al.* 1994). Different results have been

obtained for laboratory and wild *M. edulis* x *M. trossulus* hybrids, where Zouros *et al.* (1994) detected disruption to DUI in 40% of males and in 2% of females from laboratory crosses (mussels examined were three years old), yet Saavedra *et al.* (1996) found no evidence for disruption to DUI in hybrids from a wild population. However, Saavedra *et al.* (1996) found very few F_1 and F_2 individuals and the majority of hybrids identified were backcross individuals and so their results cannot be directly compared to those of Zouros *et al.* (1994). Hence, there is no previous evidence to suggest that disruption of DUI is more common in laboratory crosses than in nature. In most of the backcross individuals analysed by Saavedra *et al.* (1996) the mtDNA came from the species that contributed the majority of the individual's nuclear genes and the F and M mitotypes in F_2 and backcross males were of the same species origin. This suggests that crosses which would produce heterospecific combinations of mtDNA and nDNA or of F and M mitotypes either do not occur or that their progeny do not survive to adulthood (Saavedra *et al.* 1996). However, DUI has not previously been studied in hybrid larvae and there is insufficient evidence to conclude whether the high levels of DUI disruption observed in the present study are a feature of laboratory crosses or whether disruption of DUI is associated with increased mortality.

Saavedra *et al.* (1997) and Zouros (2000) have outlined a mechanism for the regulation of DUI which incorporates three factors, W, X and Z (see Figure 7.1). Factor W labels the sperm mitochondrion and is assumed to be produced in spermatogenesis. Factor X interacts with factor W to result in the elimination of sperm mitochondria from the egg, as in animals with Standard Maternal Inheritance (SMI) of mitochondria, and is thought to be produced during oogenesis. Factor Z is a feature of animals with DUI and is controlled by a nuclear gene that is expressed during oogenesis. Factor Z is produced in varying quantities by different females and interacts with the other factors in some way which prevents the elimination of sperm mitochondria. Factor Z might inactivate factor X or alternatively it might interact with factor W to make the sperm mitochondria insusceptible to elimination. This is a simple modification of the system which exists in SMI animals.

Figure 7.1 Schematic illustration of the mechanism of DUI proposed by Saavedra *et al.* (1997) and Zouros (2000). See text for explanation. (Modified from Zouros 2000).



The results of the present study give some insight into which interactions might break down in hybridisation. The M-positive females in the *M. edulis* female x *M. galloprovincialis* male families might result from less efficient elimination of sperm mitochondria due to a failure of the *M. edulis* factor X to recognise the *M. galloprovincialis* factor W, as has been suggested to explain paternal leakage of mtDNA in hybrid mouse crosses (Kaneda *et al.* 1995). However, in the *M. galloprovincialis* female x *M. edulis* male families, recognition of the *M. edulis* factor W by the *M. galloprovincialis* factor X must have occurred because the M mitotype was eliminated in 89% of the larvae examined. The presence of M-negative males in these families might result from a failure of the interaction between factor Z and factor W, which would normally prevent the elimination of sperm mitochondria. This provides further support for the hypothesis that two separate mechanisms are responsible for regulating DUI (Zouros *et al.* 1994; Saavedra *et al.* 1997) and implies that the occurrence of incompatibilities between species is assymetrical.

The disruption of DUI observed in the *M. galloprovincialis* female x *M. edulis* male families, where a high proportion of M-negative males were detected, has the potential to limit introgression of the *M. edulis* M mitotype into *M. galloprovincialis*. Widespread introgression of *M. edulis* F mitotypes into Mediterranean *M. galloprovincialis* populations has been detected by Rawson and Hilbish (1998) but introgression of *M. edulis* M mitotypes appeared to be less prevalent. The findings of the present study offer a possible explanation for this in the form of disruption to the retention of the *M. edulis* M mitotype in hybrids. In contrast, Rawson and Hilbish (1998) failed to find evidence of mtDNA introgression from Mediterranean *M. galloprovincialis* to *M. edulis*. The present study provides no evidence for disruption of DUI limiting the introgression of the *M. galloprovincialis* M mitotype, hence other factors must be involved. Rawson and Hilbish (1998) suggested that directional selection in favour of *M. galloprovincialis* nuclear alleles, which has been shown to occur in hybrid populations (Skibinski and Roderick 1991), might result in assymetric mtDNA introgression from *M. edulis* to *M. galloprovincialis* if selection tended to remove those mussels with predominantly *M. edulis* nuclear genotypes.

Another possible explanation is that the main pattern of water movement is eastward from the Atlantic into the Mediterranean (Perkins *et al.* 1990) which could give rise to predominantly one way dispersal of gametes and larvae. Further studies would be necessary to determine whether either of these explanations play a significant role in restricting gene flow between *M. edulis* and *M. galloprovincialis*.

The present study demonstrates that hybridisation between *M. edulis* and *M. galloprovincialis* does not affect the inheritance of the M mitotype at fertilisation but that it can disrupt the mechanism that is responsible for the transmission of the M mitotype to males as well as that which inhibits the retention of the M mitotype in females. Laboratory crosses involving *M. trossulus* might provide further insights into the regulation of DUI. Research into the effect of DUI disruptions on survival is necessary to determine the extent to which disruption of DUI in hybrids is important in the maintenance of genetic integrity for species in the *M. edulis* complex.

Chapter 8. General Discussion

Previous studies have detected disruption of DUI to varying degrees in mussels from natural hybrid populations (Rawson, Secor and Hilbish 1996; Saavedra *et al.* 1996). There has been little evidence for disruption of DUI in *M. edulis* x *M. galloprovincialis* hybrids (Rawson, Secor and Hilbish 1996), limited breakdown of DUI seems to occur in some *M. edulis* x *M. trossulus* hybrid populations (Zouros *et al.* 1994) but has not been detected in others (Saavedra *et al.* 1996), and in *M. galloprovincialis* x *M. trossulus* hybrids disruption of DUI appears to be more common (Rawson, Secor and Hilbish 1996). The pattern of disruptions observed implies that *M. edulis* is more closely related to *M. galloprovincialis* than either is to *M. trossulus*, and that *M. trossulus* is closer to *M. edulis* than to *M. galloprovincialis*, at the loci regulating the inheritance of the M mitotype (Rawson, Secor and Hilbish 1996). These relationships are consistent with those deduced from allozymes and phylogeny of the M and F mitotypes for the three species (Rawson, Secor and Hilbish 1996), and also with the observation that hybridisation is more widespread between *M. edulis* and *M. galloprovincialis* than *M. edulis* and *M. trossulus* and is relatively rare between *M. galloprovincialis* and *M. trossulus* (e.g. Sanjuan *et al.* 1994; Rawson, Secor and Hilbish 1996; Saavedra *et al.* 1996; Comesana and Sanjuan 1997; Suchanek *et al.* 1997; Comesana *et al.* 1999).

The DUI system in *Mytilus* and the tissue-specific distribution of the M and F mitotypes in males may impose greater requirements for compatibility between nuclear and mitochondrial genomes than in species with standard maternal inheritance (Saavedra *et al.* 1996). For introgression to occur, compatibility of one species' nuclear DNA with the other species' mtDNA would be required in addition to compatibility of one species' F mitotype with the other species' M mitotype in hybrid males (Saavedra *et al.* 1996). If DUI is disrupted by hybridisation, then the level of introgression among *Mytilus* taxa may be affected, which could be an important factor in the maintenance of genetic integrity for each taxon (Rawson, Secor and Hilbish 1996). Previous studies have been hampered by uncertainties about the precise genetic and geographic

background of the mussels analysed. In some studies of natural populations only very low numbers of hybrids have been detected, limiting the information which could be obtained about the extent to which DUI is disrupted in hybrids. In addition, no previous study has analysed hybrid larvae for disruption to DUI and hence it has not been possible to determine whether it is the inheritance or elimination of the M mitotype which is disrupted in hybrid mussels.

The availability of an increasing amount of nDNA sequence data for *Mytilus* has led to advances in the field of species identification in the *M. edulis* complex. Several PCR-based nDNA markers have been developed which are claimed to distinguish all three species by readily detectable PCR product length variation (Inoue *et al.* 1995; Rawson *et al.* 1996). The usefulness of these markers for identifying populations of the three species was evaluated in the present study. The Me 15/16 marker (Inoue *et al.* 1995) was found to amplify and identify species more reliably than the Glu-5' marker (Rawson *et al.* 1996) and both nDNA markers were diagnostic or almost diagnostic for the UK *M. edulis* and Mediterranean *M. galloprovincialis* populations. However, both nDNA markers showed considerable disagreement with the results of allozyme studies for the Nova Scotia *M. edulis* and *M. trossulus* populations and to an even greater extent for the Gulf of Gdansk *M. trossulus* population. The Gulf of Gdansk population is thought to be outside of the *M. edulis* x *M. trossulus* hybrid zone in the Øresund region at the entrance to the Baltic Sea. Mussels from this population have previously been identified as pure *M. trossulus* by allozymes (Wenne and Skibinski 1995) but the present study showed polymorphism for nDNA alleles typical of *M. edulis* in addition to those expected in *M. trossulus*. Further disagreement between allozymes and the nDNA markers was observed in the Nova Scotia *M. edulis* and *M. trossulus* populations which were both polymorphic for *M. edulis* and *M. trossulus* –associated nDNA alleles. It was unclear whether the disagreement was due to the markers not being completely diagnostic or the populations not being pure species. If the latter applies, it appears that the Me 15/16 and Glu-5' markers detect introgression of nuclear alleles between species which has not been revealed by allozyme loci. Similar observations were made by direct comparison of allozymes and the Glu-5' and

ITS nDNA loci in an *M. edulis* x *M. trossulus* hybrid zone in Atlantic Canada by Comesana *et al.* (1999) and indirectly in another study which detected Glu-5' genotypes typical of *M. edulis* in Gulf of Gdansk (Baltic Sea) *M. trossulus* (Borsa *et al.* 1999).

Differential introgression points towards differential selection across loci, in this case implying that the hybrid zones might be more permeable to nDNA loci than to allozymes due to greater selection on allozyme loci. The commonly assayed allozyme loci in mussels have previously been considered to be neutral with the exception of the LAP locus (Koehn *et al.* 1976; Koehn 1978; Koehn *et al.* 1980; Koehn 1983; Beaumont *et al.* 1988; Väinölä and Hvilsom 1991; Gardner *et al.* 1993) but the neutrality of allozymes in general has been a topic of much debate. For example, nDNA and mtDNA revealed a pronounced population subdivision in the oyster *Crassostrea virginica* on the southeastern coast of the USA, whereas allozyme frequencies showed no such division, and the discordance was attributed to the action of balancing selection on the allozyme loci (Karl and Avise 1992). However it is equally feasible that the adhesive protein gene, in which the Me 15/16 and Glu-5' markers reside, might also be subject to selective effects. If introgression of *M. edulis* genes into Baltic Sea *M. trossulus* has occurred recently, this hybrid zone offers an opportunity to study the effects of selection on different types of markers. Analysis of *M. edulis* populations geographically closer to the hybrid zone than those used in the present study would be necessary to determine whether symmetrical introgression from *M. trossulus* to *M. edulis* is occurring.

Completely diagnostic markers for species identification in the *M. edulis* complex may not exist. The results of the present study highlight the need for the use of a range of markers when classifying mussels as pure species or hybrids, as advocated by Boecklen and Howard (1997), however this is difficult with larvae where the small amount of template DNA available restricts the number of markers which can be applied. Recent advances have included the development of PCR-based techniques for mtDNA and nDNA analysis of individual mussel larvae (Corte-Real *et al.* 1994; Sutherland *et al.* 1998). These

techniques have been further developed in the present study, enabling the detection of F and M mitotype specific mtDNA markers and the Me 15/16 nDNA marker in individual 3 h to 4 week old larvae of *M. edulis*, *M. galloprovincialis*, *M. trossulus* and their hybrids. The inheritance of the Me 15/16 marker was investigated in pure species and hybrid crosses and the results corresponded with Mendelian inheritance of alleles. This marker should be of future use in the identification of larvae for studies of hybridisation and dispersal, although it is not yet known whether or not the Me 15/16 primers amplify from any other species.

Sutherland *et al.* (1998) demonstrated that the M mitotype enters all *M. edulis* eggs at fertilisation and that it is eliminated from those larvae expected to become females between 18 and 24 h after fertilisation. The present study has confirmed this for further *M. edulis* families, although elimination of the M mitotype was detected between 24 and 48 h after fertilisation, slightly later than observed by Sutherland *et al.* (1998). This difference was most likely attributable to variation in rearing temperatures (14°C in the present study and 20°C in Sutherland *et al.* (1998)'s study) resulting in slower development of larvae in the present study than in that of Sutherland *et al.* (1998). The present study has demonstrated that the pattern observed in *M. edulis* is also found in *M. galloprovincialis*, in which elimination of the M mitotype was detected between 18 and 72 h after fertilisation. The findings of the present study for *M. edulis*, *M. galloprovincialis* and their hybrids provide further evidence that that the sperm enters all eggs at fertilisation, thereby validating an assumption of Saavedra *et al.* (1997)'s model of the mechanisms of mtDNA inheritance and sex determination in *Mytilus*.

The present study has confirmed that DUI is disrupted by hybridisation between *M. edulis* and *M. galloprovincialis*. The results demonstrated that *M. edulis* x *M. galloprovincialis* hybrid larvae inherit the F and M mitotypes as normal but the mechanisms which usually ensure that the M mitotype is retained in males and eliminated in females were disrupted in an estimated 40% of the hybrid larvae analysed. The disruptions of DUI observed in the two types of hybrid crosses

were of a contrasting nature. In offspring from crosses between *M. galloprovincialis* females and *M. edulis* males the results indicated that approximately 81% of larvae expected to be males did not contain the M mitotype. In nature, this type of disruption would have the effect of limiting introgression of the *M. edulis* M mitotype into *M. galloprovincialis* populations. However there was no such evidence for disruption of DUI limiting introgression of the *M. galloprovincialis* M mitotype into *M. edulis*. In crosses between *M. edulis* females and *M. galloprovincialis* males the results did not reveal any presence of M-negative male offspring and the M mitotype was detected in approximately 66% of larvae expected to be females.

It is clear that through limiting introgression of the *M. edulis* M mitotype, disruption of DUI may be an important factor in the maintenance of genetic integrity of *M. edulis* and *M. galloprovincialis* but other factors must also be involved. These factors might be genetic or environmental and may not be under the influence of mtDNA. Rawson and Hilbish (1998) suggested that directional selection favouring nuclear alleles of *M. galloprovincialis* over those of *M. edulis* might remove those mussels with predominantly *M. edulis* nuclear genotypes. Such selection has been shown to occur in hybrid populations (Gardner and Skibinski 1988, 1991; Skibinski and Roderick 1991; Gardner *et al.* 1993) and would have the effect of removing potentially introgressing *M. galloprovincialis* mitotypes but not potentially introgressing *M. edulis* mitotypes. One possible environmental factor might be a bias in water movement from the Atlantic into the Mediterranean (Perkins *et al.* 1990), which might result in one way dispersal of gametes and larvae from areas dominated by *M. edulis* towards areas in which *M. galloprovincialis* predominates. Additionally, it has previously been suggested that the range of the zone of hybridisation between *M. edulis* and *M. galloprovincialis* might be limited by differential temperature tolerances of the two species and their hybrids and similarly that differential salinity tolerances might be instrumental in limiting the extent of the hybrid zone between *M. edulis* and *M. trossulus* at the entrance to the Baltic Sea (Gardner 1996).

The level of disruption observed in hybrid larvae from the laboratory crosses is more than double that previously found in adult mussels from natural *M. edulis* x *M. galloprovincialis* hybrid populations (Rawson, Secor and Hilbish 1996). It was previously assumed that either disruptions of DUI were very rare in *M. edulis* x *M. galloprovincialis* hybrids, or that individuals with disrupted DUI rarely survived to adulthood. The present study demonstrates that DUI is disrupted in an estimated 40% of larvae which survive to the age of 72 h. Hence some degree of divergence must have occurred between these two species at the loci which regulate DUI. The difference between the results obtained from larvae (this study) and from adults (Rawson, Secor and Hilbish 1996) suggests that hybrid individuals in which DUI has been disrupted might suffer greater mortality between 72 h and adulthood than mussels in which DUI has not been disrupted. Alternatively, it could be that DUI disruptions occur more frequently in laboratory crosses than in nature but there is no evidence for this from previous studies of DUI. Further laboratory crosses between *M. edulis* and *M. trossulus* and between *M. galloprovincialis* and *M. trossulus* are necessary to facilitate direct comparison of the extent to which DUI is disrupted in these three species pairs.

It has been demonstrated that many terrestrial hybrid zones are maintained by a balance between immigration of pure species and selection against hybrid individuals and the decreased fitness of hybrids in such zones is thought to be a consequence of the breakdown of co-adapted parental gene complexes resulting from hybridisation (Barton and Hewitt 1989). There are cases of increased hybrid mortality or unfitness in marine bivalves documented in the literature. For example, hybrid crosses between the oysters *Crassostrea gigas* and *C. rivularis* have a lower fertilisation rate and lower survival of fertilized eggs than pure species crosses (Allen and Gaffney 1993) and crosses of either of these species with *C. virginica* produce hybrid larvae which are inviable after eight to ten days (Allen *et al.* 1993). Hybrids of the hard clams *Mercenaria mercenaris* and *M. campechiensis* are viable but have increased susceptibility to gonadal neoplasia (Hesselman *et al.* 1988; Bert *et al.* 1993). However, in other cases hybrids have been shown to be of equal or greater fitness than parent species.

For example, Menzel (1985) found hybrid *Mercenaria mercenaria* x *M. campechiensis* to have higher growth rate and longevity than the pure species.

There is evidence from a number of studies that adult *Mytilus edulis* x *M. galloprovincialis* hybrids on average have a fitness approximately equal to *M. galloprovincialis* and higher than *M. edulis*, in terms of viability (Gardner and Skibinski 1991; Willis and Skibinski 1992; Gardner *et al.* 1993), developmental stability and developmental rate (Gardner 1995), growth rate (Gardner *et al.* 1993), fertility and fecundity (Gardner and Skibinski 1990) and resistance to parasites (Coustau *et al.* 1991). Beaumont *et al.* (1993) carried out laboratory hybridisations between *M. edulis* and *M. galloprovincialis* and compared survival rates and abnormality levels in pure species and hybrid larvae during their early stages of growth. These authors found no significant differences between pure species and hybrid larvae up to three days after fertilisation, but between three and nine days the proportion of normal veliger larvae which did not survive was significantly greater in hybrid crosses. However, the hybrid larvae which did survive subsequently grew significantly faster than the pure species larvae (Beaumont *et al.* 1993). It may be that there is considerable variation in the fitness of hybrid *M. edulis* x *M. galloprovincialis* mussels and only those which have relatively high fitness survive to adulthood to be analysed in studies of natural populations, whereas those with lower fitness suffer early mortality.

If offspring from crosses which exhibit disruption of DUI have low probabilities of survival this might be the outcome of interspecific incompatibilities between nuclear genes, between F and M mitotypes, or between nuclear and mitochondrial genes. The disruptions of DUI in the F_1 hybrids in the present study indicate that incompatibilities do exist but the type of incompatibilities cannot be determined from F_1 hybrids because they contain an equal amount of nuclear genes from each species and because F_1 males must contain F and M mitotypes from different species.

Previous studies have investigated the effects of incompatibilities between nuclear and mitochondrial genomes in other organisms. Evidence for a lack of deleterious effects of nuclear / mtDNA incompatibilities comes from many cases of mtDNA introgression among species documented in the literature (e.g. Ferris *et al.* 1983; Powell 1983; Avise *et al.* 1984; Lamb and Avise 1986; Harrison *et al.* 1987; Marchant 1988; Satta *et al.* 1988; Gyllensten *et al.* 1991). Studies of introgression in *Drosophila* have found that individuals with nuclear and mitochondrial genomes from different species or strains appear to develop normally, but incompatibilities between nuclear genes from different species have been shown to lead to hybrid breakdown (Zeng and Singh 1993; Goulielmos and Zouros 1995). Further evidence supporting nuclear control of species recognition mechanisms come from studies on mice. The mechanism for elimination of paternal mtDNA in mice is thought to involve two nuclearly encoded factors, one in the egg cytoplasm and the other in the outer surface of the sperm mitochondria (Kaneda *et al.* 1995) and is very similar to that proposed for *Mytilus* by Saavedra *et al.* (1997) and Zouros (2000). Elimination of paternal mtDNA results from recognition of the sperm factor by the egg factor, but this recognition breaks down in interspecific crosses where elimination of paternal mtDNA is less efficient. In crosses involving male mice with nuclear and mitochondrial DNA from different species, Kaneda *et al.* (1995) have shown that the recognition of the sperm factor by the egg factor depends on the male's nuclear rather than mitochondrial genome, and deduced that the failure to eliminate paternal mtDNA in interspecific crosses is due to nuclear / nuclear rather than nuclear / mitochondrial incompatibilites. Further to this, Shitara *et al.* (2000) have shown that the male recognition factor is present in mouse sperm mitochondria but not in liver mitochondria, which suggests that the sperm mitochondria are selectively labelled in some way during spermatogenesis. A similar mechanism is thought to occur in cows and monkeys and the molecule which labels sperm mitochondria for elimination in these animals is thought to be ubiquitin (Sutovsky *et al.* 1999), a recycling marker protein which in binding to a variety of cytoplasmic and nuclear proteins appears to target their selective degradation (Finley and Varshavsky 1985).

Further research is necessary to determine whether similar mechanisms occur in *Mytilus* and to investigate the additional possibility of incompatibilities between F and M mitotypes from different species. Analysis of F_2 hybrids would provide an opportunity to study possible F / M mitotype incompatibilities independently from nuclear incompatibilities and analysis of backcross individuals with a known background would provide information about possible incompatibilities between nuclear and mitochondrial genes from different species. Some information is already available for *M. edulis* x *M. trossulus* hybrids, in which evidence has been found for incompatibilities between nuclear and mitochondrial DNA from different species and between F and M mitotypes from different species (Comesana *et al.* 1999; Saavedra *et al.* 1996). It is not yet known whether these incompatibilities also exist between *M. edulis* and *M. galloprovincialis* and between *M. galloprovincialis* and *M. trossulus*. F_2 individuals are rarely detected in natural hybrid populations and their identification is complicated, requiring the use of a range of markers (Saavedra *et al.* 1996; Comesana *et al.* 1999). Laboratory crosses would facilitate easier identification of F_2 and other hybrid classes, but rearing F_1 individuals in controlled conditions in the laboratory or in the field to produce F_2 individuals would be difficult and costly in terms of both time and resources.

Although the sex of a larva cannot be determined and hence the presence of M-negative males or M-positive females in the families studied cannot be confirmed, the findings of the present study suggest that occurrences of both M-negative males and M-positive females are associated with hybridisation but that the two forms of disruption do not necessarily occur together. This provides further support for the hypothesis that the regulation of DUI involves a complex system of interactions which control the retention of the M mitotype in males independently from its elimination in females. However, disruption of DUI in hybrids cannot be the only factor limiting introgression between *M. edulis*, *M. galloprovincialis* and *M. trossulus*. It is likely that a range of both genetic and environmental factors are important in determining the location and extent of hybrid zones and hence in the maintenance of genetic integrity for the three species.

Appendix A. The Polymerase Chain Reaction

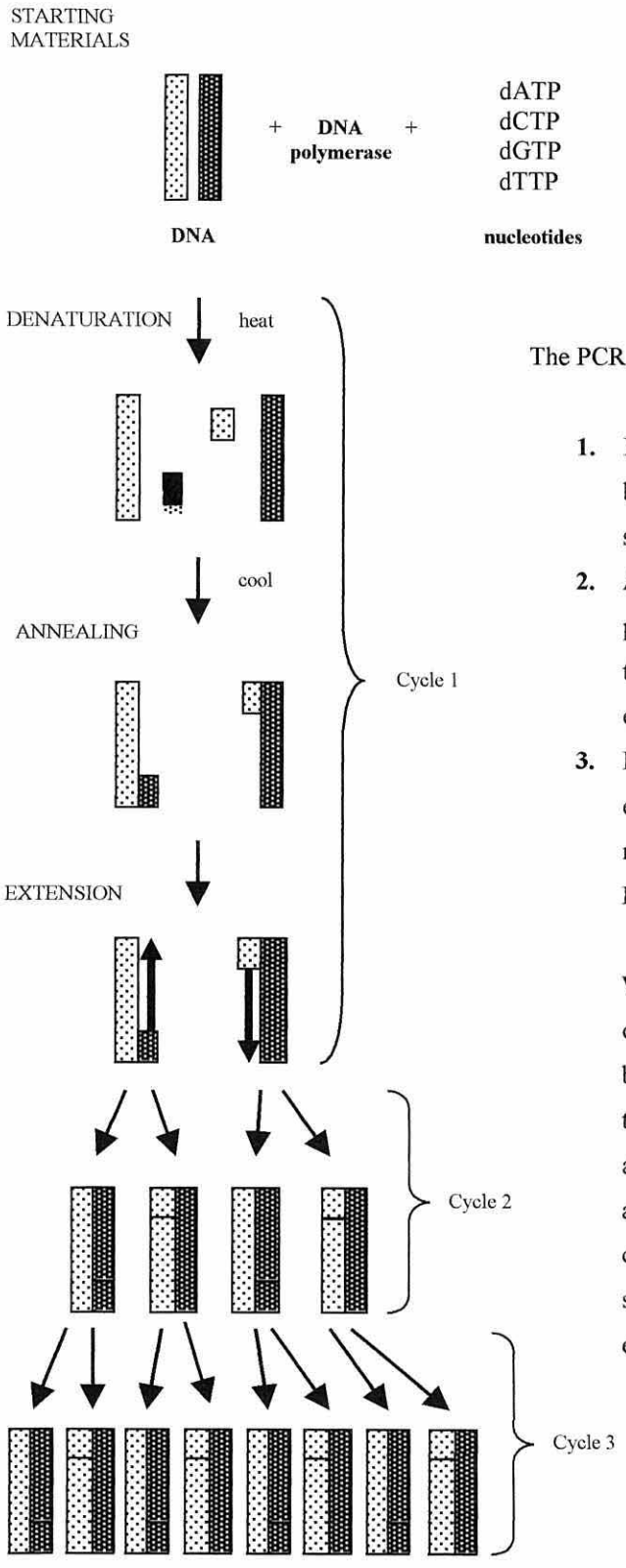
The polymerase chain reaction (PCR) is a technique by which any specific segment of DNA can be quickly amplified *in vitro*. DNA is amplified by PCR in an enzyme reaction which undergoes multiple incubations at different temperatures (Figure A.1). The starting material for PCR is a solution of double-stranded DNA containing the nucleotide sequence that is "targeted" for copying. The reaction mixture must also include a pair of oligonucleotide primers, a DNA polymerase enzyme and a supply of all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), collectively termed dNTPs.

Oligonucleotide primers are short, single-stranded molecules of DNA obtained by chemical synthesis. Primer sequences are chosen so that they bind by complementary base-pairing to opposite DNA strands on either side of the "target" sequence to be amplified.

The DNA polymerase binds to single-stranded DNA and synthesizes a new strand complementary to the original strand. DNA polymerases require a short region of double-stranded DNA to get started. In PCR this is provided by the oligonucleotide primers which create short double-stranded regions by binding on either side of the DNA sequence to be amplified. In this way the priemrs direct the DNA polymerase to copy only the target DNA sequence. Thermostable DNA polymerases are used in order to withstand the high temperature (usually 94°C) needed to separate the DNA strands in the denaturation phase of the PCR cycle. The most commonly used enzyme is *Taq* DNA polymerase from *Thermus aquaticus*, a bacterium present in hot springs.

The dNTPs correspond to the four bases present in DNA (adenine, guanine, thymine and cytosine) and are substrates for the DNA polymerase, which uses dNTPs as building blocks to synthesize new strands of DNA.

Figure A.1 The polymerase chain reaction (PCR) components and the PCR cycle (modified from Campbell 1993).



The PCR procedure:

1. **Denaturation** The DNA is briefly heated to separate its strands.
2. **Annealing** Cooling allows the primers to bind to the ends of the target sequence, one primer on each strand.
3. **Extension** The DNA polymerase extends the primers by adding nucleotides, using the longer DNA strands as templates.

Within a short time, the amount of the target DNA sequence has been doubled. The solution is then heated again, starting another cycle of denaturation, annealing and extension. The cycle is repeated until the target sequence has been replicated enough times.

Appendix B. DNA Detection Protocols

Silver Staining Protocol (modified from Skibinski *et al.* (1994))

Solutions were made up immediately prior to use. For each solution, the required volume of sterile water was poured into a conical flask and the other ingredients were added in the order given below. Acetic acid and formalin were added in a fume hood. Amounts given are per gel, for 17 cm x 16 cm x 1.5 mm gels.

		Per gel	Final percentage
Solution A.	Sterile water	360 ml	
	Ethanol	40 ml	10%
	Acetic acid	2 ml	0.5%
Solution B.	Sterile water	200 ml	
	Silver nitrate	0.2 g	0.1%
Solution C.	Sterile water	300 ml	
	Sodium hydroxide pellets	4.5 g	1.5%
	Sodium borohydride	0.03 g	0.01%
	Formalin (37% formaldehyde)	1.2 ml	0.15% formaldehyde

NB. fully dissolve the sodium hydroxide pellets before adding the sodium borohydride

Gels were washed in the solutions, in a large tray, as follows:

Solution A. 2 x 3 minutes (use ½ solution for each wash, save 2nd half for later)
Solution B. 10 minutes
Rinse twice in distilled water
Solution C. Until bands appear (approx. 5 – 10 minutes)
Return to 2nd half of Solution A

SYBR Gold Staining Protocol

SYBR Gold nucleic acid gel stain (Molecular Probes) was used according to the manufacturers instructions: 50 µl SYBR Gold stock was added to 500 ml 1 x TBE. The gel was washed in this solution for 30 minutes in a tray covered in aluminium foil to protect from light.

Internal labelling of M1/2 PCR product with ^{33}P

A slight variation was made to the usual PCR protocol when internally labelling M1/2 PCR products with α-^{33}PdCTP. Mix A was made up as usual (Section 2.7) and aliquotted out into the PCR microtubes. Then 3 µl lysate was added to each microtube and the microtubes were centrifuged briefly. Mix B was made up as usual, but including 0.015 µl of α-^{33}PdCTP (10 mCi/ml) per microtube, and was aliquotted out into the microtube lids. The tubes were loaded into the thermocycler and the temperature cycle started as outlined in the general PCR amplification protocol (Section 2.7).

3'-end labelling of 100 bp DNA Ladder with ^{33}P

GibcoBRL/Life Technologies 100 bp DNA Ladder stock was 3'-end labelled with α-^{33}PdCTP using T4 DNA Polymerase. The GibcoBRL protocol was followed:

For the exonuclease reaction (degradation of DNA from both 3'-ends), the following were added to a 0.5 ml microtube, on ice:

5x T4 DNA polymerase reaction buffer	3 µl
100 bp DNA ladder (1 µg/µl)	5 µl
T4 DNA polymerase	20 units (4 µl)
Sterile water	3 µl

The contents of the microtube were mixed gently but thoroughly, centrifuged briefly and incubated at 25°C in a thermocycler for 2 minutes, before returning to ice.

For the resynthesis reaction, the following were added to the microtube, on ice:

Sterile water	4 µl
5x T4 DNA polymerase reaction buffer	3 µl
dATP (2 mM)	2.5 µl
dGTP (2 mM)	2.5 µl
dTTP (2 mM)	2.5 µl
α-^{33}PdCTP (10 mCi/ml)	0.5 µl

The contents of the microtube were mixed gently but thoroughly, centrifuged briefly and incubated at 37°C in a thermocycler for 2 minutes. 2.5 µl of 2 mM dCTP was added and the microtube was incubated for a further 2 minutes at 37°C. The reaction was stopped by adding 1.25 µl of 0.5M EDTA and the microtube was centrifuged for 10 s. 6.25 µl Bromophenol blue loading dye was added to bring the volume of the microtube contents to 40 µl. For electrophoresis on 5% polyacrylamide gels, 2 µl of this labelled ladder stock was loaded per lane, diluted with 5 µl sterile water.

Gel drying and autoradiography

The gel and plates were removed from the electrophoresis rig and placed on to blue roll. The glass plates were separated and the top plate was removed, leaving the gel lying flat on the bottom plate. The gel was covered with Whatman paper, peeled away from the bottom glass plate and laid flat on blue roll, Whatman paper down. The gel was then covered with cling film and dried in a Gel Dryer for approximately 30 minutes. The vacuum pump was left running for a further 30 minutes, until the gel had cooled to approximately 30°C, to prevent the gel from cracking. The cling film was removed and the gel

was monitored for radioactivity. The gel, on Whatman paper, was placed inside a light-tight cassette for transportation to a darkroom. An autorad was placed on to the gel and the light-tight cassette was resealed. The autorad was exposed for 18-48 hours, before developing. Sigma Developer and Fixer solutions were used as recommended by the manufacturer. Developing time was 2-5 minutes and fixing time was double the developing time.

Appendix C. DNA Analysis Recipes

Electrode & gel buffer: TBE pH 8.3	per litre (10 x working conc.)
Tris (hydroxymethyl) aminomethane	108 g
Boric acid	55 g
0.2 M EDTA	100 ml

Add sterile water to dissolve and adjust to pH 8.3 with HCl
Adjust volume to 1 l with sterile water
Autoclave

0.2 M EDTA pH 8.0	per 100 ml
EDTA	7.44

Add sterile water to dissolve and adjust to pH 8.0 with NaOH
Adjust volume to 100 ml with sterile water
Autoclave

0.5 M Tris/HCl pH 8.0	per 100 ml
Tris (hydroxymethyl) aminomethane	6.06 g

Add sterile water to dissolve and adjust to pH 8.0 with HCl
Adjust volume to 100 ml with sterile water
Autoclave

TE buffer pH 8.0	per 100 ml
0.5 M Tris/HCl pH 8.0	2 ml
0.2 M EDTA pH 8.0	0.25 ml

Adjust volume to 100 ml with sterile water
Autoclave

CTAB buffer	per 100 ml	molarity
NaCl	8.182 g	1.4 M
EDTA	0.744 g	20 mM
0.5 M Tris/HCl pH 8.0	20 ml	100 mM

Adjust volume to 100 ml with sterile water

Autoclave

Leave to cool to room temperature

Add 2 g CTAB (Hexadecyltrimethylammonium bromide) and allow to dissolve

Lysis solution (to release DNA from larvae)	per 100 ml	molarity
Tris (hydroxymethyl) aminomethane	90.8 mg	7.5 mM
NH_4Cl	20.1 mg	3.75 mM
KCl	28.0 mg	3.75 mM
$MgCl_2$	30.5 mg	1.5 mM

Adjust volume to 100 ml with sterile water

Autoclave

Aliquot (15 µl per microtube), adding 2 µg proteinase K to each tube

Freeze at -20°C until required

Bromophenol blue gel loading dye	per 10 ml
Glycerol	3 ml
Bromophenol blue	50 mg
Sterile water	7 ml

Autoclave

100 bp DNA ladder aliquots

GibcoBRL 100 bp ladder stock	5 µl
TE buffer pH 8.0	95 µl
Bromophenol blue gel loading dye	20 µl

Mix thoroughly

Aliquot (12 µl per gel lane for agarose, 6 µl per gel lane for polyacrylamide)

pBR322 *Msp*I DNA ladder aliquots

Advanced Biotechnologies pBR322 *Msp*I ladder stock	10 µl
TE buffer pH 8.0	90 µl
Bromophenol blue gel loading dye	20 µl

Mix thoroughly

Aliquot (12 µl per gel lane for agarose, 6 µl per gel lane for polyacrylamide)

Agarose gels	per gel
Agarose	0.8 – 4 % w/v
TBE buffer 10x stock	1 in 10 dilution with sterile water

Microwave until boiling

Polyacrylamide gels	per gel: 5 %	8%
Acrylamide/Bisacrylamide 37.5:1, 30 % solution	8.3 ml	13.3 ml
TBE buffer 10x stock	5 ml	5 ml
Adjust volume to 50 ml with sterile water		
10% Ammonium persulphate solution	400 µl	400 µl
TEMED	44 µl	44 µl

Appendix D. Allozyme Analysis Recipes

Starch gels	per gel
Potato starch	33 g
Electrode buffer	25 ml
Sterile water	250 ml

Electrode buffers

TME pH 7.4 (for PGM and GPI)	g per litre	molarity
Tris (hydroxymethyl) aminomethane	12.1	0.1 M
Maleic acid	11.6	0.1 M
EDTA (di-sodium salt)	3.72	0.01 M
$MgCl_2$	4.06	0.01 M
Sterile water to dissolve		
Adjust to pH 7.4 with NaOH		
Adjust volume to 1 l with sterile water		

Tris/Citrate pH 8.0 (for MPI)	g per litre	molarity
Tris (hydroxymethyl) aminomethane	18.17	0.15 M
Sterile water to dissolve		
Adjust to pH 8.0 with citric acid		
Adjust volume to 1 l with sterile water		

Staining recipes

Phosphoglucomutase (PGM) EC 2.7.5.1.	per gel
$MgCl_2$	5 mg
NADP (sodium salt)	5 mg
Glucose 1-phosphate (disodium salt)	50 mg
0.1 M Tris/HCl pH 8.0	25 ml
Glucose 6-phosphate dehydrogenase	10 µl
Methyl thiazolyl tetrazolium (MTT, 5 mg ml^{-1})	1 ml
Meldola blue (0.8%)	50 µl
2% agar solution (60°C)	25 ml

Glucose-6-phosphate isomerase (GPI) EC 5.3.1.9.	per gel
$MgCl_2$	5 mg
NADP (sodium salt)	5 mg
Fructose 6-phosphate (barium salt)	20 mg
0.1 M Tris/HCl pH 8.0	25 ml
Glucose 6-phosphate dehydrogenase	10 µl
Methyl thiazolyl tetrazolium (MTT, 5 mg ml^{-1})	1 ml
Meldola blue (0.8%)	50 µl
2% agar solution (60°C)	25 ml

Mannose-6-phosphate isomerase (MPI) EC 5.3.1.8.	per gel
$MgCl_2$	5 mg
NADP (sodium salt)	15 mg
Mannose 6-phosphate	50 mg
Pyruvic acid (sodium salt)	20 mg
0.2 M Tris/HCl pH 7.5	25 ml
Phosphoglucose isomerase (PGI)	20 µl
Glucose 6-phosphate dehydrogenase	25 µl
Methyl thiazolyl tetrazolium (MTT, 5 mg ml^{-1})	1 ml
Meldola blue (0.8%)	50 µl
2% agar solution (60°C)	25 ml

Staining buffers

0.1 M Tris/HCl pH 8.0 (for PGM and GPI)	g per litre
Tris (hydroxymethyl) aminomethane	12.1
Sterile water to dissolve	
Adjust to pH 8.0 with HCl	
Adjust volume to 1 l with sterile water	

0.2 M Tris/HCl pH 7.5 (for MPI)	g per litre
Tris (hydroxymethyl) aminomethane	24.2
Sterile water to dissolve	
Adjust to pH 7.5 with HCl	
Adjust volume to 1 l with sterile water	

2% Agar solution	g per 100 ml
Agar	2
Adjust volume to 100 ml with dH_2O	

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