An investigation of population differentiation in the scallop *Pecten maximus* (Bivalvia: Pectinidae) using molecular techniques.

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by

Diana Angela Heipel
(Dipl.-Biol.)

Port Erin Marine Laboratory
School of Biological Sciences
University of Liverpool
Port Erin
Isle of Man

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ABSTRACT

The great scallop *Pecten maximus* (Family Pectinidae, Class Bivalvia, Phylum Mollusca) is a north Atlantic species which is heavily fished around the Isle of Man. Management for sustainable exploitation requires knowledge of the extent to which commercial fishing grounds are reproductively self-sustaining or supplied with larvae originating from further afield. The degree of genetic differentiation between locations provides insight into the pattern and scale of effective larval dispersal.

Genetic differentiation of *P. maximus* populations originating from six different locations around the Isle of Man (Bradda Inshore, Chickens Rock, East Douglas, Ramsey, The Targets and Peel) was investigated in two consecutive year-classes (3-years-old in 1995 and 1996). Comparison was extended to two samples taken outside the Irish Sea, from Mulroy Bay (Co. Donegal, Eire) and Plymouth (south west England).

Samples were analysed using both randomly amplified polymorphic DNA (RAPD) and restriction analysis of PCR-amplified mitochondrial DNA fragments. 51 polymorphic RAPD-bands generated by 13 primers were scored as present or absent and subjected to a phenotypic analysis of molecular variance (AMOVA) and multivariate analyses (PCOORD and UPGMA clustering). Correlation of phenotypic distance, based on RAPD banding pattern, with geographic distance was investigated by means of Mantel tests.

Due to length-variation of the mitochondrial DNA molecule in *P. maximus*, restriction analysis could not be carried out on the whole molecule. Instead, two mitochondrial DNA fragments, one including the 12S rRNA and 16S rRNA genes (3 kb), the other of unknown sequence (2 kb), were subjected to restriction analysis with six restriction endonucleases. Cloning of additional fragments was attempted. The fragments were analysed separately as well as combined. Haplotype and nucleotide diversity within populations, and nucleotide divergence between populations, were calculated. Mean nucleotide sequence divergence was corrected for within-population polymorphisms and visualized by UPGMA cluster diagrams. Molecular analysis of variance (AMOVA) was carried out.

Data obtained with both RAPD and mt-RFLP's showed only low levels of population differentiation. Significant small-scale structure was revealed between Douglas and the other Manx populations. Statistically significant results also indicated the distinction of the population from Mulroy Bay from all other populations. Interrelationships between the Manx locations were not similar in the different year-classes, and the two cohorts clustered separately on the basis of nucleotide divergence for the combined mitochondrial fragments. Analysis of molecular variance (AMOVA), and haplotype and nucleotide diversity, showed high levels of variability to be present in all populations. This is probably due to the dynamic hydrographic conditions in the Irish Sea, which ensure extensive mixing of the planktonic larvae.

The length-variation in the mitochondrial DNA molecule is caused by varying numbers of copies of non-coding repeated elements. Primers for PCR-amplification of a repeated element were designed and a preliminary screening for restriction-site polymorphisms was carried out.

CHAPTER ONE: GENERAL INTRODUCTION

1.1. Biology of *Pecten maximus*

The great scallop *Pecten maximus* (L.) is a North Atlantic species which belongs to the Family Pectinidae (Class Bivalvia, Phylum Mollusca); this family includes ca. 400 living species (Brand, 1991). Other members of the family fished in European waters are the queen scallop *Aequipecten opercularis* (L.), the black scallop *Chlamys varia* (L.), the Iceland scallop *Chlamys islandica* (Mueller) and the St. James scallop *Pecten jacobeus* (L.) (Ansell *et al.*, 1991). In the Irish Sea, only *P. maximus* and *A. opercularis* are exploited commercially. *C. varia, C. distorta, C. tigerina, C. striata, C. furtiva* and *C. similis* are present, but less abundant (Bruce *et al.*, 1963) and too small to be of commercial interest.

1.1.1. Distribution

P. maximus occurs along the Atlantic coast of Europe from northern Norway to Portugal as well as off West Africa, around the Azores, Madeira and the Canary Islands. At both southern and northern limits, distribution is governed by water temperature, which influences spawning, larval development and settlement (Kinne, 1970). According to Brand (1991), scallop species have an aggregated distribution within their geographical range that can be perceived at different spatial scales: 'grounds' are areas large enough to support a commercial fishery, (e.g. the North Irish Sea or the Western Channel). The average density of animals in grounds is generally between 0.1 and 10 scallops m^{-2}. Within each ground are areas of several km^2 where scallops are more abundant, known as 'beds'. They themselves consist of 'patches', a few square metres in area, of accumulations of scallops.

P. maximus can be found from very shallow waters to depths of some 200 m, being most common at 20 - 45 m (Brand, 1991). It prefers bottoms of sand, muddy sand and sandy gravel, which allow the excavation of a depression in which to recess, with the upper, flat (left), shell valve level with the sea bed and often camouflaged by sediment. Scallops, especially juveniles, are preyed on by crabs, e.g. *Carcinus*

maenas, *Cancer pagurus* and starfish, *Asterias rubens* (Lake *et al.*, 1987; Minchin, 1991). In some regions, octopus have been reported to be major scallop predators (Cochard *et al.*, 1991)

1.1.2. Morphology

The right (lower) shell valve is of concave shape. Both valves are sculptured with 15-17 radiating ribs. The two valves are joined by a rubbery ligament at the hinge and in life open about 20° (Franklin *et al.*, 1980). As monomyarian bivalves, scallops lack the anterior adductor muscle, and the valves are closed by the large posterior adductor muscle, which has come to occupy a more central position. It consists of a cross-striated (phasic) part, used in the swimming escape response, and a smaller, smooth (tonic) part with high paramyosin content, which allows the maintenance of large tensions for long periods with little expenditure of energy ('catch' state) (Chantler, 1991), e.g. when closing the valves for defence. Contractions of the phasic adductor muscle enable the scallop to swim over small distances by jet propulsion through expulsion of water from the mantle cavity. For recessing in the seabed, water jets are repeatedly ejected and directed at the sediment by adjustment of the mantle velum (Baird, 1958). If the sediment is unsuitable, random swimming movements are used to find soft grounds allowing recessing, but distances covered are very limited. The adductor muscle also acts as a storage site for metabolic reserves in the form of proteins and glycogen, and consequently reaches its highest weight when gonadal activity is lowest (Mackie, 1986; Wanninayake, 1994). The scallop is a filter feeder, living mainly on planktonic algae and detritus, which are captured by mucus and cilia on the surface of two large orange coloured gills. Food particles enter the digestive system through the mouth and are transported into the oesophagus and stomach. The stomach is surrounded by the greenish-black digestive gland, the major site of extracellular digestion. The intestine loops through the gonad and traverses the pericardium and ventricle, from where the rectum leads down the posterior edge of the adductor muscle to the anus. Two ganglionic concentrations are visible. One innervates the adductor muscle, ventral mantle region, gills and some viscera, while the other innervates the rest of the body. Along the margins of the

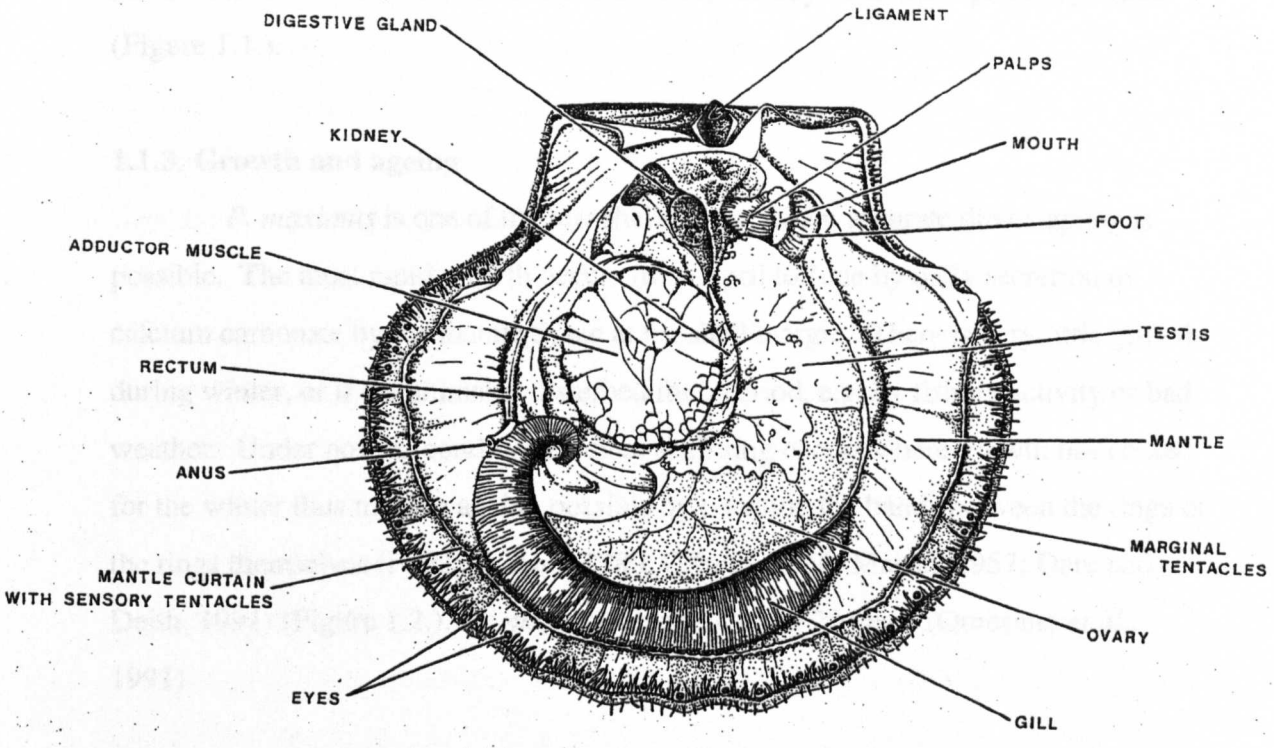


Figure 1.1. Structure of the scallop *P. maximus* (from Mason, 1983).

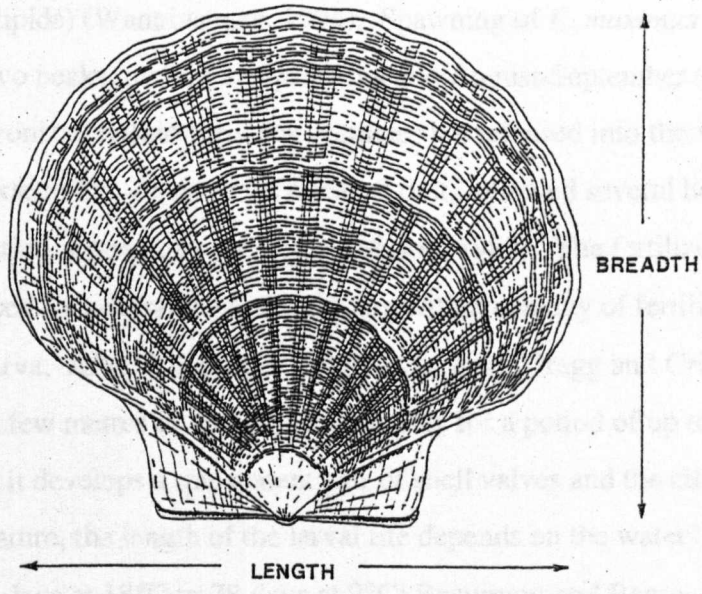


Figure 1.2. Top view of scallop shell with growth rings (from Mason, 1983).

mantle are rows of sensory tentacles and numerous tiny, iridescent green, eye spots (Figure 1.1.).

1.1.3. Growth and ageing

P. maximus is one of the few shellfish for which accurate direct ageing is possible. The most rapid growth occurs from April to June by daily secretion of calcium carbonate by the mantle tissue at the shell margin. There is very little growth during winter, or if the animal is disturbed for a period, e.g. by fishing activity or bad weather. Under normal conditions a clear shell ring occurs where growth has ceased for the winter thus making ageing possible by counting the bands between the rings or the rings themselves (Franklin *et al.*, 1980; Gibson, 1956; Mason, 1957; Dare and Deith, 1991) (Figure 1.2.). *P. maximus* lives for up to 22 years (Orensanz *et al.*, 1991).

1.1.4. Life cycle

P. maximus is hermaphroditic, the gonad consisting of the orange, distally located ovary, and the proximal white testis. The gonads develop during the winter months, using metabolites stored in the adductor muscle (protein, glycogen) and in the digestive gland (lipids) (Wanninayake, 1994). Spawning of *P. maximus* in the Irish Sea occurs in two peaks, mainly in April-May and August-September (Mason, 1958, 1983). Synchronized within the stock, gametes are released into the water and fertilisation occurs externally. As sperm is released first, followed several hours later by eggs, self-fertilisation is generally precluded (Mason, 1983). The fertilized eggs remain near the sea bed for several days, developing within one day of fertilization into a trochophore larva. After 2-3 days, a veliger is formed (Cragg and Crisp, 1991), which drifts within a few metres of the surface, possibly for a period of up to six weeks, during which it develops a transparent pair of shell valves and the cilia used for propulsion. In nature, the length of the larval life depends on the water temperature (e.g. 24 days at 18°C to 78 days at 9°C; Beaumont and Barnes, 1992) and food availability, and is perhaps also influenced by genetic factors (Paulet *et al.*, 1988). At the end of this pelagic phase the larva develops a foot and sinks to the sea

bed where it moves around testing surfaces upon which to settle. This 'pediveliger' stage has a shell length of 0.25 - 0.30 mm (Le Pennec, 1974; Franklin *et al.*, 1980). Eventually, it attaches to an appropriate benthic substrate by means of sticky threads secreted by the foot gland and metamorphoses into a juvenile scallop, now called a 'spat' (larvae are regarded as having metamorphosed when the gill buds and post-larval shell are visible) (Figure 1.3.). Settlement is influenced by the availability of suitable primary settlement sites such as hydroids and bryozoans, which provide a clean, silt-free surface (Brand *et al.*, 1980). At about 10 mm shell length the juvenile scallop releases the byssus and recesses into the seabed (Franklin *et al.*, 1980).

The reproductive cycle is determined by environmental factors including temperature, light, salinity, tides, and food availability, in co-ordination with the nervous and hormonal systems (Sastry, 1975). The time of spawning is therefore different for populations occurring in different latitudes and climates. The first maturity of *P. maximus* seems to be size- rather than age-dependent. In warm seas spawning at an age of less than one year is frequent (Mason, 1957; Broom, 1976). However, in the colder Manx waters, most *P. maximus* first spawn in the autumn following the deposition of the second growth ring, when about two years old (Mason, 1957; 1958). The seasonal pattern of subsequent spawnings varies in different locations. The northern-most *P. maximus* populations in Norway show a closely synchronised spawning in June (Strand and Nyland, 1991), while further south, in West Scotland, spawning still occurs in one main period, but is not synchronised between all individuals in the population (Mackie, 1986; Ansell *et al.*, 1991). In the Irish Sea, studies have reported two main spawning peaks, in spring (April - May) and in autumn (August - September) (Mason, 1958; Stanley, 1967; Duggan, 1987). However, Wanninayake (1994) noted only a single spawning peak in June - July for the Port St. Mary Offshore population. In the English Channel a primary spawning of *P. maximus* takes place in July, followed by a secondary spawning at the end of August (Paulet *et al.*, 1988; Ansell *et al.*, 1988). In contrast, in the Bay of Brest, repeated periods of maturation of cohorts of gametes throughout

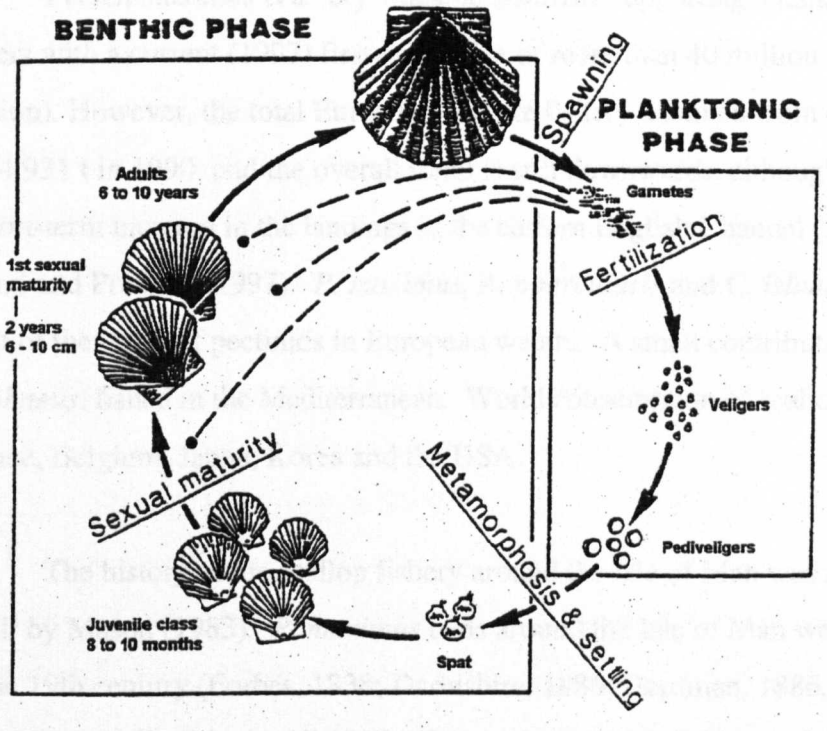


Figure 1.3. Life cycle of the great scallop *P. maximus* (from Devauchelle and Mingant, 1991)

the year, which were often resorbed rather than spawned, were reported by Lubet *et al.* (1987, 1991). It appears that further south in the geographical range, more variation and less synchronisation in gonadal development is the rule (Ansell *et al.*, 1991).

1.2. Pectinid fisheries

Pecten maximus is a very valuable shellfish, supporting a fishery in European waters with a current (1997) first-sale value of more than 40 million ECU (about £ 28 million). However, the total European capture fishery declined from 48,571 t in 1973 to 14,931 t in 1990, and the overall trend is still downwards, although there has been a short-term increase in the landings in the eastern English Channel (23,000 t in 1993; Brand and Prudden, 1997). *P. maximus, A. opercularis* and *C. islandica* account for most of the catch of pectinids in European waters. A small contribution is made by *P. jacobaeus*, fished in the Mediterranean. World consumption of scallops is mainly in France, Belgium, Japan, Korea and the USA.

The history of the scallop fishery around the Isle of Man was reviewed in detail by Mason (1983). *P. maximus* beds around the Isle of Man were first reported in the 19th century (Forbes, 1838; Darbishire, 1886; Herdman, 1886, 1895) but were not commercially fished until 1937, when a visiting Irish fishermen brought the potential of the scallop beds off Bradda Head to attention (Smith, 1938). At the same time the fishery for cod and plaice was decreasing rapidly, and the Isle of Man fleet could not effectively compete with other national fleets in catching herring, so that exploitation of scallop beds provided a new source of income (Brand *et al.*, 1991). Within a few years, management of scallop stocks became necessary, with the first closed season (1st May to 30th September) and minimum legal landing size (4 ½ inches shell length) established by the Escallop Control and Maximum Prices Order in 1943 (Brand *et al.*, 1991). Today, the closed season extends from 1st June to 31st October, and the minimum legal landing size is 110 mm, allowing the scallops to spawn two to three times before becoming of market size. Since 1969, the queen scallop *A. opercularis* has also been a valuable fishery for the Isle of Man. Fished

initially during the summer closed season for scallops, queen scallops are now fished throughout the year (Brand *et al.*, 1991). No specific restrictions apply to the queen scallop fishery.

Until 1953 *P. maximus* were mainly fished around Bradda Head, Peel Head, Maughold Head and in Ramsey Bay. After a survey by Mason (1959) located scallop beds off Chicken Rock, west of the Calf of Man, on Kirkmichael Bank, 3-5 miles west of Port Erin and south-east of Douglas, the Isle of Man fleet expanded considerably. In the 1970s, the fishery extended further south and east of the Island, mainly due to the introduction of spring tooth bar dredges, which allowed the exploitation of rougher grounds (Brand *et al.*, 1991). Today, scallop fishing takes place all around the Isle of Man, extending far into the open sea south of the Island.

Data on landings of scallops on the Isle of Man go back to 1969. Until 1985, there was a steady rise in total landings to 2100 tonnes live weight, followed by a decline to less than 650 tonnes in 1993 (Figure 1.4.). There was a brief recovery in 1987, but the overall trend is downwards. Constantly increasing effort is now necessary to maintain catches, at the same time further reducing scallop abundance and the profitability of fishing. According to Allison (1993), up to 55 % of the recruiting year-class on the inshore west fishing grounds could be removed by fishing in a single season. Depletion of the older year-classes endangers the breeding stock, since the replenishment of overfished beds becomes dependent on the strength of the recruiting year-class (Brand *et al.*, 1991; Allison, 1993). Failure of recruitment in one year could thus result in the collapse of the fishery (Allison, 1993). To prevent this happening, attempts have been made to conserve the exploited beds almost from the beginning of the Manx scallop fishery. The introduction of the closed season, minimum legal landing size, and restrictions on the size of dredges and fishing vessels (boats have to be smaller than 15 m to fish within 3 miles of the Isle of Man), are measures which have ensured continued exploitation up to the present, although they have failed to prevent a steady decline in the stocks.

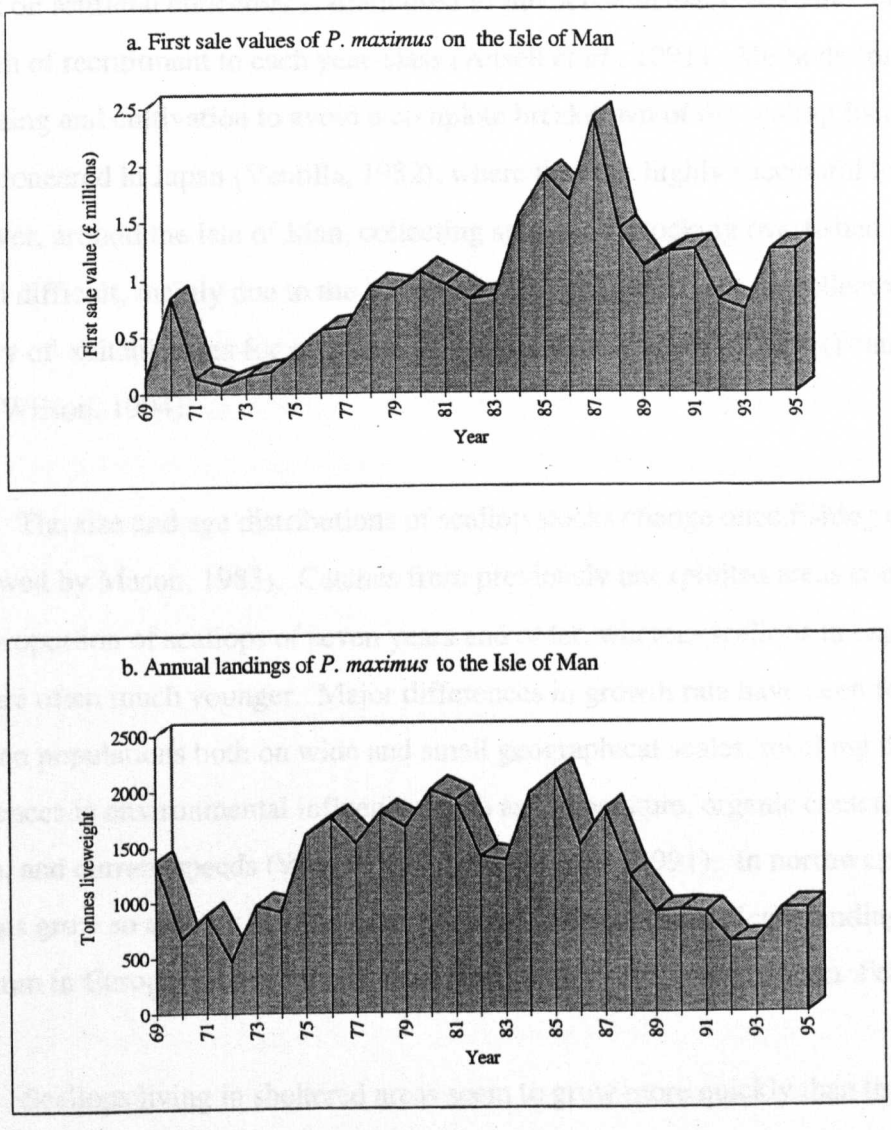


Figure 1.4. First sale values (a) and total annual landings (b) of *P. maximus* landed on the Isle of Man, 1969 - 1993. From the Isle of Man Department of Agriculture, Fisheries and Forestry.

Spat collectors of various designs have been employed for many years to investigate the seasonal and spatial pattern of settlement, and to provide spat for aquaculture and restocking purposes (Brand *et al.*, 1980). The strength of settlement of spat on artificial collectors is often used to predict at an early stage the future strength of recruitment to each year-class (Ansell *et al.*, 1991). Methods for restocking and cultivation to avoid a complete breakdown of the scallop fisheries have been pioneered in Japan (Ventilla, 1982), where they are highly successful today. However, around the Isle of Man, collecting spat and restocking overfished beds has proved difficult, mainly due to the low density of spat settlement on collectors and the scarcity of suitable sites for suspended culture around the Isle of Man (Brand *et al.*, 1980; Wilson, 1994).

The size and age distributions of scallop stocks change once fishing occurs (reviewed by Mason, 1983). Catches from previously unexploited areas contain a high proportion of scallops of seven years and older, whereas scallops in exploited beds are often much younger. Major differences in growth rate have been found between populations both on wide and small geographical scales, resulting from differences in environmental influences such as temperature, organic content of the seston, and current speeds (Wilson, 1987; Ansell *et al.*, 1991). In northwest Spain, scallops grow so quickly that they can already have reached the legal landing size (100 mm in Europe) before they have spawned (G. Roman, pers. comm. Feb. 1997).

Scallops living in sheltered areas seem to grow more quickly than those in exposed areas (Gibson, 1956), affecting the age at which they reach the minimum legal landing size, which can vary from 3 - 7 years on different grounds in the north Irish Sea (Brand and Prudden, 1997). Differences in growth rate among Isle of Man *P. maximus* populations is probably due to decreasing food availability and lower temperature with increasing depth (Mason, 1957, 1983). Scallops from shallow beds grow faster than those in deeper water; for example the inshore grounds Targets, Ramsey, Bradda Inshore, and Chickens, have high growth rates, whereas grounds further offshore have lower growth rates (Mason, 1983; Brand and Prudden, 1997).

As differences in growth rates did not persist following experimental transplantation, they are unlikely to be genetically determined (Dao *et al.*, 1985; Huelvan, 1985).

In contrast, variation in the breeding cycle of populations is probably due to genetic adaptation, and independent of environmental influences. A number of studies reported differences in the reproductive cycle of individuals originating from different locations (Paulet *et al.*, 1988; Cochard and Devauchelle, 1993; Connor, 1978; Lubet *et al.*, 1987; Taylor and Venn, 1979). Mackie and Ansell (1993) transplanted *P. maximus* from Scotland and from the Bay of Brest into the Bay of St. Brieuc. Whereas the storage cycle was influenced by environmental factors, the transplanted animals maintained their reproductive cycles according to their areas of origin, suggesting that these populations are genetically distinct and isolated, although allozyme studies carried out earlier did not suggest this (Beaumont *et al.*, 1993).

1.3. Introduction to population genetics

A population may be defined as a group of individuals of the same species living within a sufficiently restricted geographical area that any member can potentially mate with any other member (Hartl, 1987). Members of a species are never evenly distributed in space, as there is always some sort of aggregation, due to habitat discontinuity, or caused by social behaviour (herding, flocking etc.). Population genetics focuses on the breeding in such units, which ultimatively results in evolutionary adaptations. The observation of genetic subdivision between populations is an instantaneous view of the process of evolution.

Each individual within a sexually reproducing species possesses a unique genotype, and the degree of genetic differences between individuals defines the scale of genetic variation within a population. New variation enters the population by mutation and gene flow, the latter arising from migration of reproductively successful individuals.

The Hardy-Weinberg (HW) principle defines the relationship between allele frequencies and genotypic composition in a population under idealised conditions (diploid organisms with sexual reproduction, non-overlapping generations, random mating, infinite population size, no migration, mutation, or selection). The allele frequencies and therefore the genotypic composition of the population will be constant. The frequency of heterozygote of two alleles with frequencies p and q will be $2pq$, while the frequencies of the respective homozygote will be p^2 and q^2. If individuals within a finite population mate at random with each other, genetic composition and allele frequencies within the population change between generations by chance, a process known as random genetic drift. Genetic drift in the absence of gene flow may result in the loss of alleles from a population and the fixation of alternative alleles, causing a reduction in heterozygosity.

A subdivided population has three levels of complexity: individuals, subpopulations, and the total population. F-statistics are concerned with the extent to which the observed proportions of heterozygotes and homozygotes depart from the expected proportions under HW equilibrium (Wright, 1965; 1978). The fixation index F_{ST} measures the reduction in heterozygosity of a population due to differences in allele frequencies between subpopulations (Nei, 1977). In an ideal population with no mutation, migration, or selection, the value of F_{ST} can be interpreted as a direct measure for genetic drift. Natural populations, however, always undergo mutation, selection and migration, and F_{ST} has therefore to be interpreted with caution. Genetic drift has a greater influence in small populations; in the extreme case of only a few individuals from established populations founding a new population, or when a population encounters bottlenecks, genetic drift leads quickly to a reduction in heterozygosity. Where populations are isolated, with only negligible gene flow occurring, very different allele frequencies can develop amongst them, especially when the population size is small. The same happens in large populations, but it takes considerably longer. The evolutionary opposite is the fusion and inter-breeding of formerly isolated populations, which results in reduced homozygote frequency (Wahlund's principle). The reduction in the frequency of homozygotes due to

population fusion can be expressed in terms of the variance in allele frequency among the original populations.

With the first applications of allozymes to population genetics, the existence of genetic variation between and within many natural populations was revealed (Harris, 1966; Lewontin and Hubby, 1966). The Darwinian view that organisms become progressively adapted to their environment by accumulating beneficial mutants and that variation between populations is maintained by natural selection could not satisfactorily explain the the unexpectedly high levels of molecular variability. With the introduction of the neutral theory, Kimura (1968) brought another facet into the discussion. It was suggested that the main cause of evolutionary change at the molecular level is random fixation of selectively neutral or nearly neutral mutants rather than positive Darwinian selection (Kimura and Ohta, 1971; Kimura, 1983). The neutral theory is not antagonistic to the view that natural selection plays a role in determining the course of adaptive evolution, but it assumes that only a minute fraction of DNA changes in evolution are adaptive in nature, while the great majority of phenotypically silent molecular substitutions exert no significant influence on survival and reproduction and drift randomly within the species (Kimura, 1983). Mutations that are selectively neutral produce such small effects on the ability of their carriers to survive and reproduce that they are completely equivalent in terms of natural selection; the fate of neutral alleles is determined largely by the process of random genetic drift. There is, however, some evidence that natural selection acts on particular allozyme loci, potentially countering the influence of genetic drift; the relation of high multilocus heterozygosity and increased fitness often observed in molluscs is presumably due to allozyme polymorphisms underlying physiological differences (Karl and Avise, 1992). The fact that different mitochondrial genes exhibit different mutation rates may also indicate some degree of selection.

The long period of time for which many marine larvae can drift in the water column gives them an extensive dispersal capability, especially when originating from open-water populations. The gene flow arising from wide-scale larval dispersal would

be expected to suppress population differentiation. Indeed, population genetic studies have shown that extensive panmictic units exist in many aquatic species (Palumbi, 1992). However, there are also exceptions, where populations show genetic differentiation on a small geographical scale (Palumbi, 1995). Hydrographic features such as fronts, gyres and tidal circulation systems, and other, more subtle factors, like differences in light, food availability, and water temperature, can potentially restrict distribution and lead to differences in size, age, and genetic structure between grounds and beds. On the other hand, special behavioural adaptations can overcome, or even take advantage of these restrictions. There is some evidence that larvae actively migrate into particular water layers, which could aid retention over home beds; larvae of *Placopecten magellanicus* are known to undertake small amplitude vertical migrations, possibly in response to light (Kaartvedt *et al.*, 1987; Silva and O'Dor, 1988), or temperature (Tremblay and Sinclair, 1990). Furthermore, Cragg (1980) found that *P. maximus* larvae pass through three phases of swimming behaviour before metamorphosis: trochophores and early veligers swim upwards and tend to accumulate at the surface; after three days, larvae can alternatively swim up in a vertically orientated spiral, and sink; pediveligers can in addition swim close to the substratum or crawl on it. Increasing pressure causes an increasing swimming response in larvae older than ten days. According to Beaumont and Barnes (1992), *P. maximus* spat smaller than 500 μm have the ability to actively alter their sinking rate. The exact mechanisms used are uncertain; suggestions are that the secretion of byssus threads or extension of the foot could allow spat to vary their rate of descent. Recently, Manuel *et al.* (1996) showed that *P. magellanicus* veligers obtained from different hydrographic regimes exhibited significantly different vertical migration patterns and depth distribution, which could assist retention over, or return to, adult beds. This behavioural difference could be due to behavioural selection in local populations (Manuel *et al.*, 1996). Active swimming and vertical migration could potentially influence the direction of transport by water currents.

1.4. Pectinid population genetics

In fisheries, populations are usually referred to as 'stocks', although the relation between the two terms is far from clear. A stock has been defined as 'the largest group of animals that can be shown to be genetically connected through time' (Ovenden, 1990), or simply as animals occupying a physical region, or having the same meristic or morphometric attributes (Waldman *et al.*, 1988). Characterization of stock structure is an essential element in the management of fisheries. Production and harvest estimates are based on data of growth and mortality; only a harvestable surplus, replenished by annual recruitment, should be removed. Localised overfishing of a panmictic species might not seriously affect future fishery, since replenishment by recruitment from neighbouring areas can take place. However, in the case of self-sustaining populations, overfishing can lead to the extinction of the breeding stock and collapse of the fishery in that area. To ensure continued exploitation, the management of a scallop fishery therefore requires knowledge of the extent to which the stocks on particular fishing grounds and beds are reproductively self-sustaining or supplied by larvae originating from further away. This will be influenced by the scale and pattern of larval dispersal, which is very difficult to monitor directly, as larvae are too small to be tagged and it is laborious to determine the species of bivalve larvae correctly. Indirect techniques are therefore generally used to estimate widespread larval movement and the relationship between potential and realized dispersal. One important line of indirect evidence is the degree to which the natural stocks on different grounds and beds are genetically divergent, reflecting the balance between diversifying processes such as genetic drift, mutation, and local selection versus the homogenizing effect of gene flow (Slatkin, 1985).

With the aim of providing a sound genetic understanding of scallop stock differentiation for fishery management, previous workers have investigated allozyme variation (a protein solution, obtained by homogenising tissue from an individual, is electrophoresed through a starch gel, and an enzyme-specific reaction reveals a locus whose alleles may migrate differently due to differences in charge), and, more recently, DNA based molecular techniques.

Performing allozyme electrophoresis on *A. opercularis*, Mathers (1975) found that the glucose phosphate isomerase (GPI) locus differed between populations on the West and the East coasts of Ireland. The analysis of 115 individuals of *A. opercularis* exhibited five different phenotypes. Populations from Galway and Dublin differed significantly: Galway individuals showed four phenotypes dependent upon the three alleles *A, B* and *C*. Dublin populations consisted of three phenotypes dependent upon the alleles *B, C* and *D*. 34.5 % of the Galway individuals were BB homozygotes, but no BB occurred in Dublin. 53.3 % Dublin were *CC* homozygotes, in contrast to 9.0 % *CC* in Galway. Dublin was in general less variable than Galway. The study gives no information about year-classes. Genotype frequencies were found to be in Hardy-Weinberg equilibrium within samples and differences between populations were attributed to restricted gene flow. Beaumont (1982) screened individuals of *A. opercularis* stocks around the British Isles for differences at four loci (leucine amino peptidase (LAP), phosphoglucomutase (PGM), octopine dehydrogenase (ODH), structural proteine locus (PT-A). The LAP locus was monomorphic, and allele frequencies at the PGM and ODH loci did not show differences between populations. Variation at only the PT-A locus (three alleles) lead to the distinction of Scottish Coast, West Brittany and West Irish coast, English Channel and Irish Sea populations, which were considered as being self-sustaining, based on values for Nei's (1972) genetic identity (Figure 1.5.). There was no distinction between populations within the Irish Sea, which were therefore regarded as one panmictic population. In the Irish Sea population allele *3* dominated, the Channel population showed allele *3* and allele *1*, while allele *1* was also found in Brittany and West Ireland, and allele *2* was commonest in the North of Britain. All samples were in agreement with HW expectations, and again, restricted gene flow was concluded to be the cause of population differentiation. Beaumont and Beveridge (1984) found an allele which did not occur in populations around the Isle of Man in *A. opercularis* from Anglesey. Macleod *et al.* (1985), calculating the frequencies of two loci (phosphoglucose isomerase (GPI) and 6-phosphogluconate dehydrogenase (PGD) in five Manx populations (Laxey, Douglas, Port St. Mary, Chicken Rock, Peel) of

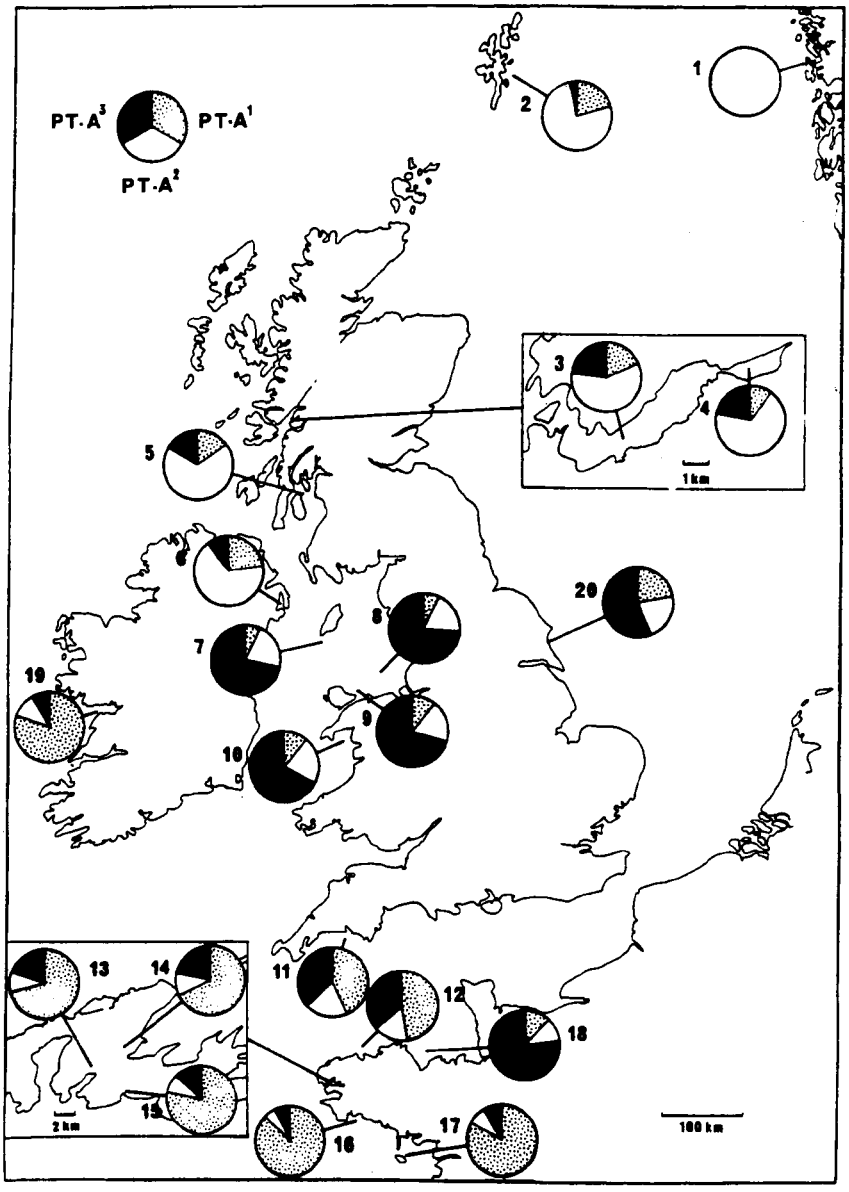


Figure 1.5. Distribution map of allele frequencies at the PT-A locus in *A. opercularis*. Sample sites are numbered as follows: 1, Bergen; 2, Shetlands; 3 and 4, Lower and Upper Loch Creran; 5, Clyde; 6, Strangford Lough; 7, Isle of Man; 8, Liverpool Bay; 9, Anglesey; 10, Cardigan Bay; 11, Plymouth; 12, Roscoff; 13, 14, and 15, Rade de Brest; 16, Concarneau; 17, Belle Ile; 18, St Brieuc; 19, Galway; 20, Bridlington. From Beaumont (1982).

A. opercularis, found uniformity of allele frequencies between all beds and year-classes, except for GPI allele *4*, which occurred in the 1979 year-class only. (Mathers (1975) found allele *4* at low frequencies in the western Ireland population.)

In contrast, Lewis (1992), investigating 12 sites around Britain, 7 of which were within the Irish Sea, found evidence based on 4 loci for two distinct populations of *A. opercularis* west and east of the Isle of Man (Figure 1.6.). He suggested consistent recruitment patterns within populations. Based on the same data, but excluding two sites (Kilmore Quay and Kyle of Lochalsh), Lewis and Thorpe (1994) found significant inter-site heterogeneity, which was consistent through year-classes of *A. opercularis*, indicating self-sustaining populations. This is the only study to date reporting differentiation between beds around the Isle of Man, with two loci showing heterogeneous allele frequencies between Manx samples, and is supported by discriminant function analysis.

C. islandica populations from off Jan Mayen, Spitsbergen, Bear Island and northern Norway were found to be at least partially geographically isolated, based on allozyme studies by Fevolden (1989, 1992).

Mitochondrial DNA RFLPs revealed differentiation between populations of *A. irradians* (Blake and Graves, 1995), which were confirmed by shell morphological differences (Wilbur and Gaffney, 1997). In contrast, the study by Blake and Graves (1995) indicated a common gene pool for populations of *A. gibbus.*

Not many genetic studies on population differentiation of *P. maximus* have been carried out to date. Huelvan (1985) analysed data from nine polymorphic loci of *P. maximus,* but found no significant genetic differentiation across samples from Scotland, Ireland and Brittany. Beaumont *et al.* (1993) compared the allele frequencies of eight polymorphic enzymes in 13 *P. maximus* populations from Scotland and Brittany, including data from Huelvan (1985), but could not reveal any differentiation at the population level. They found an overall deficiency of

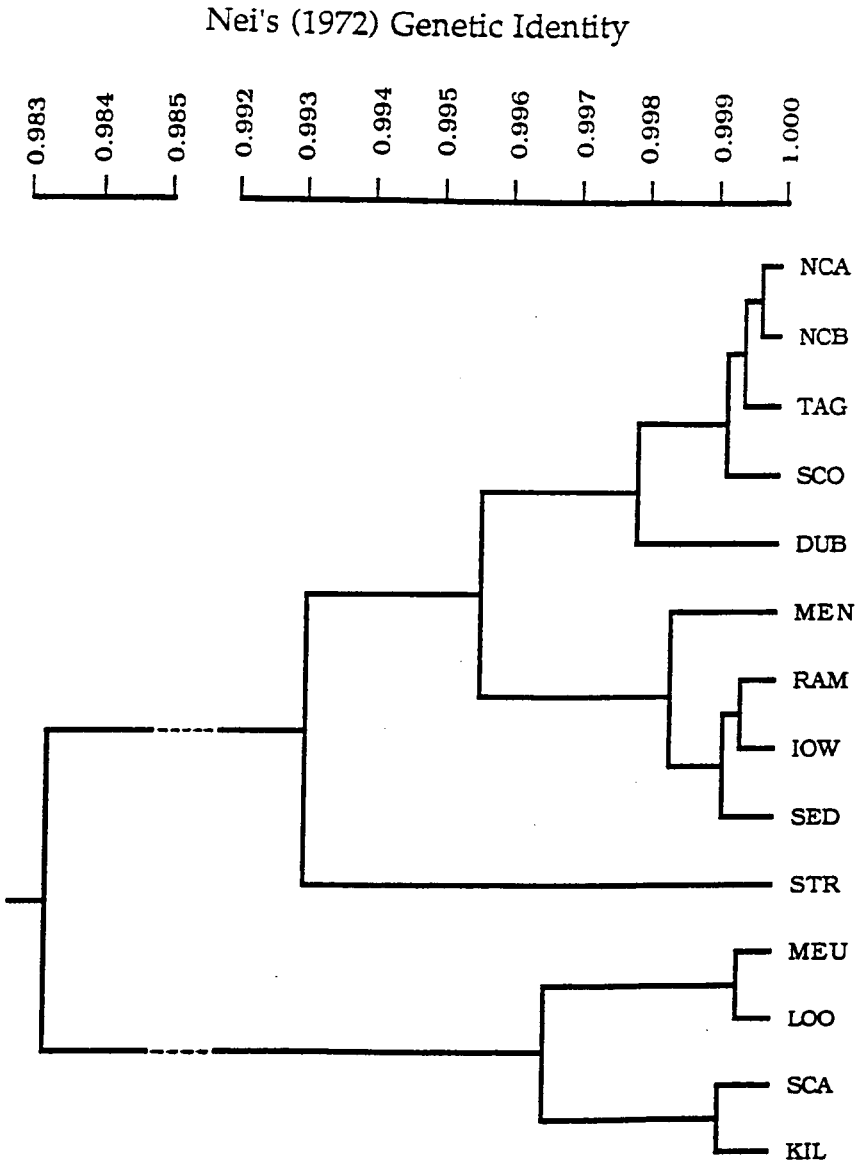


Figure 1.6. UPGMA cluster analysis from Nei's (1972) genetic identity statistic on *A. opercularis* populations. Abbreviations: NCA, NCB - North Calf; TAG - Targets; SCO - Kyle of Lochalsh; DUB - Dublin; MEN - Colwyn Bay; RAM - Ramsey Bay; IOW - off Douglas; SED - south east Douglas; STR - Strangford Lough; MEU - Meustone; LOO - Looe; SCA - Scarborough; KIL - Kilmore Quay. From Lewis (1992).

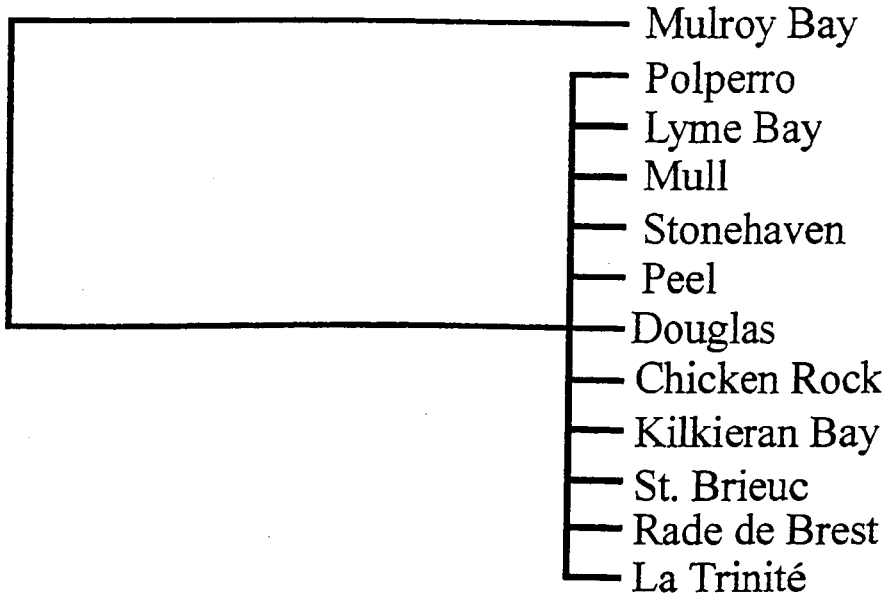


Figure 1.7. Consensus tree of UPGMA cluster dendrograms based on nucleotide divergence between populations of *P. maximus*. From Wilding *et al.* (in press).

heterozygotes. The populations in Bay of Brest and Bay of St. Brieuc did not show differences in allele frequencies.

P. maximus specimens derived from Mulroy Bay were found to be distinct from other British populations in PCR-based RFLP analyses of mtDNA (Wilding *et al.*, in press). In this study, no genetic differentiation between Irish Sea stocks was apparent (Figure 1.7.).

No significant variation in allele frequencies has been found in an allozyme study of populations from Hordaland and Trondelag (Norway). Comparison with data from Beaumont *et al.* (1993) for populations from the UK and France revealed genetic differentiation on a larger geographical scale. The authors (Igland and Naevadal, 1995) however pointed out that the two studies can not be compared directly, since there is no verification that the same alleles were revealed.

In summary, the few studies carried out on population structure of *P. maximus* have failed to reveal population differentiation on a small geographical scale. So far, this would imply that the populations of *P. maximus* within the Irish Sea represent a panmictic unit. In contrast, parallel studies of *A. opercularis, A. irradians* and *C. islandica* have revealed differentiation on relatively small geographical scales (Beaumont, 1982; Lewis and Thorpe, 1994; Fevolden, 1992; Blake and Graves, 1995). The failure to detect differentiation between *P. maximus* populations could be the consequence of the limitation of the allozyme methodology in which only a few loci were available for analysis. As a single locus revealed population structure in *A. opercularis* (Beaumont, 1982), it can not be ruled out that the inclusion of data from other loci would do the same for *P. maximus*. Transplantation experiments by Mackie and Ansell (1993) suggest genetic differentiation between *P. maximus* stocks, at least on a broad geographical scale. Furthermore, data on reproductive cycles indicate that populations may to some degree be self-sustaining (Paulet *et al.*, 1988; Cochard and Devauchelle, 1993); it might, therefore, be a matter of finding the right markers to reveal genetic differences.

1.5. Aims of this study

Analysing variation in DNA sequence is expected to provide resolution at a higher level than allozyme studies and is therefore more likely to detect potential genetic population structure. The aim of this study is to investigate the degree of genetic similarity between *P. maximus* populations from different fishing grounds around the Isle of Man. Samples from Mulroy Bay and Plymouth are analysed for comparison. Population differentiation is assessed by means of two DNA-based approaches:

1) randomly amplified DNA (RAPD), a method which screens the whole genome for variation in DNA sequence;

2) restriction analysis of PCR-amplified mitochondrial DNA fragments.

Comparison of two consecutive year-classes is used to try to elucidate underlying recruitment processes and to assess temporal stability.

Chapter 2 includes a brief overview of molecular techniques used in population genetics, describes the basic techniques used in this study and gives protocols for the general procedures applied. Chapter 3 concerns the RAPD experiments, and Chapter 4 the work on mitochondrial DNA. In order to develop primers for mitochondrial DNA, cloning of several mtDNA fragments was attempted, which is dealt with in Chapter 5. An outstanding feature of scallops is their unusally large mitochondrial genome due to the existence of 'repeated elements'; preliminary investigations of these are described in Chapter 6. Chapter 7 summarizes the results of both RAPD and mtDNA-RFLPs and discusses the contribution of this work to understanding of the genetic structure of scallop populations.

CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.1. General introduction to DNA-based techniques for population genetics

2.1.1. Advantages of DNA-based methods

From the 1960s allozyme electrophoresis was the dominant method for the analysis of genetic variation in natural populations, to be displaced only recently by methods investigating variation at the level of the DNA molecule. Although allozyme analysis still has the advantage of being a cheap and easy method, enabling the screening of large sample sizes in a short space of time, and supplying data as simple codominant Mendelian characters, the technique has only a limited resolution and accuracy. It does not allow a representative sampling of the whole genome, as its application is mainly restricted to genes which code for soluble enzymes of metabolic pathways, excluding most genes for structural enzymes and all non-coding sequences, therefore probably not representing a random sample of the genome. Most allozyme studies assume that polymorphisms have arisen as predicted by the neutrality theory, although there is some indication that natural selection acts on particular loci (Karl and Avise, 1992). Such balancing selection on allozyme polymorphisms could counter the influence of genetic drift. Also, much of the potential variability can be missed due to the degeneracy of the genetic code or if mutations result in substitutions of amino acids carrying the same charge (16 of the 20 amino acids are neutral in charge). It has been estimated that maximally only one third of all amino acid substitutions can potentially be detected by allozymes (see e.g. Thorpe, 1982; Hoelzel, 1992). Mutations resulting in non-functional protein products are generally not scored (null alleles). Further problems with this technique include the low resolution of gels and subjectivity when scoring gels.

The ability to directly examine nucleid acid variation provides the ultimate level of resolution, potentially enabling even the detection of single base substitutions

and mutations in non-coding sequences. DNA-level variation is therefore a less biased estimator of genetic variation than information based on gene products.

A number of DNA-based techniques have been developed in recent years for examining genetic variation in natural populations, e.g.: restriction fragment length polymorphisms of nuclear DNA (RFLPs), mtDNA haplotypes (RFLPs on the whole mitochondrial genome or on a PCR-amplified fragment, PCR-RFLP), multilocus DNA fingerprinting, random amplified polymorphic DNA (RAPD), microsatellites and macrosatellite variable number of tandem repeats (VNTR), and sequencing of specific regions. In particular, methods which screen the whole genome randomly provide more polymorphic markers and show higher variation. However, the higher cost and labour must be taken into account when using DNA-based methods instead of allozymes.

In the following sections, a brief overview of commonly used techniques for population genetic studies is given.

2.1.2. Sequencing

DNA sequence variation is ideally assayed by direct determination of the nucleotide sequences representing independent alleles. Although nucleotide sequence determination is the most direct and comprehensive method, it is also still the most expensive way of analysing genetic variation and has been mainly employed in phylogenetic and evolutionary studies. This might change in the near future, as cycle sequencing and automatic sequencing allows the screening of large sample sizes at relatively low cost.

2.1.2.1. Principle of sequencing with chain terminators (Sanger *et al.*, 1977)

A primer for the target sequence is annealed to single-stranded DNA. The mixture is divided into four subsamples, each containing the four deoxynucleotides (dNTPs) plus a single dideoxynucleotide (ddNTP, 'terminators', lacking the 3'-OH group and therefore unable to bind further deoxynucleotides). Each subsample

contains a different ddNTP at low concentration. The primer or one of the dNTPs are radioactive or otherwise labeled. Incubation with DNA polymerase at the appropriate temperature (e.g. 3 min at 70°C) leads to the extension of the primer-targeted sequence by attachment of nucleotides to a free 3'-OH. The reaction is stopped when a ddNTP is incorporated. Fragments of different length are electrophoretically separated in a polyacrylamide gel, whereby each subsample is run in a different lane (four lanes per reaction, ACGT). Because it is known which subsample contains which ddNTP, the DNA sequence can be read directly.

In automatic sequencing instruments, gel-electrophoresis, reading, and entering of data to computer are automated. Usually, terminators or sequencing primers are labelled with fluorescent dyes. Template preparation and sequencing reaction still have to be carried out manually.

2.1.2.2. Cycle sequencing (linear amplification, direct sequencing)

Conventional sequencing requires a laborious and expensive template preparation and amplification of target sequences by cloning into microbial vectors. Before PCR was invented each individual had to be cloned separately for population surveys. Cycle-sequencing couples PCR (see 2.2.1.) and sequencing, requiring cloning DNA of one individual only for the design of primers. PCR-amplification provides sufficient DNA to support a sequencing analysis. Once primers are developed, large numbers of individuals can be screened for sequence differences. In many cases, one of the PCR-amplification primers can be directly employed, or a third, internal primer is used. This method has opened the door for population genetic studies based on sequencing.

2.1.3. Restriction fragment length polymorphism (RFLP)

RFLPs can be analysed in nuclear, plastid or mitochondrial DNA. Nuclear DNA is isolated, cut with restriction enzymes (see 2.2.5.), size-fractionated on gels, southern blotted to a filter, hybridised with probes from the region of interest, and bands are visualised by autoradiography. Mitochondrial or plastid DNA does not

necessarily require southern transfer and hybridising. When only relatively few fragments are generated by restriction digests, they can be seen as distinct bands on gels. Ethidium bromide staining is sufficient when the amount of DNA is large enough. MtDNA restriction analysis is described in Chapter 4.

2.1.4. Randomly amplified polymorphic DNA (RAPD)

The RAPD technique (Williams *et al.*, 1990) involves PCR amplification of genomic DNA fragments, typically using a single decamer primer of arbitrary sequence to "screen" the whole genome, priming the sequence at both ends of each fragment, and producing a banding pattern of anonymous DNA fragments that may be highly polymorphic. Bands of characteristic length arise when complementary inverted priming sites flank a DNA sequence short enough (< 2.5 kb) to be amplified. RAPD primers of different sequence therefore yield different amplification products. The RAPD technique has been employed in this study and is explained in detail in Chapter 3.

2.1.5. Microsatellite markers

Microsatellites (or SSRs, simple sequence repeats; microsatellite variable number of tandem repeats, VNTRs) are stretches of DNA with tandem repeats of short sequence motifs (2-4 bp) that are interspersed in many eukaryotic genomes (Tautz, 1989). They often occur throughout an organism's genome, and potentially provide numerous, highly variable, independent loci which are inherited in a codominant (Mendelian) and neutral fashion and are easy to score, making them a powerful tool for population genetic studies.

2.1.5.1. Designing microsatellites

The first step is to cut nuclear DNA by digestion with a restriction enzyme. The restriction fragments are separated by gel electrophoresis, and fragments of a length suitable for sequencing (300 - 600 bp) are isolated from the gel. These fragments are ligated into plasmids which have been cut open with an appropriate restriction enzyme. *Escherichia coli* bacteria are transformed with the plasmids and

plated out to produce colonies. The desired repeat sequence, e.g. $(xx)_n$, $(xxx)_n$, or $(xxxx)_n$ is synthesized and labelled to serve as a probe (on a replica filter) for screening the colonies for this sequence. Clones which are positive for the repeat sequence are grown in liquid medium, plasmids are isolated and the insert is sequenced. PCR primers which enclose a microsatellite locus are designed. The PCR reaction is used to amplify the alleles of the microsatellite sequence in native DNA from any individual. After electrophoresis of the PCR-product through a sequencing gel, size and repeat number of the locus can be determined from the fingerprint-like banding pattern. Alternative alleles are expected to differ by multiples of repeat nucleotides.

In contrast to RAPDs, more stringent PCR reaction conditions make reproducibility problems less likely. However, one must first go through the laborious phase of finding specific primers, which makes development of this method expensive (3-4 times the relative cost of RAPDs: Jarne and Lagoda, 1996) and time consuming. Alternative approaches are microsatellite-primed PCR and randomly amplified microsatellites (RAMS).

Microsatellite-primed PCR uses unanchored oligonucleotide primers consisting of microsatellite-complementary sequences (Perring *et al.*, 1993; Meyer *et al.*, 1993). However, in a critical evaluation of the method, Weising *et al.* (1995) find that it resembles RAPDs closely in its susceptibility to slight changes in reaction conditions. Moreover, RAPD-like mismatch priming seems to occur frequently. The RAMS technique (Ender *et al.*, 1996) uses a RAPD-based strategy for finding microsatellite loci in anonymous genomes. It thereby avoids genomic library construction and screening and the need for larger amounts of DNA. Using RAPD-primers, parts of the genome are randomly amplified and separated through gel electrophoresis. A labelled repeat sequence is used to probe (Southern analysis) for microsatellite sequences amongst the RAPD-PCR products. Positive RAPD-fragments are gel-extracted, cloned and sequenced to design primers for sequences adjacent to the microsatellites.

2.1.5.2. Analysing microsatellite data

Observed values for mutation in microsatellite loci are two to three orders of magnitude higher than values reported for allozymes (Jarne and Lagoda, 1996). The cause for this high rate is thought to be polymerase slippage at DNA replication (Levinson and Gutman, 1987). Analysis of microsatellite data for population differentiation depends much on the application of the correct mutation model. Several models are currently distinguished (Estoup, 1995): the infinite allele model (IAM), in which each mutation creates a new allele; the two-phase/step model, in which most mutations change allele size by one repeat unit, but the rest change allele size by several repeat units; the K-allele model, in which k allelic states are possible; and the stepwise mutation model (one-step model, SSM), in which mutation adds or subtracts with equal probability single repeat units to/from the allele (for review see Jarne and Lagoda, 1996). It is important to identify the appropriate model for the study, since they make different predictions about the expected heterozygosity. These models assume no constraints on allele size, but Garza *et al.* (1995) found some evidence that there might be constraints. Slatkin (1995) states that the mutation process at microsatellite loci does not conform to the K-allele model with low mutation rates, because for many microsatellite loci rates may exceed 10^{-3} per generation. As the mutation rates are high and the mutational process does not erase information about the ancestral state, the assumptions made in using F_{ST} are not satisfied.

Although it has been assumed that microsatellite loci are selectively neutral, there is some recent indication that microsatellites located in exons are under selective pressure (Sutherlands and Richards, 1995; Watkins *et al.*, 1995). Mutation in priming sites may produce null alleles (Jarne and Jagoda, 1996) which may necessitate the redesigning of primers to enable the collection of a complete data set (Paetkau and Strobeck, 1995).

A comparison of microsatellites, PCR-RFLPs, RAPD and allozymes is given in Table 2.1.

Table 2.1. Comparison of different DNA profiling techniques and allozymes (after Jarne and Lagoda, 1996; Schierwater *et al.*, 1994).

	Microsatellites	PCR-RFLPs	RAPD	Allozymes
Isolation of new loci/probes	manageable, needs about several months time	laborious	not necessary	(screening of limited number of stainable enzymes)
DNA template in ng	~ 20	~ 20	~ 20	(relatively much tissue necessary)
Degraded template	possible	possible	not possible	(Degradation of proteins potentially problematic)
Accuracy of detection	high	high	medium	low
Reproducibility	very high	very high	medium	medium
Dominance, co-dominance	co-dominant	co-dominant (nuclear DNA)	dominant	co-dominant
Neutrality	yes	yes	yes	dubious
No. of variable loci analysed	5 - 20	10 - 100?	10 - 100	10 - 50
No. of alleles/locus	1 - 50	2	2	1 - 5
Relative cost per individual (no scale)	3 - 4	2 - 3	1	1
Non-radioactive detection possible	yes	yes	yes	yes
Mutation rate	0.1 % per generation	unknown	not predictable	much lower than microsatellites
Species specificity	work in related species	primers probably work in related species	for every new species the optimal primers have to be determined	work in related species

2.2. Commonly used basic methods in molecular biology

2.2.1. Polymerase chain reaction (PCR)

2.2.1.1. Principles of the PCR

Several of the molecular techniques mentioned above are based on the polymerase chain reaction (PCR), an *in vitro* method for enzymatic synthesis of specific DNA sequences. PCR was invented in 1983 by Kary B. Mullis as a method for amplification of human ß-globin DNA (Mullis *et al.*, 1986). The Klenow fragment of *E.coli* DNA polymerase originally used was found to lose its activity under the high temperature conditions necessary for denaturation (separation of complementary strands) of DNA. Thus it was replaced by thermostable DNA polymerase isolated from the thermophile bacterium *Thermus acquaticus* ("*Taq*"-polymerase). Stated briefly, PCR involves combining a DNA sample with two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA, plus deoxynucleotide triphosphates (dNTPs) and *Taq* DNA polymerase, in a suitable buffer. Repeated cycles of heating to 94°C (denaturing the DNA strands), cooling to 55°C (typical temperature for annealing of primers) and heating to 72°C (polymerisation = extension), the optimum temperature for the *Taq* polymerase, lead to an exponential amplification of the desired sequence, defined by the 5´ends of the primers (Ehrlich, 1991) (Figure 2.1.). Because the primer extension products of one cycle serve as a template in the following cycle, the number of copies doubles with every cycle (20 cycles yield a million-fold amplification). Therefore, once primer sequences are available, this method offers an alternative to the molecular cloning of DNA-fragments. PCR does not require the time-consuming preparation and purification procedures for extraction of plasmids or the high safety standards which are vital while working with bacteria (see Chapter 5). Since many sequences are conserved between species, it is possible to amplify regions with primers originally designed for another species, circumventing the need for prior sequencing. If sequence data for a species are available from a database, primers can also be designed based on this published sequence information.

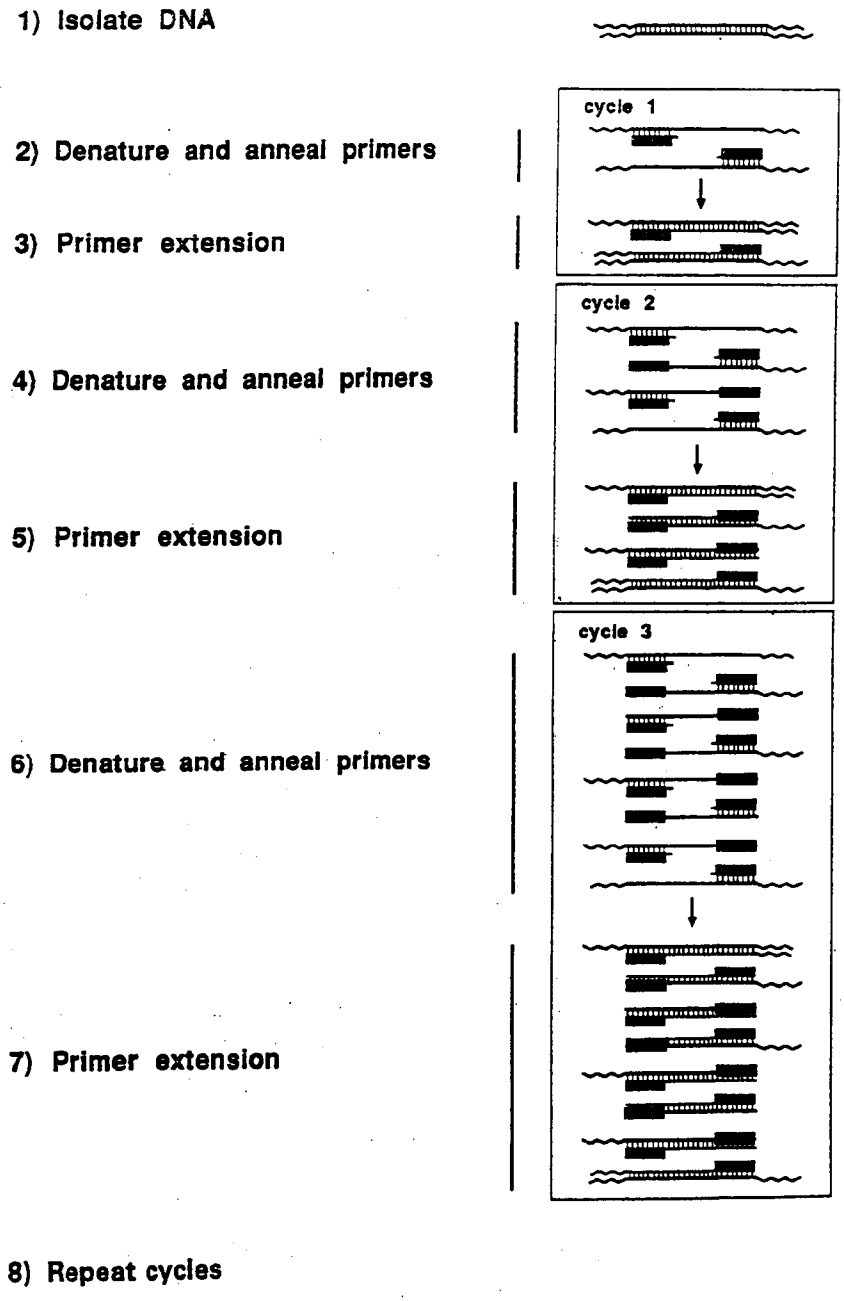


Figure 2.1. General protocol of the polymerase chain reaction for amplifying DNA (from Avise, 1994).

2.2.1.2. Designing PCR-primers

Designing primers involves certain fundamental considerations. As a rough guide, Hoelzel and Green (1992) proposed that primers should be 17 - 24 bases long, with 40-60 % GC content. Homology with the template should be 100 % at the 3' end of the primer (where polymerisation is initiated), whilst the 5' end can be completely non-homologous. Complementarity at the 3' ends of the two primers should be avoided to prevent the formation of primer-dimers, which compete for reagents. It is also beneficial to incorporate dinucleotides of C or G at the 3' ends. The annealing (melting) temperature can be calculated in °C by the following formula: (2 x A+T) + (4 x G+C) - 5 (Hoelzel and Green, 1992), or by roughly adding 2°C each for A's and T's, 4°C for G's and C's (Palumbi *et al.*, 1991), but it must be taken into account that the duplex stability also depends on the type of sequence (for example the duplex TT/AA is more stable than GA/TC (Hoelzel and Green, 1992)). Melting temperatures of the two primers should be within a few degrees of each other. Primer concentration should be non-limiting, normally around 25 pmoles of each primer per 50 µl reaction volume.

2.2.1.3. Long fragment PCR

The limiting factor for amplification of long fragments is the termination of the polymerisation due to misincorporation of nucleotides before the product is complete (Barnes, 1994b). This problem can be overcome by employing a polymerase with proofreading function. In addition, the use of relatively long primers permits high stringency annealing at high temperatures, and thus a reduced risk of false priming.

To date, few publications concern the amplification of fragments longer than 15 kb. Barnes (1994a) first described the use of a 16:1 mixture of a Taq-polymerase and a polymerase with 3'-exonuclease proofreading activity (*Pfu* or *Deep Vent*) for amplification of a 35 kb fragment from a λ bacteriophage template (for PCR conditions see Table 2.2.). Kainz *et al.* (1992) amplified a 15.6 kb fragment of bacteriophage λ template by means of a two-step PCR and *Tub* polymerase. Fragments of up to 42 kb of bacteriophage λ template and up to 22 kb from the β-

globin gene cluster from human genomes have been amplified by Cheng *et al.* (1994). Amplification of the entire insect mitochondrial genome in two reactions is described by Roehrdanz (1995). Nelson *et al.* (1996) describe long-PCR with primers for the 16S rRNA gene for the assay of full-length mitochondrial DNA. For a summary of long-distance PCR conditions see Foord and Rose (1994). The longer the amplified mtDNA fragment, the more potential sequence information can be expected from a subsequent restriction analysis.

2.2.2. Total DNA extractions

Only small quantities of DNA are necessary for PCR-amplification. In theory, a single molecule of template DNA is enough, although it is desirable to have a higher template concentration, because this makes the amplification of contaminating (non-target) DNA less likely. Moreover, if the PCR amplification is not particularly specific or efficient, or the target sequences are present infrequently among a large number of cells, the yield will be inadequate without enough starting DNA (Higuchi, 1992). Nevertheless, it is possible to perform PCR on DNA extracted from small, non-lethal samples including fish scales, feathers, blood, single hairs, or from small organisms such as invertebrate larvae, and from tissue containing only little undegraded DNA, like preserved specimens (Ehrlich, 1991).

Total DNA can be easily isolated by digesting the tissue with a proteinase to break down proteins, and SDS to disrupt cell membranes, then separating the polar (hydrophilic) molecules (e.g. nucleic acids) from apolar (hydrophobic) molecules by extraction with phenol-chloroform; during centrifugation, the nucleic acids remain in the aqueous phase, apolar components drift in the phenol phase and amphipolar molecules aggregate at the interface between aqueous and phenol phases. Extraction of the aqueous phase with chloroform ensures the complete removal from the sample of the phenol, which would otherwise destroy enzymes involved in subsequent reactions like PCR or restriction digests. DNA can be precipated from the aqueous phase by adding ethanol, which competes with the DNA for the water molecules in the solution. To remove excess salts, which have been precipitated with the DNA, the

Table 2.2. Suggested conditions for long PCR as compared to classical PCR (after Barnes, 1994b).

Long PCR	Classical PCR
combination of two DNA polymerases, one with proofreading function	only one type of DNA polymerase, no proofreading function
Robocycler	P.E. original or 480 cycler
pH 9.2	pH 8.3
16 mM $(NH_4)_2SO_4$, no KCl	50 mM KCl
3.5 mM $MgCl_2$	1 - 2 mM $MgCl_2$
5 s 95 °C melt	60 s 95 °C melt
2 ng lambda DNA	20 ng lambda DNA
33 µl reaction volume	100 µl reaction volume
20 cycles	30 cycles
68 °C extension temperature	72 °C extension temperature
33-nucleotide primers	20 - 22 nucleotide primers
11 - 24 min extension	3 - 10 min extension
hot start	cold start

pellet is washed with 70 % ethanol. Because ethanol can inhibit enzymatic reactions like PCR or restriction digests, the pellet is dried and re-dissolved in water or TE.

Extraction of mitochondrial DNA first requires the isolation of intact mitochondria from cell homogenates by differential centrifugation. After the mitochondria have been lysed, mitochondrial DNA can be obtained in a way similar to total DNA extraction, by phenol-chloroform extraction and ethanol precipitation (see Chapter Four).

2.2.4. Gel electrophoresis

DNA samples (total DNA extractions, PCR-products, restriction fragments etc.) can be separated by means of agarose gel electrophoresis. Agarose is a linear polymer of D-galactose and 3,6-anhydro-L-galactose (Hoek, 1978), and is extracted from red algae (Rhodophyta, genera *Gelidium, Pterocaldia, Gracilaria*). The size of the pores in an agarose gel, and therefore its resolution, is determined by its concentration.

When subjected to an electric field, DNA migrates through an agarose gel from the negative to the positive electrode (cathode to anode) mainly due to the negative charge of the phosphate groups (the charges of the bases are negligible due to their pK-values). The electric mobility of a DNA fragment is inversely proportional to the logarithm of the number of base pairs (up to a certain limit: Stryer, 1988). A buffer solution provides a constant pH value, which ensures the dissociation of the charge-carrying groups and therefore constant charges on the molecules.

The resolved fragments can be visualised by ethidium bromide staining of the gel. Ethidium bromide intercalates between the base pairs of DNA. This complex absorbs UV-light (254 nm) and re-emits visible light to reveal amplified fragments as fluorescent orange bands. A band containing only 50 ng of DNA can readily be seen.

2.2.5. Restriction digests

A restriction endonuclease is an enzyme that cleaves DNA wherever a particular sequence of bases occurs. Each type of restriction enzyme recognises a different sequence (Table 2.3.). The enzymes are found in many species of bacteria, where they function in the restriction and modification system, which enables the bacterium to distinguish between its own and foreign DNA and to destroy the latter. The bacterium's own DNA is protected against the action of restriction endonucleases by methylation: the enzyme methyltransferase transfers methyl groups from s-adenosylmethionine to specific bases within the recognition sequence (Stryer, 1988). Restriction endonucleases can only cut at unmethylated sites. They are typically named after the organism from which they have been isolated. Three types are to be distinguished:

Type I endonucleases have both methyltransferase and endonuclease activity and require ATP and $MgCl_2$ as cofactors. They recognize a sequence of typically 15 bp and either methylate it or cut the DNA at a distance of ca. 1000 bp from the recognition site.

Type II endonucleases recognize a specific, palindromic sequence (typically 4 - 8 base pairs long) and cut within this sequence, whereby they produce defined blunt (square) or sticky (unequal) ends in double-stranded DNA. As short restriction sites are more common than longer ones, enzymes with short recognition sites digest or cut the DNA more frequently than enzymes with longer recognition sites. Four-base cutter enzymes cut on average every $4^4 = 256$ base pairs, six-base cutter enzymes every $4^6 = 4096$ base pairs (Tegelstrom, 1992). Thus, four-base cutters sample the genome more effectively and are more likely to detect differences between the genomes under analysis. The disadvantage of four-base cutters is their production of small fragments, which may be difficult to detect on gels. Also, if diversity between populations is high, every individual may be found to have a different haplotype.

Type III endonucleases also recognize a specific sequence; they cut in the direct vicinity, but the two ends are not compatible (Winnacker, 1985).

Molecular biology mainly uses type II endonucleases. When DNA is cut by a restriction enzyme in a 'digest', which consists of the DNA, a buffer (see Table 2.4.), H_2O and the restriction enzyme, the pattern of the fragments obtained can differ between individuals or species due to mutational base changes within the recognition site of the restriction enzyme or length polymorphism between restriction sites. Restriction analysis can be carried out on either nuclear or mitochondrial DNA. RFLPs on nuclear DNA need to be visualized by hybridisation with labelled probes, due to a high background of other DNA fragments, whereas mtDNA RFLPs can be detected by ethidium bromide staining. By using a number of different restriction enzymes, a picture can be built of the amount by which the mtDNA of each sampled animal differs from every other. Each type of mtDNA discovered amongst the samples is called a mitochondrial haplotype. Double digests of circular DNA molecules of known size, like the mitochondrial or plastid genome or plasmids, with two different restriction enzymes, can determine the relative location of restriction sites. It is then possible to construct a map of the circular genome showing the relative positions and number of the restriction sites for each individual.

Table 2.3. Features of restriction endonucleases employed in this study (Name, bacterium, recognition site, and buffer type (from Boehringer-Mannheim product information sheets)).

Restriction endonuclease	**isolated from**	**recognition site**	**buffer**
Acc I	*Acinetobacter calcoaceticus*	GT'(A or C)(T or G)AC	A
Alu I	*Arthtrobacter luteus*	AG'CT	A
Asn I	*Arthrobacter spezies NCM*	AT'TAAT	B
Ava I	*Anabaena variabilis*	C'PyCGPuG	B
Bam HI	*Bacillus amyloliquefaciens*	G'GATCC	B
Cfo I	*Clostridium formicoaceticum*	GCG'C	L
Cla I	*Caryophanon latum L*	AT'CGAT	H
Dra I	*Deinococcus radiophilus*	TTT'AAA	M
Eco RI	*Escherichia coli BS 5*	G'AATTC	H
Eco RV	*Escherichia coli J62 pLG74*	GAT'ATC	B
Hae III	*Haemophilus aegyptius*	GG'CC	M
Hind III	*Haemophilius influenzae Rd com10*	A'AGCTT	B
Hinf I	*Haemophilius influenzae*	G'ANTC	H
Hpa I	*Haemophilus parainfluenzae*	GTT'AAC	A
Msp I	*Moraxella* species	C'CGG	L
Mva I	*Micrococcus varians RFL 19*	CC'(A or T)GG	H
Pvu II	*Proteus vulgaris*	CAG'CTG	M
Rsa I	*Rhodopseudomonas sphaeroides*	GT'AC	L
Sma I	*Serratia marcescens SB*	CCC'GGG	A
Taq I	*Thermus aquaticus YTI*	T'CGA	B
Tru 9 I	*Thermus ruber 9*	T'TAA	M

All listed enzymes were supplied by Boehringer-Mannheim, UK. Taq I and Tru 9 I have an optimum cleavage temperature of 65°C, all others cleave best at 37°C.

Table 2.4. Buffer composition and final concentration (mM, 1:10 diluted buffer); DTE= Dithioerythriol; DTT=Dithiothreitol. From Boehringer-Mannheim 1996 Biochemicals Catalogue.

Buffer components	**A**	**B**	**L**	**M**	**H**
Tris-acetate	33	-	-	-	-
Tris-HCl	-	10	10	10	50
Mg-acetate	10	-	-	-	-
$MgCl_2$	-	5	10	10	10
K-acetate	66	-	-	-	-
NaCl	-	100	-	50	100
DTE	-	-	1	1	1
DTT	0.5	-	-	-	-
2-Mercaptoethanol	-	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

2.3. Protocols for general molecular techniques applied in this study

2.3.1. Total DNA extractions from different types of *Pecten* tissue

For both methods (RAPD and PCR-RFLP) applied in this study, it was necessary to first extract total DNA. This was carried out according to a protocol based on Okamura *et al.* (1993). A piece approximately 2 x 2 mm in size was cut from the centre of the adductor muscle and transferred into a 1.5 ml Eppendorf tube containing 500 µl TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8), 50 µl Tris-HCl (1 M, pH 8), and 25 µl 25 % sodium dodecyl sulphate (SDS, BDH). 25 µl proteinase K (20 mg/ml, Biometra, Maidstone, UK) was added and the samples were digested at 55°C for 3 hours. After cooling the tubes for a few minutes at room temperature, 700 µl phenol-chloroform (Biometra) was added to each tube, and the tubes were rotated on a mixing machine for 10 minutes, then centrifuged at maximum speed (13000 *g*) in a table-top microcentrifuge (Heraeus Biofuge 13) for a

further 10 minutes. The top (aqueous) layer, containing the DNA, was taken off using a wide-orifice pipette tip and placed in a new Eppendorf tube. 600 µl chloroform-isoamyl alcohol (24:1, Biometra) was added, the tubes were again rotated for 10 minutes and centrifuged for 10 minutes. The supernatant was placed in a new tube and 1000 µl (approx. 2.5 volumes) absolute ethanol (Biometra) was added and mixed with the supernatant by inverting gently several times. 6 µl of 3 M sodium acetate (Sigma) was added, and the tubes were left in a freezer at -70°C for 1 hour. After pelleting by centrifugation at maximum speed for 5 minutes, the sample was washed twice with 200 µl 70 % ethanol, dried for 15 - 30 minutes at 37 °C in an incubator and re-dissolved in 50 µl sterile, UV-treated water. For long-term storage, samples were kept at -70°C, for medium-term storage at -20°C and samples which were in daily use were stored at +4°C, to avoid break-down of DNA through excessive freeze-thaw cycles.

To test the yield of total DNA from different types of *P. maximus* tissue, total (nuclear and mitochondrial) DNA was extracted from adductor muscle, gill, mantle and female and male gonad. Different types of tissue were stored under different conditions: frozen at -20°C, -70°C, and -196°C (liquid N_2). The quality and quantity of DNA was compared to that obtained from fresh tissue by running samples on an agarose gel with added ethidium bromide. Fresh tissue always gave a higher yield than tissue frozen at either -20°C, -70°C or -196°C. No noticeable difference in yield existed between samples of the same type of tissue frozen at different temperatures. Best results were obtained using fresh tissue from the adductor muscle and the male gonad. Extractions using fresh gill tissue had high mucus content, which made accurate pipetting impossible, although the DNA quality was satisfactory. Tissue from mantle and female gonad contained a large amount of protein. Gonad tissue had the disadvantage of not being available in consistent quality throughout the year. Therefore, for all subsequent experiments, total DNA was extracted from fresh adductor muscle. The DNA concentration was estimated by comparison with a standard on an agarose gel.

2.3.2. Protocol for agarose gels

Agarose gels made of multi-purpose agarose (Boehringer-Mannheim) and 1x Tris-borate-EDTA-buffer (TBE) (diluted from 5 x concentrate: 0.445 M Tris borate, pH approx. 8.3, 0.01 M EDTA, Sigma) were used for all experiments. Two differently-sized gel bath systems were available, for "mini-gels" of 6 x 10 cm size, and "long-gels", 20 x 28 cm. For mini-gels, which were usually employed to test for successful PCR, a 0.7 % gel was cast (0.21 g agarose, 30 ml 1x TBE, 3 µl ethidium bromide 10 mg/ml, BDH, Poole), whereas for long-gels 4-5.6 g agarose (1-1.4 %), 400 ml 1x TBE and 30 µl ethidium bromide were used. Electrophoresis was undertaken in 1x TBE. The buffer could be used for up to 10 gel-runs, before the bands on the gel became diffuse and started "smiling". Gels were made by boiling agarose and buffer in a microwave, adding ethidium bromide, and cooling to approximately 60°C. The mixture was then poured into a gel casting tray with combs positioned to create sample wells. After 30 minutes, the combs could be removed and the gel was submerged in buffer. Each DNA sample was mixed with 4 µl of a gel loading buffer (a mixture of ficoll, TBE and bromophenol blue (BPB), see Sambrook *et al.*, 1989) to make the sample sink into its well and to visualise the progress of subsequent electrophoresis (BPB migrates at approximately the same rate as a DNA fragment of 500 bp). Samples were loaded into wells using disposable pipette tips. At least one size standard (1kB-ladder, GibcoBRL; low-range-marker, Amresco; 100 bp-ladder, Promega) was run on every gel. Electrophoresis took place either at 80 V for up to 2 hours (mini-gels), or at 60 V for 12-15 hours (long-gels). Long-gels were destained for 4-6 hours in distilled water and photographed on a UV-transilluminator using a polaroid camera (DS34, Polaroid, Herts, UK) and monochrome negative film type 665 (Polaroid).

2.3.3. Detoxification of ethidium bromide solutions

Ethidium bromide is a powerful mutagen and suspected carcinogen. After use, solutions containing this dye were decontaminated according to the method of Lunn and Sansone (1987) which reduces the mutagenic activity in the *Salmonella* / microsome assay by approximately 200-fold (Sambrook *et al.*, 1989): the

concentration of ethidium bromide in solution was reduced to < 0.5 mg/ml by adding water. 0.2 volume of fresh 5% hypophosphorous acid (BDH/Merck, Poole, Dorset, UK) and 0.12 volume of fresh 0.5 M sodium nitrite (BDH/Merck) was added. After 24 hours, a large excess of 1 M sodium bicarbonate (BDH/Merck) was added and the solution was discarded.

Gels containing ethidium bromide and disposable labware which had been in contact with ethidium bromide were incinerated at high temperature at Noble's Hospital, Isle of Man.

2.4. Biological material

2.4.1. Research vessel survey of scallop fishing grounds around the Isle of Man

A research vessel survey of the main scallop fishing grounds around the Isle of Man has taken place twice a year since 1990, before and after the scallop fishing season (1 November - 31 May). The aim of this survey is to sample the commercial grounds to estimate scallop densities and catch per unit effort (CPUE) for comparison between fishing seasons and years and between grounds. Ageing and measurement of scallops (both great scallop, *Pecten maximus,* and queen scallop, *Aequipecten opercularis)* enables size-frequency distributions to be plotted. In addition, scallops and by-catch species are assessed for damage due to dredging.

For this purpose, the RV *Roagan* (23.4 m, 600 HP) is equipped with two "gangs" of four scallop (port side) and queenie (starboard side) spring tooth bar dredges of 0.76 m dredge width. Scallop dredges have nine teeth on the tooth bar with a width of about 21 mm and an average length of 110 mm, while queenie dredges have ten teeth with a length of 76 mm. The belly of the dredge is made of steel rings of an internal diameter of 70 mm in the scallop dredge and 57 mm in the queenie dredge. The dredges are towed four times on each ground for approximately one hour at an average speed of about two knots, to cover a total dredged distance of two nautical miles per tow (3704 m).

2.4.2. Collection sites

Pecten maximus were obtained by dredging in the course of research vessel surveys during October 1994, 1995 and 1996 from the Chickens Rock, Bradda Inshore, Peel, Targets, Ramsey and East Douglas beds (Figure 2.2., Table 2.5.), areas around the Isle of Man which are all fished regularly by commercial vessels. *P. maximus* from Bigbury Bay, Plymouth, SW. England, were dredged by the RV *Squilla* in summer 1996. Animals originating as spat from Mulroy Bay (Co. Donegal, Eire) were obtained in 1996 after ongrowing at a scallop farm in Strangford Lough (N. Ireland).

Table 2.5. (approximate) location of the Isle of Man sampling sites

Sampling site	North	West
Bradda Inshore	54° 06.24'	04° 48.20'
Chickens	53° 58.73'	04° 52.79'
East Douglas	54° 07.45'	04° 12.88'
Ramsey	54° 20.50'	04° 18.05'
Targets	54° 19.64'	04° 40.74'
Peel	54° 12.19'	04° 48.36'

2.4.3. Maintenance of animals

Only animals which were three years old in 1995 and 1996 were transported back from the RV *Roagan* to the laboratory in seawater containers. Spawning and settlement of these animals took place in 1992 and 1993. Scallops from Plymouth were three and four years old (the 1993 and 1992 year-class). All animals were kept in outside tanks with aerated, flowing seawater until DNA extraction. Scallops from Mulroy Bay were two years old (1994 year-class). They were transported in a coolbox back to the laboratory and processed immediately.

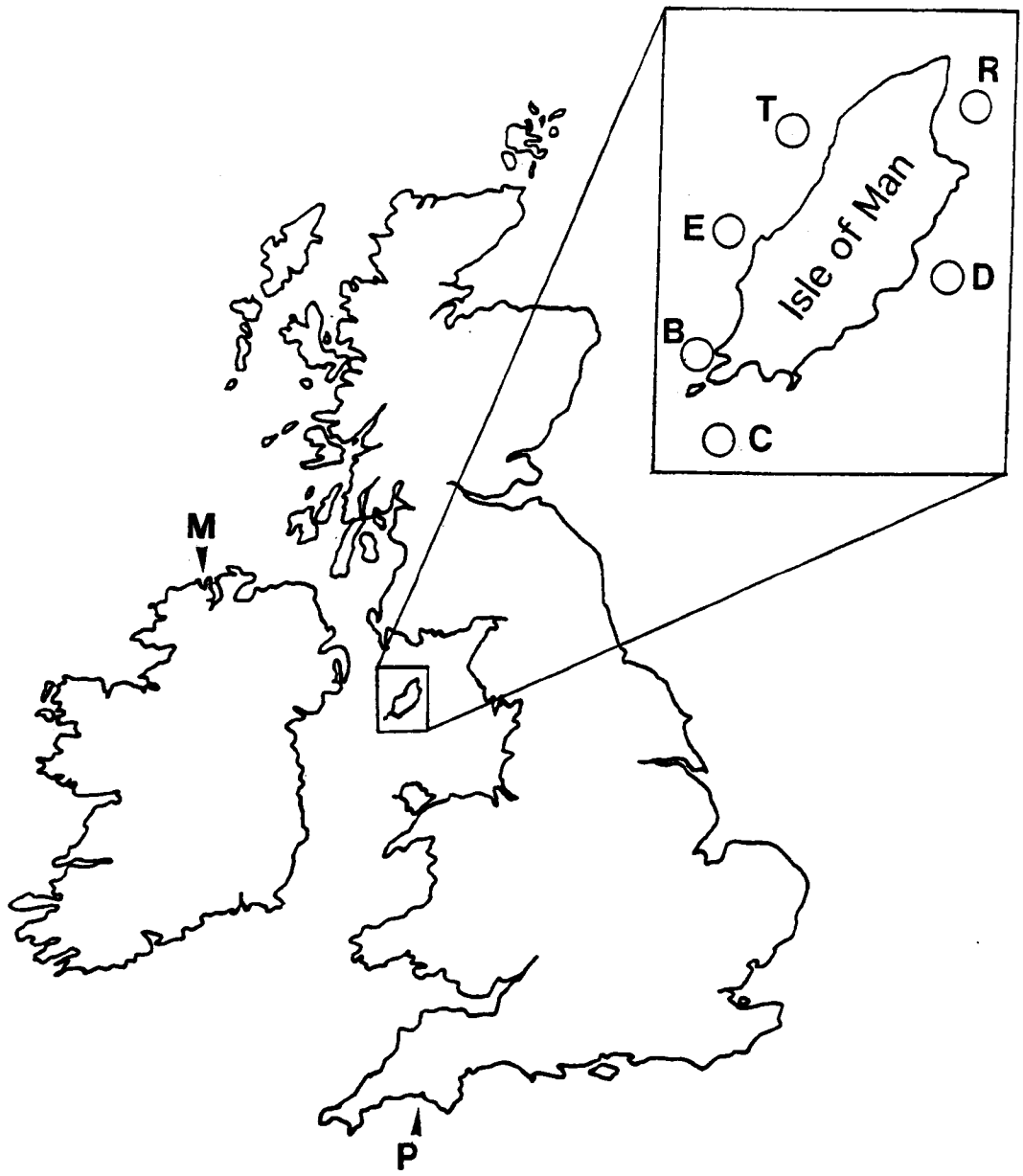


Figure 2.2. *P. maximus* sample sites around the Isle of Man, Mulroy Bay (M) and Plymouth (P). B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel.

CHAPTER THREE: RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)

3.1. Introduction

Since its introduction in 1990 by Welsh and McClelland, and Williams *et al.*, the RAPD technique has been extensively applied in studies on identification and differentiation of closely related species and conspecific populations. The strong point of this method in comparison to other molecular techniques is that the whole genome is 'screened' for mutations, resulting in a high number of polymorphic loci, while relatively little effort in terms of cost and labour is required. In contrast to normal PCR, the RAPD-PCR technique employs just one short primer (around 10 bases) of arbitrary sequence, and no sequence information of the species studied is necessary. RAPD-PCR uses low annealing temperatures (typically 36°C) during thermal cycling, which decreases the specificity of primers, and a large number of cycles. Due to the small size of the primer there is a high probability that the genome will contain multiple priming sites. When by chance the primer anneals at sites on opposite strands separated by a suitable distance for complete transcription (< 2.5 kb), fragments of this length will be amplified (Figure 3.1) (for expected total number of RAPD bands see Clark and Lanigan (1993)). Polymorphisms arise through failure to prime because of nucleotide sequence differences at a priming site (transitions, transversions), through sequence rearrangements that destroy complementarity of the pair of sites, or through insertions or deletions in the sequence between two conserved priming sites, altering fragment size and thus band position on the electrophoresis gel. Carried out with extracts of total DNA, RAPD-primers amplify parts of the nuclear genome as well as of the mitochondrial genome. The number of primers is almost limitless and primers of arbitrary sequence can be acquired commercially.

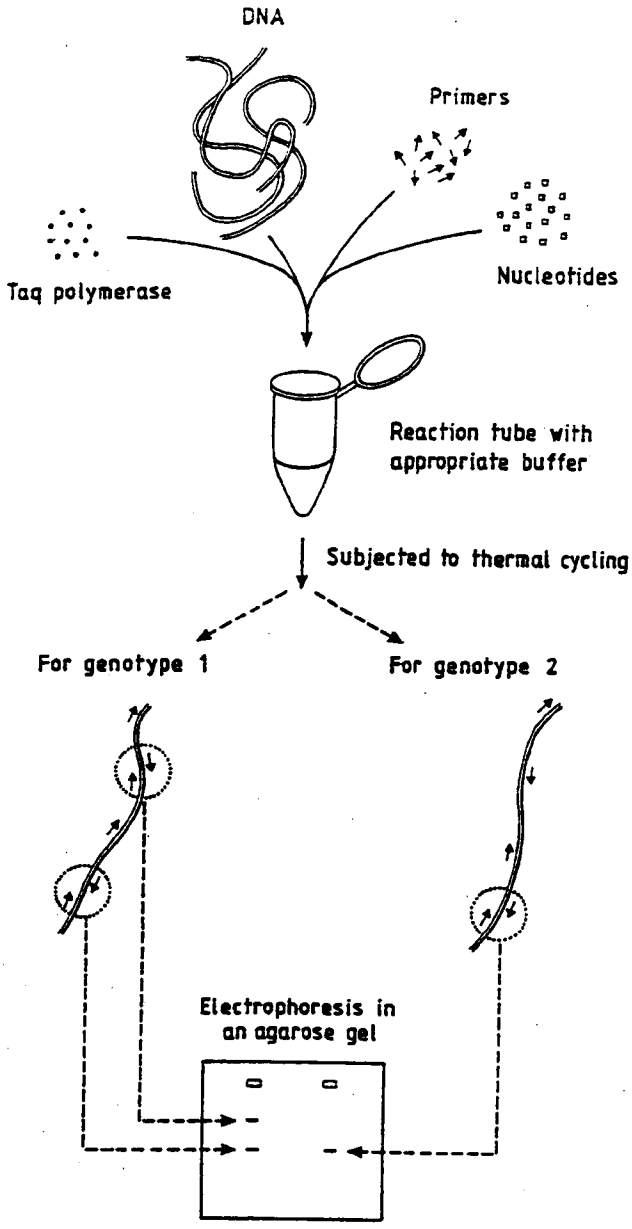


Figure 3.1. Principle of RAPD-PCR. A single type of primer is employed. In genotype 1, two regions are shown within the schematic genome at which primers bind close enough to allow amplification of the intervening sequence, and the two amplification products can be visualised as two bands in a gel. The amplification products migrate at different rates during electrophoresis if they are of differing sizes. For genotype 2, one primer binding site is missing, and this results in the production of only one amplified sequence (from Newbury and Ford-Lloyd, 1993).

A major advantage of RAPD is the high number of polymorphic markers generated, much higher than for instance with allozymes. Several studies using RAPD have revealed differences between populations where allozymes did not (reviewed by Peakall *et al.*, 1995). This is due to RAPD carrying out a more extensive sampling of the whole genome, including non-coding sequences. In addition, *all* coding sequences are equally likely to be screened, whereas allozymes are restricted to genes coding mostly for soluble enzymes of metabolic pathways. Silent mutations can also potentially be detected.

Problems with reliability and reproducibility characterise every PCR-based method, including RAPD. The low stringency PCR conditions for RAPD (short primers and low annealing temperature, possibly allowing imperfect primer-template annealing) increase the chance of amplification artifacts. This can be controlled by careful optimisation and standardisation of the protocol and the use of good quality, undegraded DNA to improve repeatability of results. Unreliable primers, which give inconsistent results, should not be used. Conservative choice of bands for scoring is also important. As bands can vary in intensity, only bands which are of consistent brightness over a range of samples should be selected for analysis. Amplification may only be reliable within a limited size-range of fragments, typically 0.3-1.5 kb.

Although it has been shown that RAPD loci are mainly inherited as Mendelian markers (Williams *et al.*, 1990; Welsh *et al.*, 1991), there are also studies indicating that nonparental, new marker bands can appear in offspring (Carlson *et al.*, 1991; Hunt and Page, 1992; Riedy *et al.*, 1992; Levitan and Grosberg, 1993). Bands of different size are normally assumed to be independent loci, but could in theory be alleles of the same locus. Hybridisation trials have indeed proved that different bands amplified by a single primer can occasionally contain homologous sequences (Smith *et al.*, 1994). In a similar way, co-migrating bands are considered homologous (Hadrys *et al.*, 1992) although exceptions have been shown to occur (Smith *et al.*, 1994). Little research has been carried out to date to confirm one or the other statement or to evaluate the extent to which RAPD markers are mostly dominant, and without

breeding studies it is usually not known whether an individual is homozygous or heterozygous for a certain band. Regarding band presence and absence as alternative allelic states at a locus (dominant and recessive, respectively), and assuming Hardy-Weinberg equilibrium, allele frequencies could be estimated from the frequency of null homozygotes (individuals lacking the band). Standard population genetic analyses, such as the calculation of F_{ST} values, could then be undertaken. However, most workers do not attempt explicitly genetic analyses of RAPD data, as the necessary conditions cannot be met (reviewed by Grosberg *et al.*,1996). Instead, banding patterns are analysed as phenotypic data.

RAPD has revealed differentiation between populations of various species (e.g. Burrows *et al.*, 1996; Carlier *et al.*, 1996; Kimberling *et al.*, 1996; Stewart and Excoffier, 1996; Nusser *et al.*, 1996; Högberg *et al.*, 1995; Peakall *et al.*, 1995; Haig *et al.*, 1994; Huff *et al.*, 1993; Russell *et al.*, 1993). The method has also been applied to the pectinid *Placopecten magellanicus* by Patwary *et al.* (1994). 222 primers were screened on one individual of *P. magellanicus*. 140 primers (59 %) yielded amplification products. 40 primers were tried on 24 scallops from 5 different beds (4-5 per bed) and 15 primers produced polymorphic bands. Analysis of the banding pattern of a single mating revealed that most, but not all bands segregated in Mendelian fashion. No bands unique to populations were found, but the frequencies of bands between populations varied. The authors concluded that RAPD can provide a useful tool for analysing the population structure of scallops.

In this Chapter, the RAPD technique is used to assess patterns of genetic diversity within and between populations of *P. maximus,* thus giving data based on a much more extensive sampling of the genome than has been possible with allozyme markers. The extent of stock differentiation between *P. maximus* samples from five commercially fished beds around the Isle of Man is investigated in two successive year-classes and comparisons made with samples from two locations in western Britain outside the Irish Sea (Mulroy Bay, Plymouth).

3.2. Material and Methods

3.2.1. Choice of primers

In a preliminary experiment, 60 primers (Operon kits F, R and Y) were tested for their banding pattern on individuals from different locations around the Isle of Man (in duplicate samples) to select primers which gave easy scorable, reproducible bands. Subsequently, two year-classes of *Pecten maximus*, each consisting of 18 individuals per location from five locations around the Isle of Man (Chicken Rock, Bradda Inshore, Targets, Ramsey, East Douglas; 90 individuals) and two outgroups (Mulroy Bay and Plymouth; 36 individuals) were subjected to RAPD analysis with 13 primers (Operon F01, F02, F04, F06, F10, R01, R03, R04, R06, R12, Y11, Y14, Y18, see Table 3.1.), from which 51 polymorphic bands were scored. In addition, two individuals of *Aequipecten opercularis* were analysed with several primers to investigate the ability of the RAPD method to distinguish between the species.

Sampling of animals and total DNA extraction is described in Chapter Two. To avoid possible location-specific biases during PCR, individual DNA extracts from all populations were allocated at random to batches of 45 for subsequent amplification. For the second year-class, RAPD of two individuals belonging to the previous year-class were run on the same gel, to facilitate the recognition of scored bands. Identity of samples was only revealed after scoring of photographs was completed.

3.2.2. RAPD-PCR protocol

The RAPD-PCR reaction was performed in a total volume of 20 µl consisting of 2 µl 10 x reaction buffer (100mM Tris-HCl, pH 9.0; 500 mM KCl; 1.0 % Triton X-100; Promega Ltd., Southampton, UK), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Sigma, Poole, UK), 3.75 mM $MgCl_2$ (Promega), 0.75 µM 10-mer primer (Operon Technologies, Inc., Alameda, CA, USA), genomic DNA and water (molecular biology grade, BDH, Poole, UK). The reaction mixture was overlaid with mineral oil (Sigma). The optimal concentration of template DNA was determined in preliminary trials.

Table 3.1. RAPD primer sequences and number of bands scored per primer.

code	sequence 5' to 3'	no. of bands scored
OPF-01	ACGGATCCTG	5
OPF-02	GAGGATCCCT	4
OPF-04	GGTGATCAGG	3
OPF-06	GGGAATTCGG	5
OPF-10	GGAAGCTTGG	3
OPR-01	TGCGGGTCCT	4
OPR-03	ACACAGAGGG	4
OPR-04	CCCGTAGCAC	3
OPR-06	GTCTACGGCA	3
OPR-12	ACAGGTGCGT	3
OPY-11	AGACGATGGG	6
OPY-14	GGTCGATCTG	3
OPY-18	GTGGAGTCAG	5

Water, mineral oil and TE were made up in 1 ml aliquots, placed on a UV transilluminator (Sigma T2202) (300 nm) for 10 min to degrade possible contaminating DNA (UV disrupts DNA by producing thymine dimers) and stored at -20°C. Each aliquot was discarded after one use. Pipetting was carried out using sterile pipette tips and sterile Eppendorf tubes. Gloves were worn at all times and every attempt was made to keep the working conditions sterile.

The same Perkin Elmer DNA thermal cycler (model TC1, Perkin Elmer, Warrington, UK) was used for all samples. The tubes were placed in random order in the PCR-machine and kept for 3 min at 94°C ('hot start') before adding 1 unit of *Taq* DNA polymerase (Promega). Amplification was carried out using the following programme (step-cycle, giving fastest available transitions between each temperature): 3 min denaturation at 94°C, 30 s annealing at 36°C, 1 min 30 s elongation at 72°C, for 3 cycles, followed by 43 cycles with 30 s denaturation at 94°C, 30 s annealing at 36°C, 1 min 30 s elongation at 72°C. An additional 5 min extension-time was allowed before the samples were finally cooled to 4°C. A negative control, in which the template DNA was replaced by water (molecular grade, BDH), was included with most sets of samples.

The PCR products were resolved by electrophoresis for 12-14 hours at 60 V on a 1.4 % agarose-gel (see Chapter Two) and photographed with a Polaroid camera (DS34, Polaroid, Herts, UK) and monochrome negative film type 665 (Polaroid). On enlarged reprints, polymorphic fragments were scored as present (1) or absent (0).

3.2.3. Data analysis

Because of the restrictions and assumptions involved (reviewed by Grosberg *et al.*, 1996; see also Lynch and Milligan, 1994; Allegrucci *et al.*, 1995; Dean and Arnold, 1996; Stewart and Excoffier, 1996), no attempt was made to estimate allele frequencies from the RAPD data in order to perform explicitly genetic analysis. In particular, in the absence of pedigree-structured data, it was not possible to confirm that the presence and absence of each band reflected a biallelic system with Mendelian

segregation. Nor was it feasible to affirm that genotype frequencies conformed to Hardy-Weinberg expectations. In fact, a number of studies have reported heterozygote deficiencies particularly in populations of bivalves (reviewed by Zouros and Foltz, 1984). Accordingly, an alternative approach was adopted (cf. Bardakci and Skibinski, 1994; Allegrucci *et al.*, 1995) in which the RAPD data were subjected to analyses based directly on patterns of phenotypic expression (band presence/ absence).

The phenotype of each individual was presented as a 51-component vector of zeros and ones. A matrix of squared Euclidian phenotypic distances between each pair of individuals was computed from the RAPD data using NTSYS-pc (Rohlf, 1993), including only those individuals for which complete banding information was available (1995 samples: Bradda Inshore, n = 18; other Manx locations, n = 17; 1996 samples: all locations n = 18; Mulroy Bay, n = 14, Plymouth n = 17). (In this context, squared Euclidian distance is equivalent to the number of bands differing in presence/absence between the two individuals). Analyses of molecular variance (AMOVA, version 1.55; Excoffier *et al.*, 1992) were performed on these matrices, separately for the two year-classes, following Huff *et al.* (1993), by partitioning the total sums of squares of the phenotypic distances into components representing variation among individuals within populations, among populations within regions and among regions of geographically adjacent populations. Significance of the observed variance components and of pairwise phi_{ST} values between samples was tested by simulation methods in which individuals were randomly allocated to populations and populations randomly allocated to regions, and the null distribution of each variance component was estimated from 1000 independant realizations of this process, avoiding the assumption of normality. Phi_{ST} is analogous F_{ST}, but based on phenotypes, not on allele frequencies (Excoffier *et al.*, 1992).

For each year-class, a hierarchical AMOVA was performed on all individuals and treating the Isle of Man, Mulroy Bay and Plymouth as three separate regions. However, the data sets used in this analysis are numerically dominated by the five

Manx locations, the other two regions each being represented by a sample from a single location. To obtain a clearer indication of the relative contribution of variation between regions, a second partitioning of variance was undertaken with a single sample of comparable size in each of the three regions. This involved reducing the Isle of Man representation to include only the first three individuals from each of the five locations, treated here as a single sample (n = 15). In addition, a third AMOVA was performed on the five complete Manx samples alone.

A matrix of (unsquared) Euclidian phenotypic distances between locations was calculated (NTSYS-pc) from band frequencies in the samples. This was carried out for each year-class separately as well as with both year-classes in the same analysis. Relationships between locations were then summarized in principal coordinates (PCOORD) and UPGMA cluster analyses (both NTSYS-pc). A PCOORD of individuals was also carried out on a similarity matrix using the index of Dice (1945) and Nei and Li (1979) (NTSYS-pc). The possible correlation of phenotypic distance and geographic distance between samples was investigated by a Mantel test using 1000 permutations to assess the significance of the test statistic (NTSYS-pc). Geographic distance between locations by sea was estimated from charts as the shortest series of straight lines around headlands. A Mantel test (1000 permutations) on matrices of Euclidian distance between Manx locations in 1995 and 1996, was applied to test for significant correlation of phenotypic distances between the year-classes.

3.3. Results

3.3.1. General results

The banding pattern of all primers employed was found to be very distinct for *Aequipecten opercularis*, indicating that RAPD analysis has the ability to distinguish between pectinid species.

Of the 60 primers tested for their banding patterns in the preliminary trials, 13 giving reliable and easily-scorable polymorphic bands were chosen for this experiment. In most PCR runs, the control sample did not show any amplification products. On a few occasions, a diffuse band of small molecular weight appeared in the control, but it was never of the same size as the scored bands. The size range of the scored bands was between 0.35 kb and 1.5 kb.

Repeat analyses (with duplicate DNA extractions and PCRs, repeats of both stages being carried out on different days) of a large number of samples (285 PCRs) confirmed in most cases the reproducibility and identity of the scored bands; problems were restricted to the case of very faint bands, especially when samples had been stored for a long period of time. In a few repeat PCRs, differences occurred at extreme band sizes (< 0.2 and > 3 kb), but these sizes were already excluded from the analysis (see above). Problems were encountered when scoring the corresponding bands on different photographs, the quality of which varied due to different brightness of the reprint. In the case of very faint bands it was difficult to decide whether to score the band as present or absent, and the negative rather than the print sometimes had to be analysed. Bands were scored as present whenever there was a band of DNA visible. Examples of the photographs of RAPD-gels which were scored are given in Plate 3.1.

In general, there was extensive variation in RAPD banding pattern even between individuals originating from the same location, and no location-specific marker was found. Each individual possessed a unique overall RAPD phenotype.

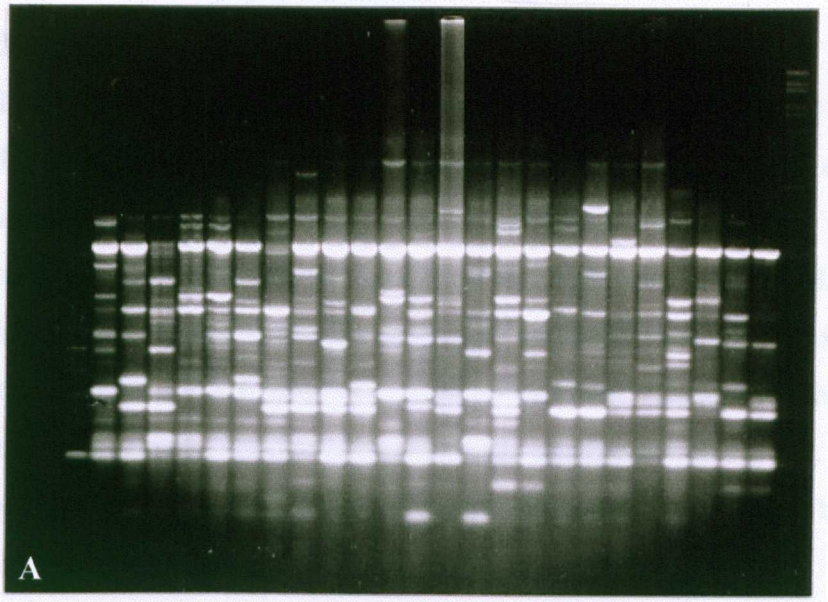
Plate 3.1.

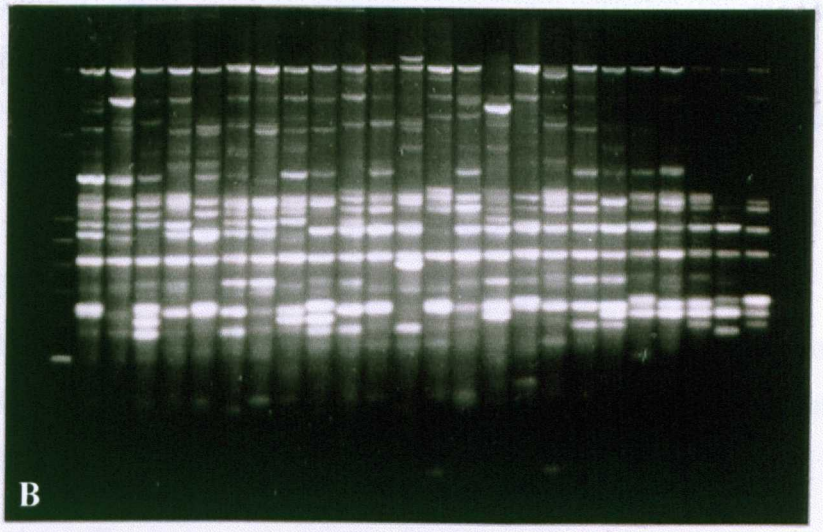
Banding pattern obtained after gel-electrophoresis of RAPD:

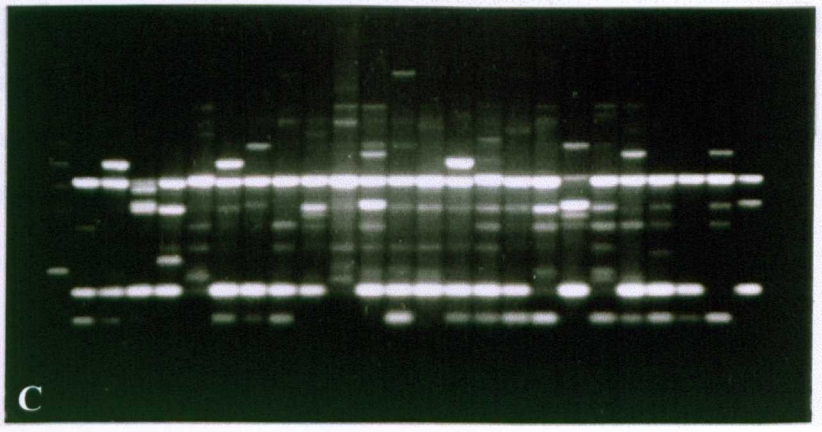
A. primer F02

B. primer R12

C. primer R06


A


B


C

3.3.2. 1995 samples

The nested AMOVA of 117 individuals (Table 3.2.) indicated that a high percentage of total phenotypic variance arose among individuals within locations, with small but significant variation among regions, as well as between locations within regions (this last category arising exclusively from the five samples in the Isle of Man region). The second analysis, with Isle of Man specimens reduced to a single regional sample, as expected indicated a higher proportion of total variance, 7.24%, between the regions. In the third AMOVA, considering the five Isle of Man samples alone, between-location variance was again significant, although still greatly outweighed by variation between individuals within locations.

After correction for multiple comparisons (Bonferroni procedure, Dunn-Šidák method, Sokal and Rohlf, 1995), pairwise between-population variance (phi_{ST}) (Table 3.3.) showed significant differences between Bradda Inshore and Ramsey, and between all Isle of Man locations and Mulroy Bay. The highest value was calculated for the comparison between Mulroy and Plymouth. Plymouth also differed significantly from all Isle of Man locations, except for Ramsey and Targets. The similarity between Bradda Inshore and Chicken Rock was indicated by a very low phi_{ST} value, with the second-lowest figure being between Ramsey and Targets. No heterogeneity of phenotypic variance between locations was revealed by the Bartlett's test available in AMOVA.

Plotting the first three axes of the PCOORD analysis based on band frequencies at all seven locations revealed a grouping consisting of the five Isle of Man locations, with the samples from Plymouth and Mulroy Bay separating on axes one and two (Figure 3.2.). Within the Isle of Man cluster, the southern locations Chickens Rock and Bradda Inshore were close together, as were the northern beds Ramsey and Targets, while East Douglas was separate (Figure 3.3.).

Table 3.2. 1995 samples. Nested analysis of molecular variance (AMOVA) for 117 individuals from 7 locations, using 51 RAPD markers. Locations were grouped into 3 regions: 1, Bradda Inshore, Chicken Rock, East Douglas, Ramsey and Targets; 2, Mulroy Bay; 3, Plymouth. Analyses were also performed on a reduced data set with the Isle of Man region represented by a single sample of 15 individuals, and on Isle of Man locations only (Region 1, 86 individuals). df = degrees of freedom; SSD = sums of squared deviations; MSD = means of squared deviations; *P*-value = probability of more extreme random variance component, estimated by permutational analysis of data matrix; NA = not applicable.

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	44.43	22.21	0.41	4.40	0.033
Among locations within regions (Isle of Man region only)	4	48.68	12.17	0.19	2.03	0.002
Among individuals within locations	110	974.32	8.86	8.86	93.57	< 0.001
All regions, Isle of Man specimens reduced to single sample of 15 individuals						
Among regions	2	38.40	19.20	0.68	7.24	<0.001
Among individuals within locations/regions	43	376.56	8.75	8.75	92.76	NA
Isle of Man locations only						
Among locations	4	48.68	12.17	0.18	1.97	0.001
Among individuals within locations	81	732.55	9.04	9.04	98.03	NA

Table 3.3. 1995 samples. Matrix of estimates of pairwise between-population variance (phi_{ST}) from AMOVA of RAPDs phenotypes. Significance levels, estimated from permutational analysis, are the probability that a random phi_{ST} value is greater than the observed value, and are corrected for multiple comparisons using the Bonferroni procedure. $^{**}p < 0.01$. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	0.0001					
D	0.0148	0.0312				
R	0.0330**	0.0191	0.0200			
T	0.0266	0.0149	0.0258	0.0103		
M	0.0798**	0.0922**	0.0782**	0.0708**	0.0820**	
P	0.0705**	0.0477**	0.0696**	0.0263	0.0337	0.0930**

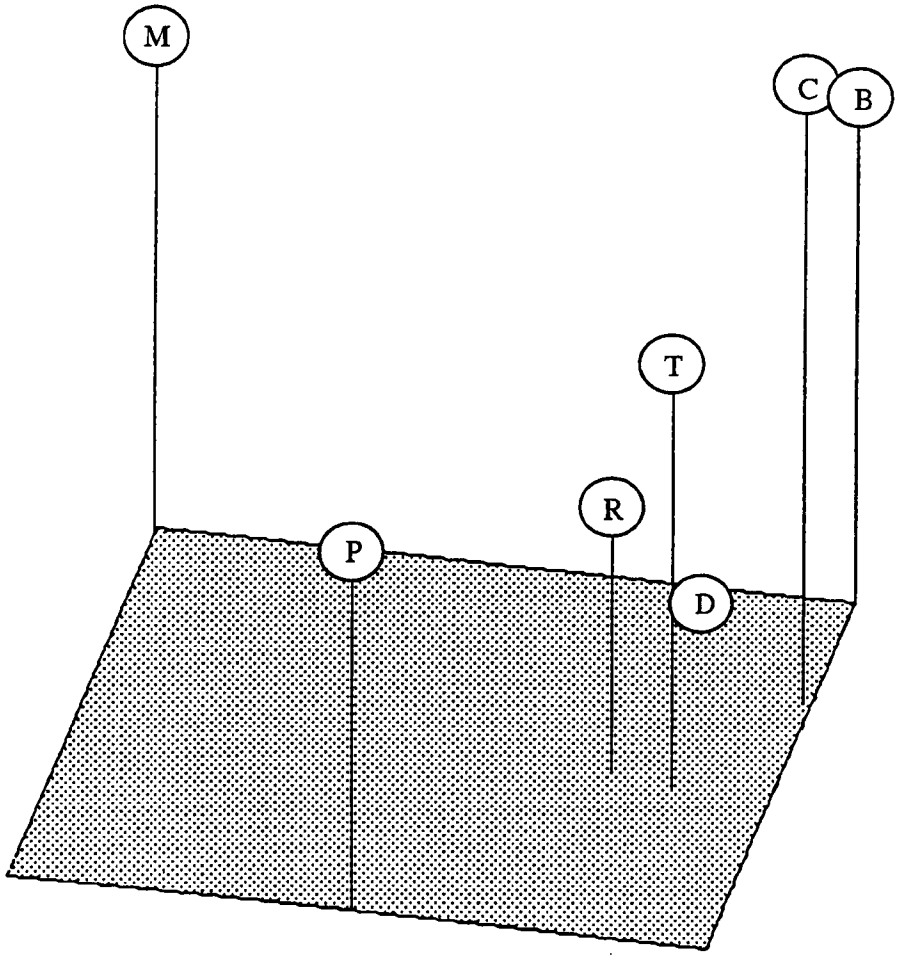


Figure 3.2. RAPD 1995. Plot of seven locations on the first three axes of principal coordinates analysis based on frequencies of 51 RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

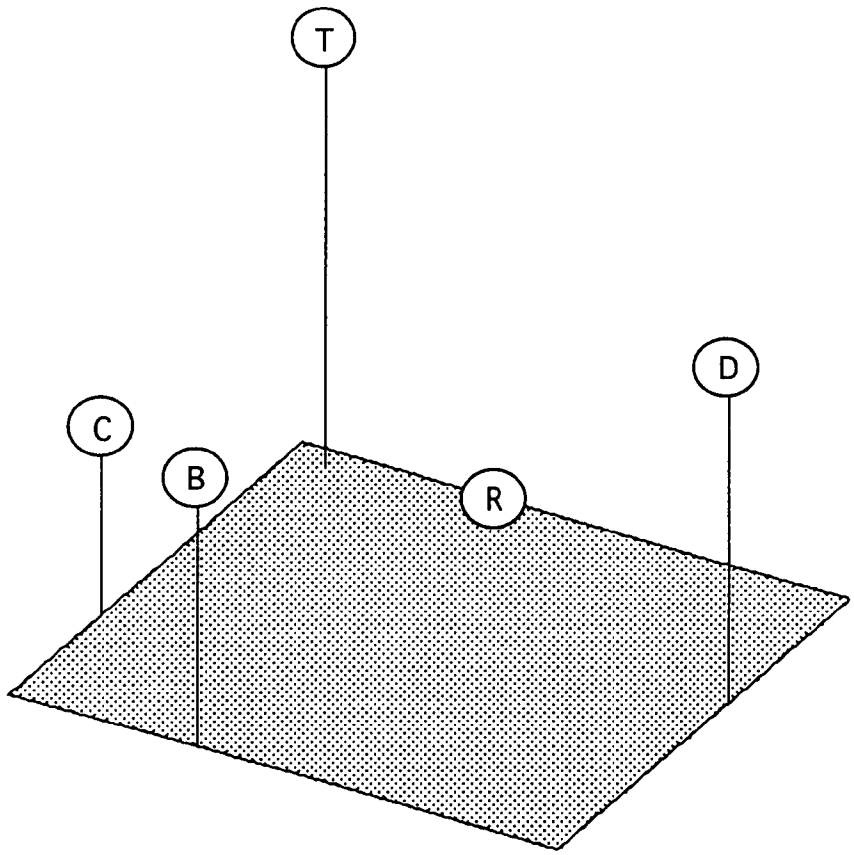


Figure 3.3. RAPD 1995. Plot of the Isle of Man locations on the first three axes of principal coordinates analysis based on frequencies of 51 RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas.

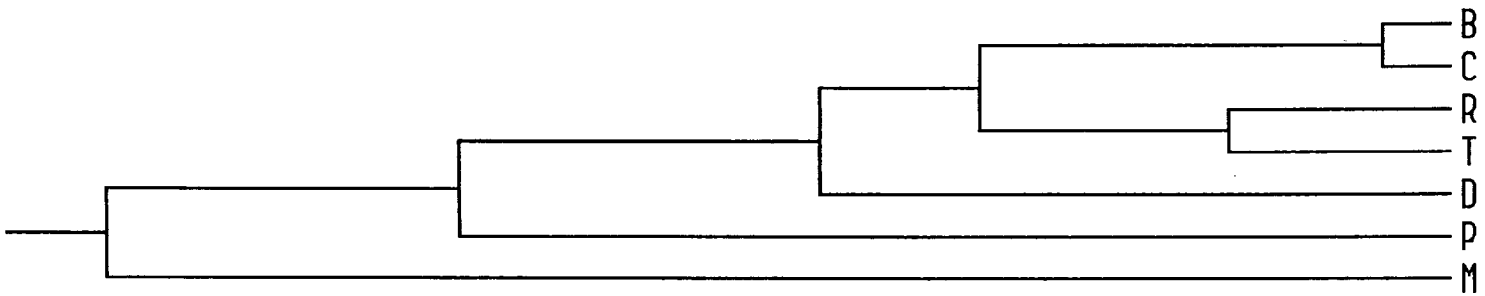


Figure 3.4. 1995 samples. UPGMA dendrogram based on Euclidian phenotypic distances from frequencies of RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

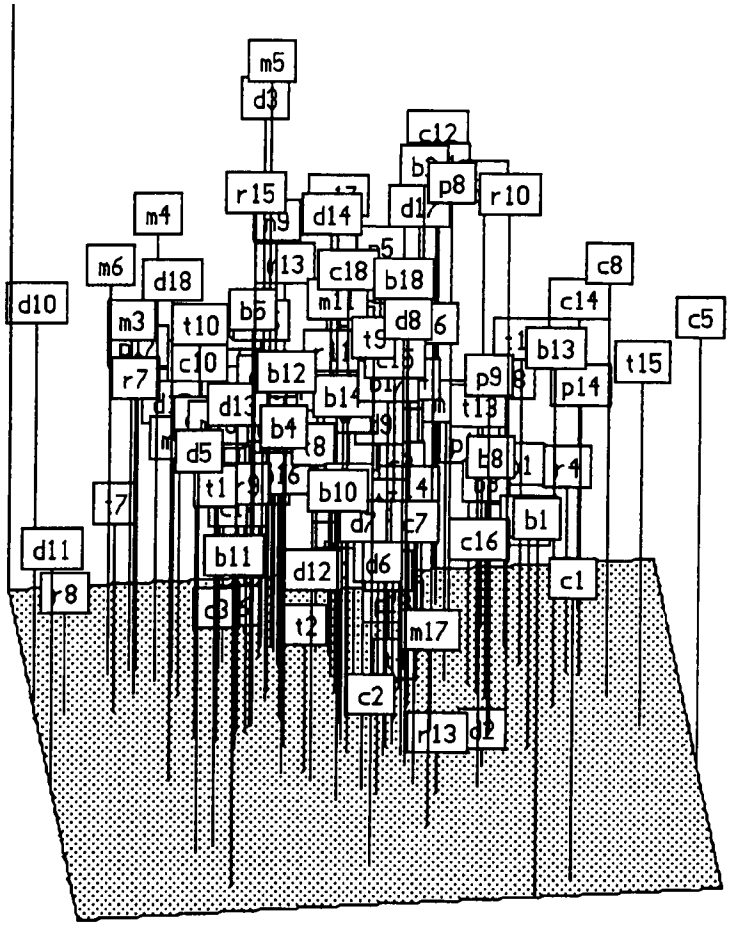


Figure 3.5.A. 1995 samples. Principle coordinates analysis based on RAPD-band frequencies of all individuals.

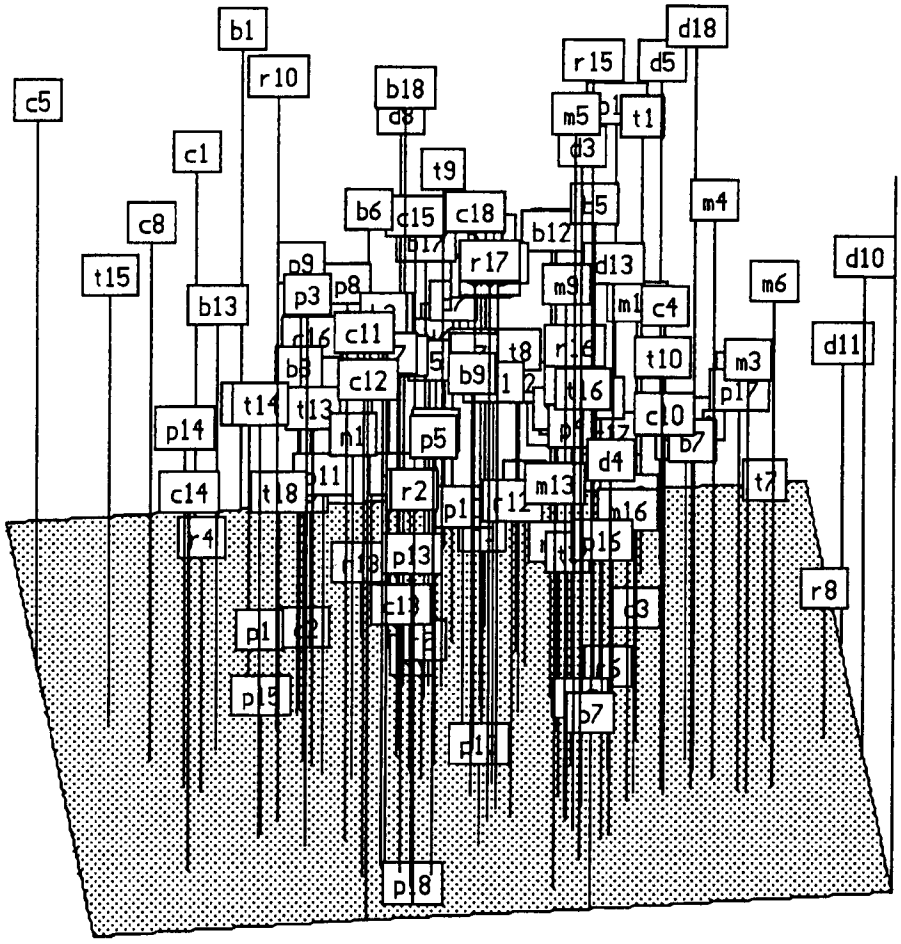


Figure 3.5.B. 1995 samples. Principle coordinates analysis based on RAPD-band frequencies of all individuals, rotated in relation to 3.5.A.

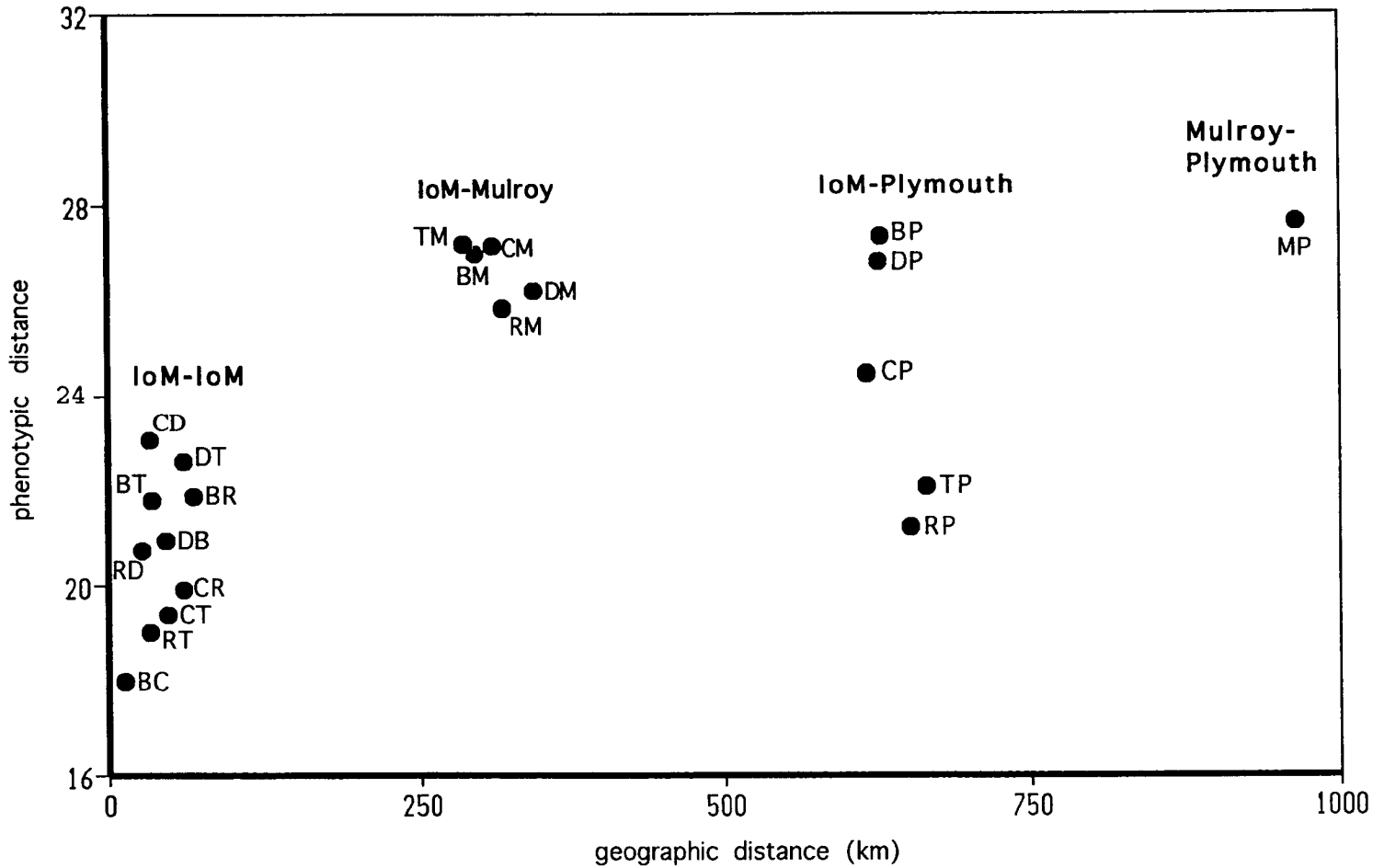


Figure 3.6. 1995 samples. Scatter-plot of phenotypic distances between locations versus corresponding geographical distances. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

The UPGMA dendrogram, also based on band frequencies at locations (Figure 3.4.), showed the same grouping: the first cluster consisting of Bradda Inshore and Chickens Rock, and the second cluster of Ramsey and Targets. East Douglas clustered last of the Isle of Man locations. Plymouth appeared less distant from the Isle of Man locations than Mulroy Bay, which was the most divergent. Because of the large number of individuals the PCOORD plot of all 117 individuals is difficult to interpret except when being rotated on the computer screen. It showed loose grouping of individuals from geographically proximate locations (Figure 3.5.A. and 3.5.B.).

The Mantel test showed no significant correlation between phenotypic and geographic distances ($p = 0.092$). Notable from the scatterplot (Figure 3.6.) is the relative phenotypic distinctiveness of the Mulroy Bay sample, and the fact that the Plymouth specimens are as similar to those from Ramsey and Targets as are some of the Isle of Man samples to each other.

3.3.3. 1996 samples

As for the 1995 samples, the nested AMOVA of the 1996 individuals indicated a high percentage of the total variance occurring among individuals within locations (Table 3.4). Small, but significant variance was present among regions. Values for variance among locations were non-significant. When the Manx specimens were reduced to a single sample of 15 individuals, a higher proportion of the total variance was present between regions, similarly to the 1995 result. Analysis of the Manx samples alone again showed a large proportion of the total variation to occur between individuals, and small, but almost significant variation between locations. Bartlett's test did not reveal heterogeneity of phenotypic variance between locations.

Table 3.4. 1996 samples. Nested analysis of molecular variance (AMOVA) for 121 individuals from 7 locations, using 51 RAPD markers. Locations were grouped into 3 regions: 1, Bradda Inshore, Chicken Rock, East Douglas, Ramsey and Targets; 2, Mulroy Bay; 3, Plymouth. Analyses were also performed on a reduced data set with Isle of Man region represented by a single sample of 15 individuals, and on Isle of Man locations only. df = degrees of freedom; SSD = sums of squared deviations; MSD = means of squared deviations; *P*-value = probability of more extreme random variance component, estimated by permutational analysis of data matrix; NA = not applicable.

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	41.10	20.54	0.41	4.40	0.024
Among locations within regions (Isle of Man region only)	4	41.80	10.45	0.08	0.95	0.071
Among individuals within locations	114	1009.10	8.52	8.85	94.04	< 0.001
All regions, Isle of Man specimens reduced to single sample of 15 individuals						
Among regions	2	36.75	18.38	0.65	7.17	<0.001
Among individuals within locations/regions	43	362.57	8.43	8.43	92.83	NA
Isle of Man locations only						
Among locations	4	41.80	10.45	0.08	0.87	0.059
Among individuals within locations	85	767.33	9.03	9.03	99.13	NA

Table 3.5. 1996 samples. Matrix of estimates of pairwise between-population variance (phi_{ST}) from AMOVA of RAPDs phenotypes. Significance levels, estimated from permutational analysis, are the probability that a random phi_{ST} value is greater than the observed value, and are corrected for multiple comparisons using the Bonferroni procedure. *$p < 0.05$, **$p < 0.01$. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	0.0121					
D	0.0300	0.0009				
R	0.0184	0.0119	0.0167			
T	0.0126	-0.0110	-0.0026	-0.0018		
M	0.0726**	0.0728**	0.0764**	0.0642**	0.0752**	
P	0.0407*	0.0211	0.0277	0.0605**	0.0262	0.09308**

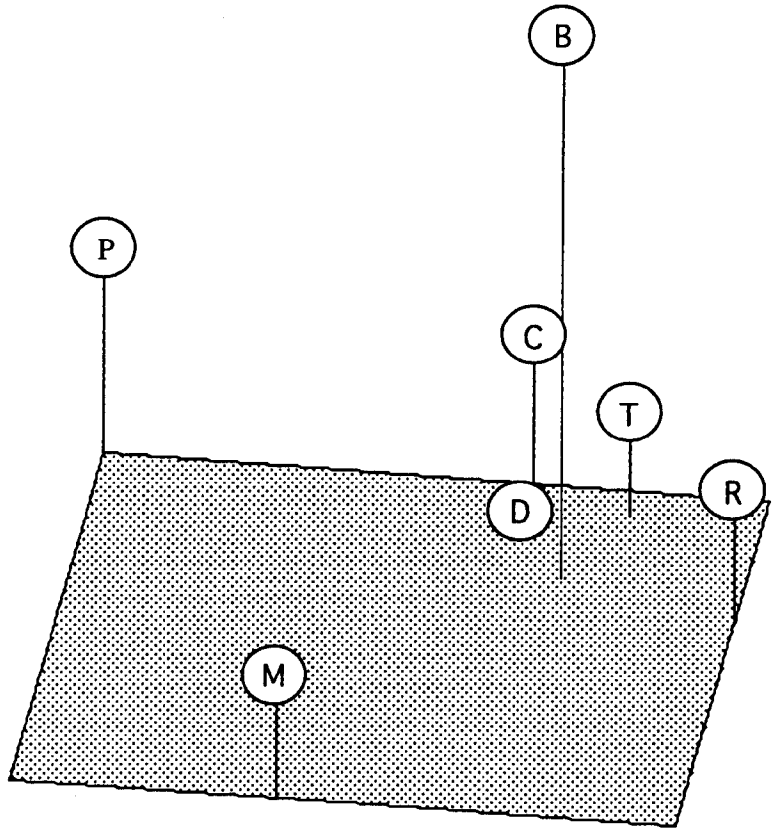


Figure 3.7. RAPD 1996. Plot of seven locations on the first three axes of principal coordinates analysis based on frequencies of 51 RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

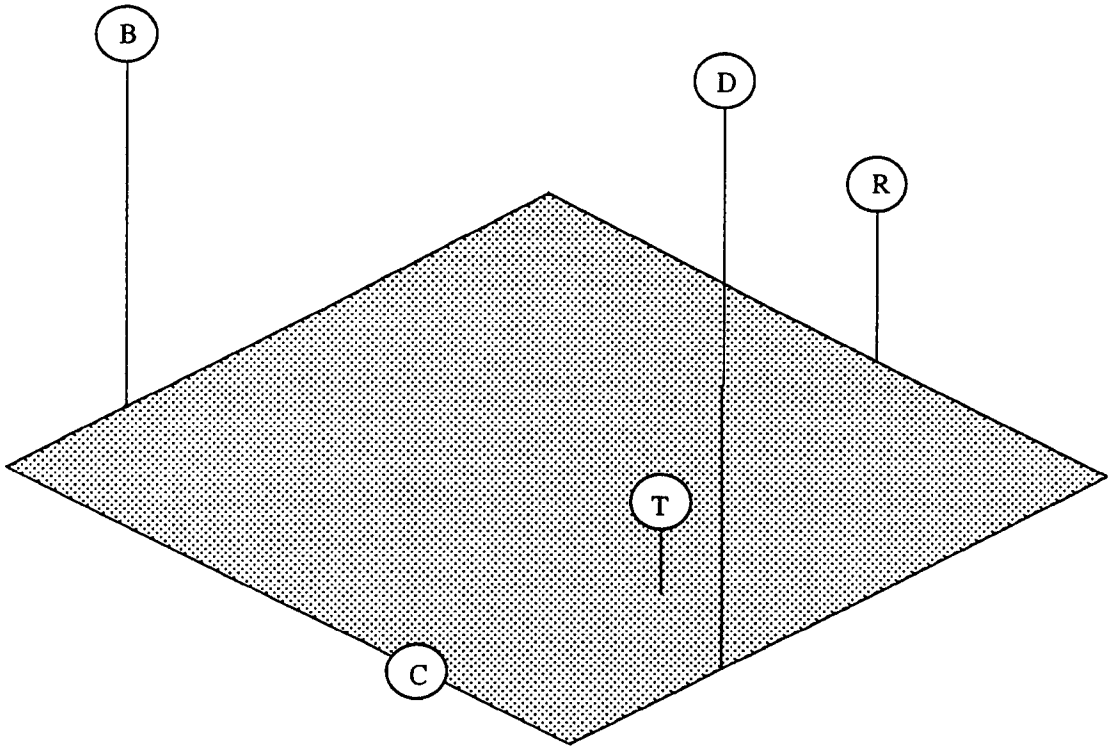


Figure 3.8. RAPD 1996. Plot of the Isle of Man locations on the first three axes of principal coordinates analysis based on frequencies of 51 RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas.

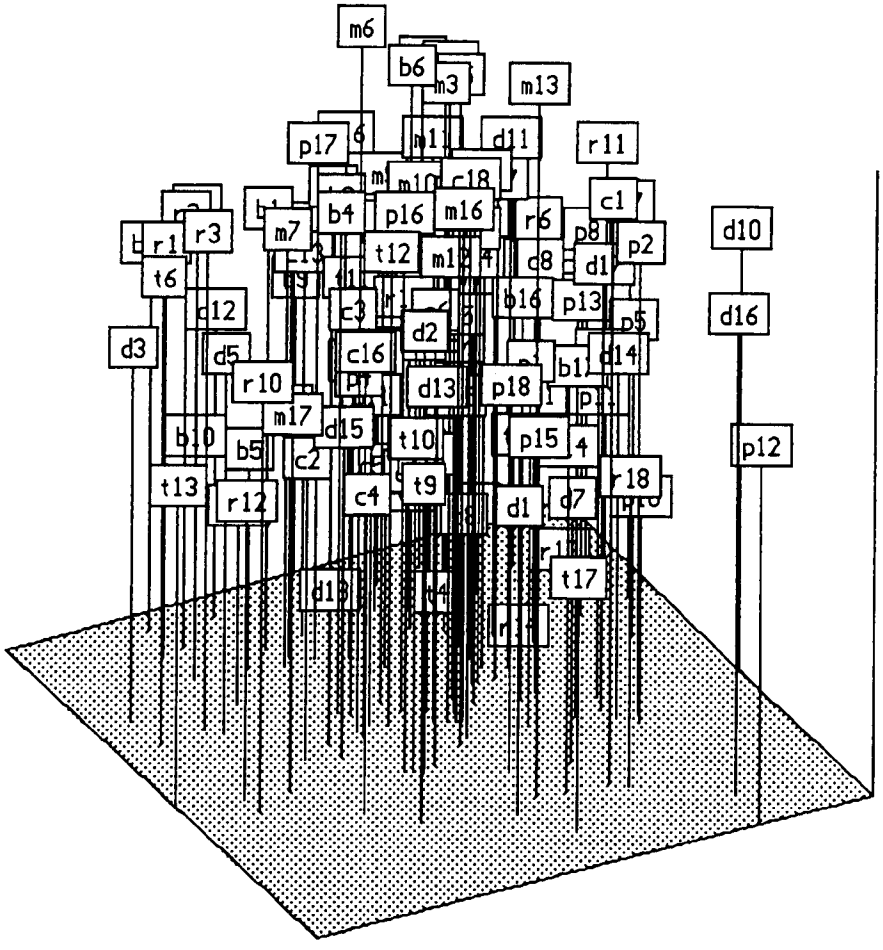


Figure 3.9.A. 1996 samples. Principle coordinates analysis based on RAPD-band frequencies of all individuals.

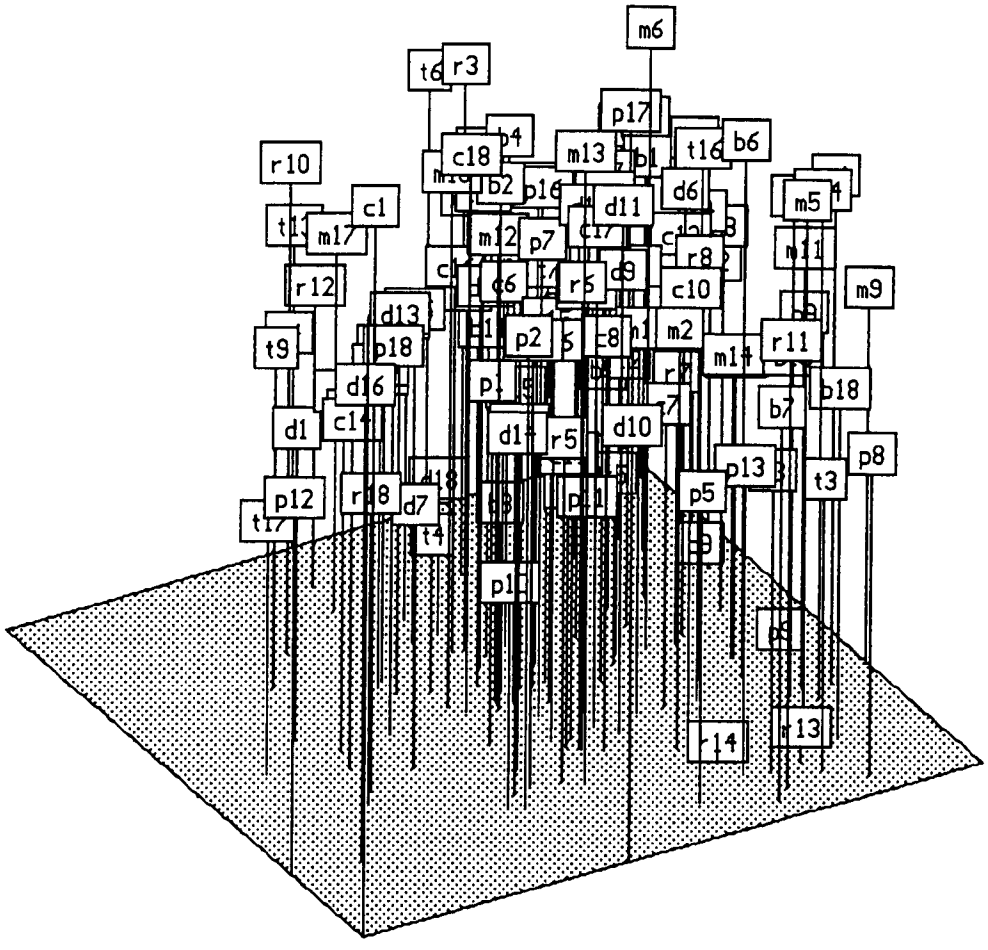


Figure 3.9.B. 1996 samples. Principle coordinates analysis based on RAPD-band frequencies of all individuals, rotated in relation to 3.9.A.

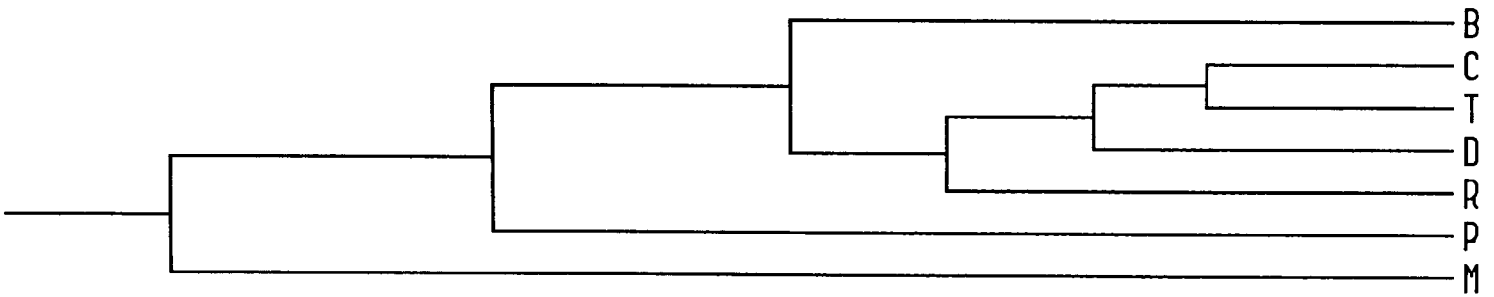


Figure 3.10. 1996 samples. UPGMA dendrogram based on Euclidian phenotypic distances from frequencies of RAPD bands. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

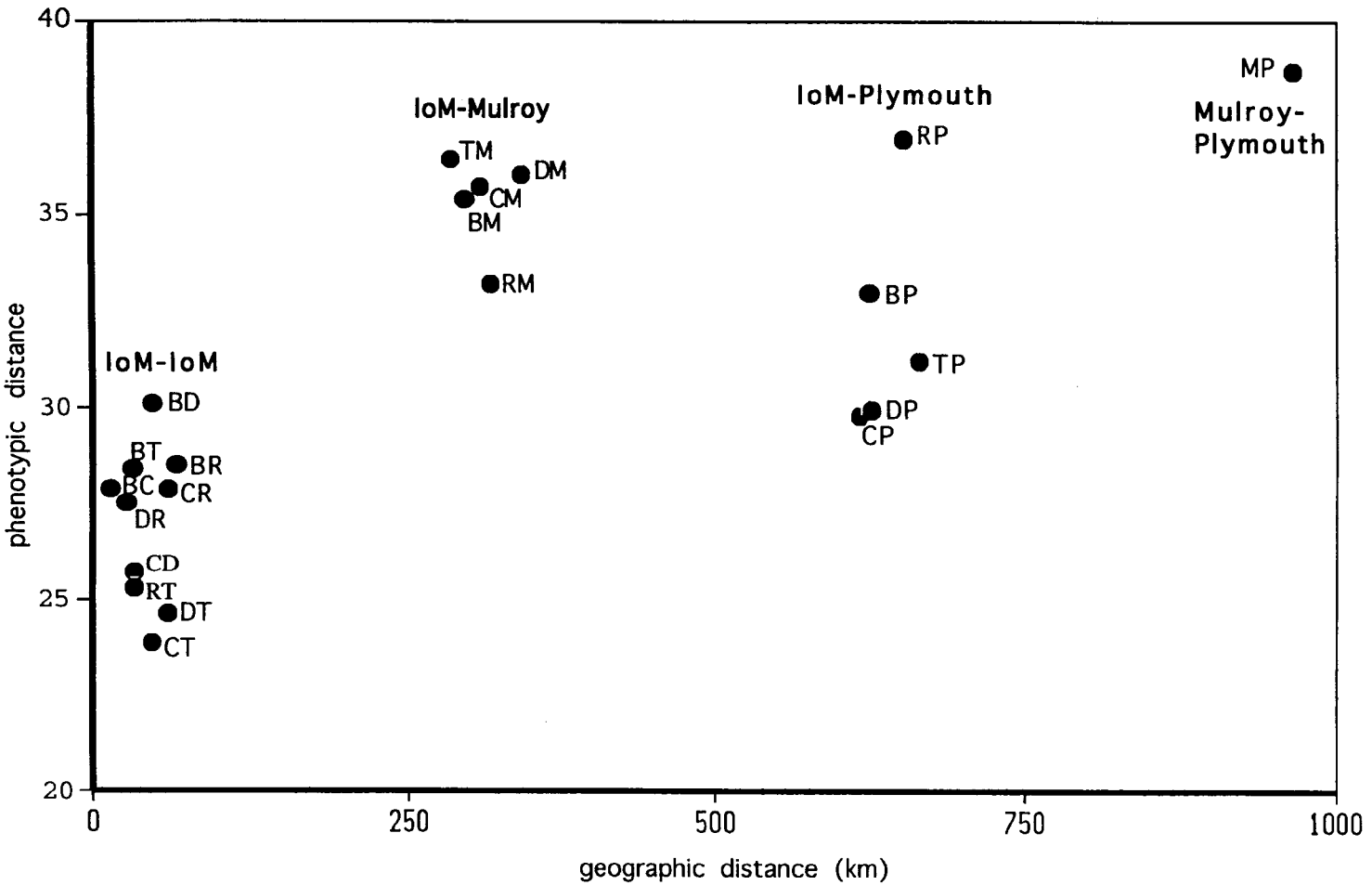


Figure 3.11. 1996 samples. Scatter-plot of phenotypic distances between locations versus corresponding geographical distances. Abbreviations: B - Bradda Inshore, C - Chickens Rock, R - Ramsey, T - Targets, D - East Douglas, P - Plymouth, M - Mulroy Bay.

Phi_{ST} values remained significant after the Bonferroni correction between all Isle of Man locations and Mulroy, between Plymouth and Bradda, Plymouth and Ramsey, and Plymouth and Mulroy (Table 3.5).

The PCOORD based on band frequencies of all locations 1996 revealed a picture similar to the 1995 samples: on the first two axes, four Manx locations were adjacent to each other, while Mulroy and Douglas were further away (Figure 3.7.). Within the Manx locations, Douglas and Bradda occupied extreme positions on the third axis. None of the Manx locations were particulary close to each other (Figure 3.8.). The PCOORD based on phenotypic distance between all 1996 individuals did not show any obvious clustering of individuals originating from the same location, except for the samples from Mulroy, which tended to occupy the upper part of the cluster, and were thus separate on the third axis (Figure 3.9.A. and 3.9.B.).

The UPGMA confirmed the distinction of Mulroy and Plymouth from the Manx samples. Within the Manx locations, Chickens and Targets clustered closest, and Douglas and Ramsey associated with them. Bradda appeared most distant from the other Manx samples (Figure 3.10.). The correlation between phenotypic and geographic distances for the 1996 samples was almost significant (Mantel test, p = 0.059) (Figure 3.11.).

3.3.4. Comparison of the 1995 and 1996 samples

The PCOORD of the samples from 1995 and 1996 together confirmed the consistent separation of Mulroy and Plymouth from the Manx populations. The Manx populations showed little resemblance between year-classes at a location. However, the 1995 and 1996 samples seemed to be distinct on the third axis (Figure 3.12.). UPGMA cluster analysis was also carried out on the samples of both year-classes. While Mulroy and Plymouth remained distinct from the Manx locations, no definitive trend could be observed within the Manx samples; Douglas 95 clearly clustered last of all the Manx samples, while Douglas 96 clustered quite early, reflecting a loss of distinctiveness at this location (Figure 3.13.). The scatterplot obtained by comparison of the distance matrices of both year-classes showed no significant correlation (Mantel test, p = 0.4905) between the two year-classes of Manx samples (Figure 3.14.).

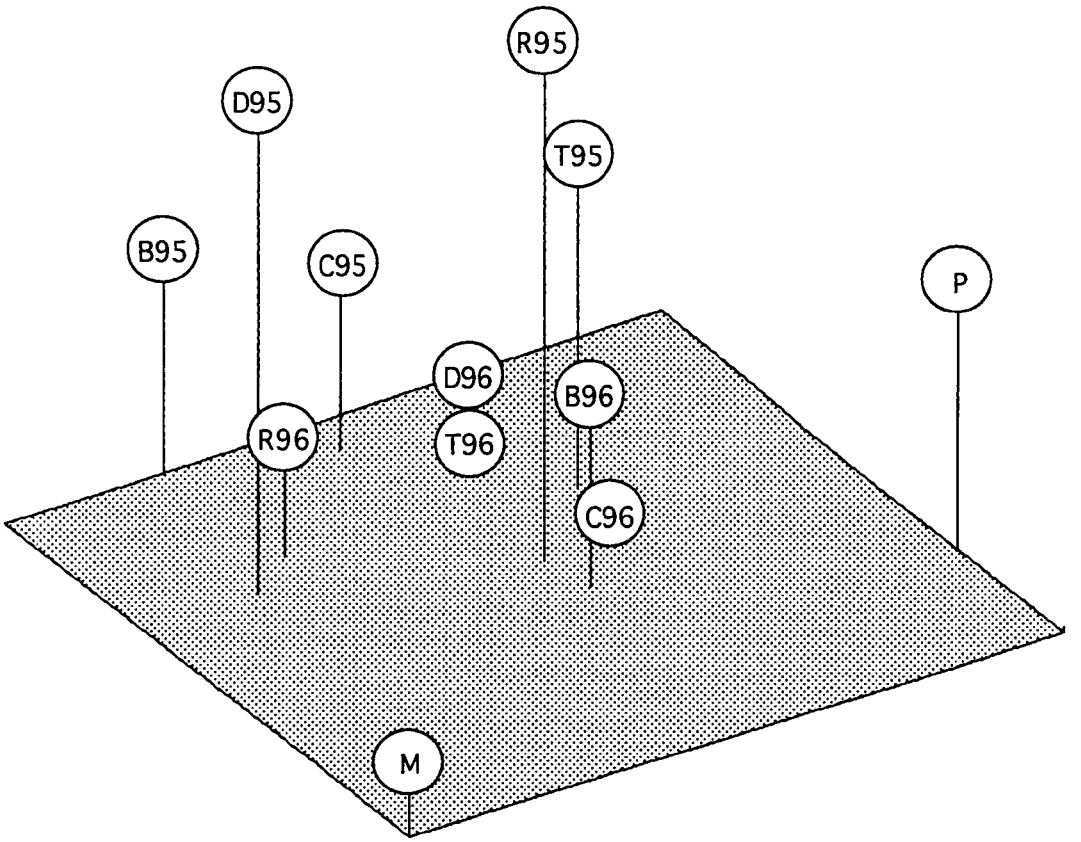


Figure 3.12. RAPD of the 1995 and 1996 samples together. Plot of all locations on the first three axes of principal coordinates analysis based on frequencies of 51 RAPD bands.

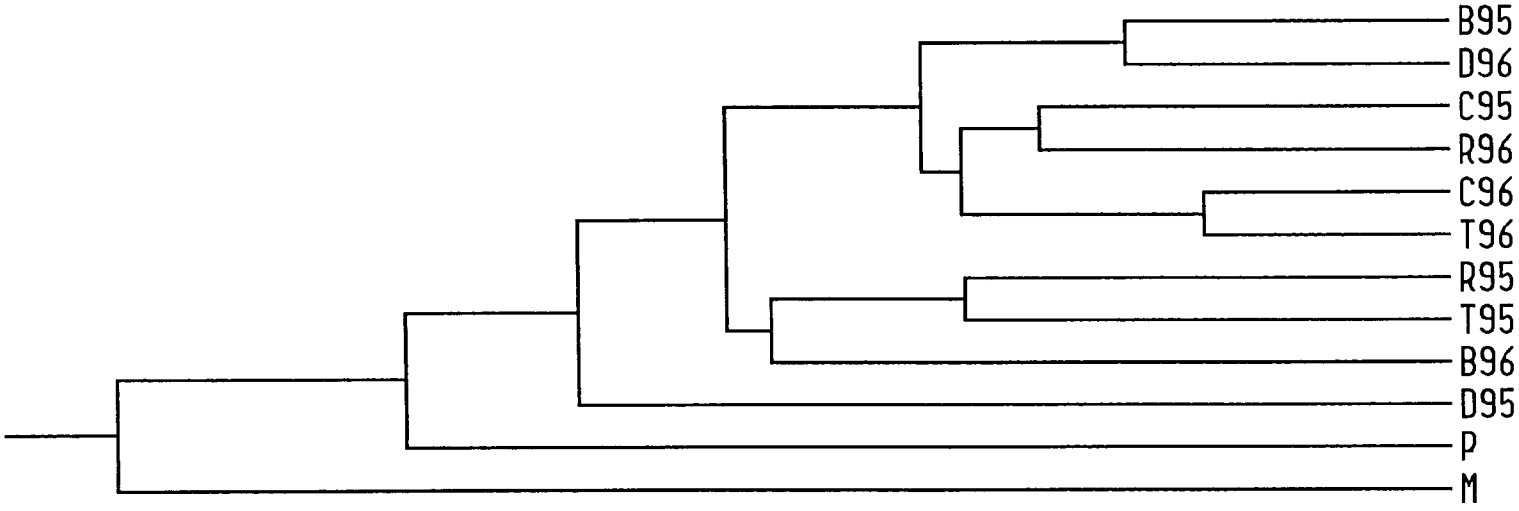


Figure 3.13. UPGMA dendrogram based on band frequencies of the combined samples from 1995 and 1996.

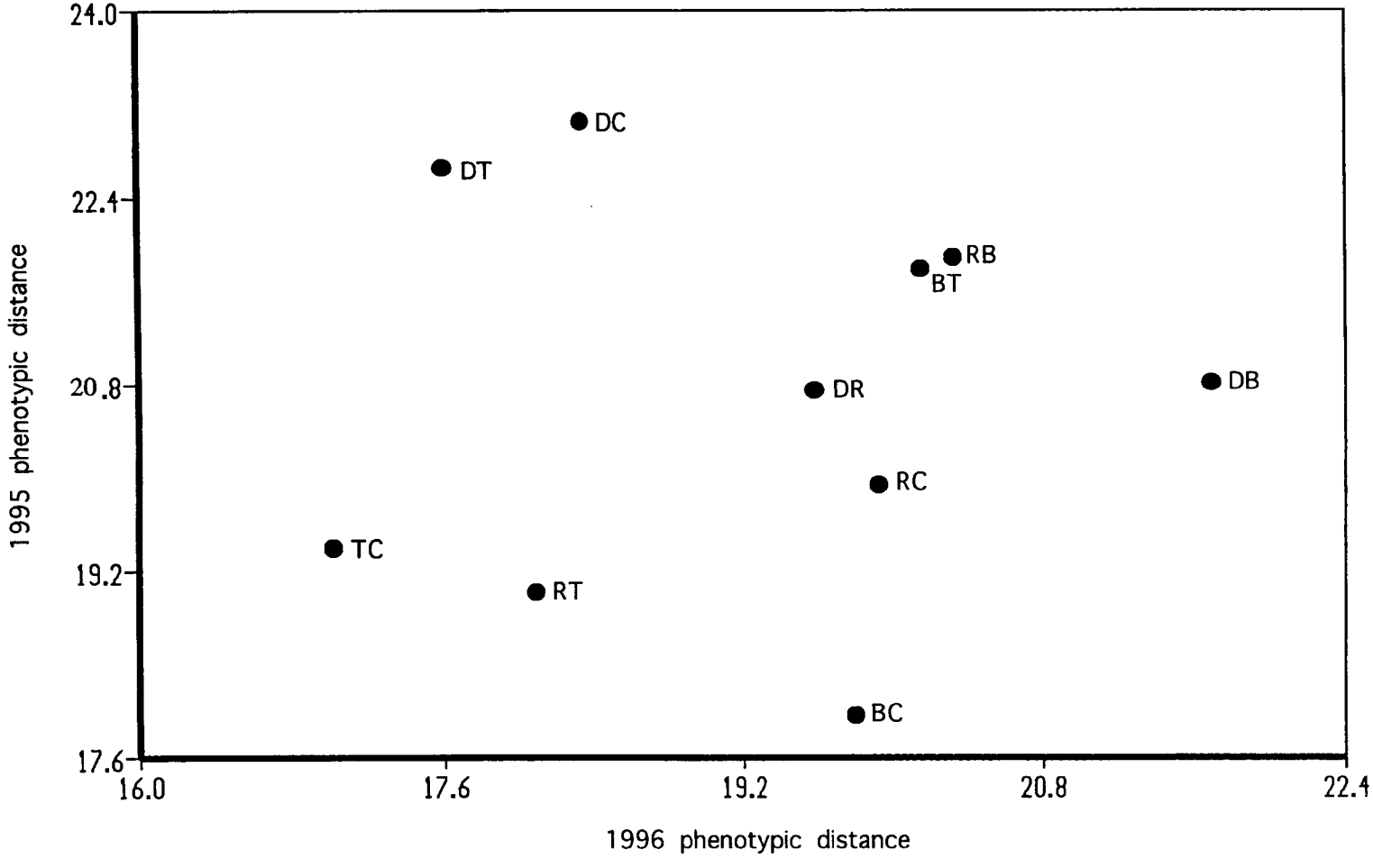


Figure 3.14. Scatter-plot showing correlation of the two year-classes.

3.4. Discussion

Although no serious problems were encountered in this study, RAPD has gained something of a bad reputation for difficulties with repeatability. Consistent results with RAPD are very much dependent on good quality, undegraded DNA. Problems can occur if working conditions are not sterile, resulting in contamination and subsequent destruction of DNA samples by bacterial DNAses, or in amplification of bacterial DNA sequences. Prolonged storage of samples between repeat PCRs or repeated freezing and thawing leads to DNA breaking down into small pieces; primers are not able to amplify the same fragments as they did in undegraded DNA, because priming sites are now separated. A decrease in the size of amplification products after PCR could indicate degraded DNA.

Bands which vary continuously in brightness should not be selected for analysis. However, as the number of samples increases, so too does the likelihood that bands of intermediate brightness will be observed. Some bands which gave a clear presence/ absence polymorphism at the beginning of the study, exhibited continuous variation in brightness as the number of samples analysed increased. Problems could also arise from the equally-weighted scoring of bands containing varying amounts of DNA. Differences in the amount of PCR-product could be caused by different numbers of copies of the amplified sequence present in the genome either at a single locus (heterozygotes /homozygotes), or even at different locations within the genome. As bands were given the same weight in the analysis, regardless of their intensity, potential information will have been lost. It is not possible to consistently estimate the degree of brightness of bands without the appropriate technical equipment; the scoring of PCR-products purely for presence/absence seemed an acceptable compromise.

The appropriate statistical analysis of RAPD data has been the subject of recent discussions. As RAPD are generally dominant markers (not codominant, as allozymes or microsatellites), they only generate phenotypic information. Therefore the genotypes of the assayed individuals can not be assessed directly, excluding the

application of genetic analyses. Not enough information on origin and inheritance, and on the average mutation rate of RAPD loci is available to make reliable estimates of allele frequencies. In any case, the precision of the analysis is always decreased compared to the analysis of codominant markers (Lynch and Milligan, 1994; Stewart and Excoffier, 1996).

There is some evidence that a considerable proportion of RAPD loci could derive from the mitochondrial genome; in a study by Aagard *et al.* (1995), nearly half of all RAPD bands scored were found to be amplified from mitochondrial DNA. This was however carried out on plants, which have a mitochondrial genome several orders of magnitude larger than animals.

In this study, variation between individuals greatly outweighted variation between populations, and due to the large number of loci scored, each individual had a unique composite phenotype. The large number of polymorphic markers (with the potential for the application of multivariate analyses) makes the method suitable for investigating family-level relationships (Levitan and Grosberg, 1993; Bishop *et al.*, 1996) and mating systems (Okamura *et al.*, 1993), but might be of disadvantage in population studies, providing too high a resolution. The high variability results in a completely different banding pattern for different species, with no bands shared in most cases (e.g. Hadrys *et al.*, 1992; Crossland *et al.*, 1993; Bardakci and Skibinski, 1994; Gibbs *et al.*, 1994; Pfenninger *et al.*, 1995; Haig *et al.*, 1997; Schierenbeck *et al.*, 1997), making RAPD perfectly suitable for distinguishing between species, but not for the construction of evolutionary trees. The major advantages of RAPD are firstly the large number of polymorphic markers generated, potentially making up for the noise introduced by the various uncertainties mentioned above, and secondly that it is a quick, cheap and easy method, compared to other techniques.

Based on AMOVA of RAPD data, several previous studies have detected population differentiation. Nusser *et al.* (1996), found significant variances between populations of the rail *Rallus longirostris* in southern California (48 individuals, four

populations). Huff *et al.* (1993) and Peakall *et al.* (1995), applying AMOVA to 48 individuals of four populations of the plant *Buchloe dactyloides*, originating from two regions, revealed highly significant differences between populations and regions. Similarly, 47 individuals of another plant, *Grevillea scapigera*, showed significant variation between seven locations (Rosetto *et al.*, 1995). Levels of variation, of a similar magnitude to the present study, of 3.7 % among and 92.4 % between populations, led Haig *et al.* (1994) to the conclusion that population differentiation exists in red-cockaded woodpeckers, *Picoides borealis,* from south east USA (101 individuals from 14 populations).

The different statistical approaches adopted in this study generally point to similar conclusions. In the 1995 samples, variance components and pairwise phi_{ST} values from AMOVA both indicate significant differentiation between regions and even between Isle of Man beds of *P. maximus*, while the multivariate analyses (PCOORD, UPGMA clustering) assist interpretation of this differentiation in terms of specific geographical trends. Together, the observations made in the 1995 samples indicate some degree of genetic differentiation between open-water populations of *P. maximus,* in contrast to the failure of allozyme studies to reveal genetic heterogeneity in *P. maximus* over extensive geographical ranges (Beaumont *et al.*, 1993). The greater number of genetic polymorphisms available from RAPD analysis as used in this study may have been crucial in revealing differentiation. However, RAPD markers may also be inherently more likely than allozyme loci to show geographical differentiation, reflecting a more representative sampling of overall genomic variation. Possible reasons for the apparently enhanced resolution of RAPD include the fact that many RAPD bands derive from non-coding parts of the genome, and are consequently unconstraint by balancing selection, and the ability of the technique to detect "silent" substitutions within coding regions (Allegrucci *et al.*, 1995; Aagaard *et al.*, 1995). (With allozyme studies silent substitutions are undetectable and only approximately 30 % of other substitutions are thought to be detected; see e.g. Thorpe, 1982; Hoelzel, 1992). A number of previous studies have reported a greater degree of

differentiation between individuals and populations with RAPD markers when compared with allozymes (reviewed by Peakall *et al.*, 1995).

The population differentiation reported for the 1995 samples, although statistically significant, is uniformly low, as suggested by the phi_{ST} values. The modest level of genetic differentiation between Isle of Man locations may partly reflect the occasional general influx of larvae from populations located far away from the Isle of Man, in addition to any local exchange: a few individuals transported by water currents from further afield could have a strong influence in preventing more distinct genetic differentiation between locations. As current patterns in the Irish Sea change from year to year (see General Discussion), it is not surprising that the analysis of the 1996 samples drew a picture different from 1995, with less population differentiation being present in 1996. Whereas Douglas was located at some distance from the other Manx locations in the PCOORD analyses of the Manx locations in 1995, it appeared less distinct in 1996, and overall less heterogeneity was detected between the other Manx locations in the 1996 samples; furthermore, the populations clustered out differently, with Bradda being the most distinct Manx population in the UPGMA of the 1996 samples. However, there were also some consistencies with the results of the 1995 samples: in the PCOORD including all populations, Douglas could again be distinguished on the third axes, and Mulroy and Plymouth were different from all Manx populations of both Manx year-classes.

The PCOORD analysis of the year-classes together showed a hint of temporal differentiation: the two Manx year-classes appeared to separate on the third axis. This weak trend was not supported by the UPGMA cluster analysis. The comparison of distance matrices did not reveal significant correlation between the year-classes.

CHAPTER FOUR: RESTRICTION ANALYSIS OF MITOCHONDRIAL DNA

4.1. Introduction: mitochondrial DNA

4.1.1. General features of mitochondrial DNA

In a typical mammalian somatic cell there are approximately one thousand mitochondria, each containing five to ten molecules of mitochondrial DNA (mtDNA) in their inner compartment between the cristae. In general, animal mitochondrial DNA is a double-stranded, circular molecule, consisting of genes for 13 proteins for oxidative phosphorylation (subunits for NADH dehydrogenase, cytochrome *b*, cytochrome c oxidase (CO), and ATPase), 22 transcription RNAs and 2 ribosomal RNAs (12S, 16S rRNA) (Figure 4.1.). A non-coding region (the D-loop, control-region or A+T-rich region) contains the initiation sites for mtDNA replication and RNA transcription. The control region ranges in size from 121 bp in sea urchins to 3.2 kb in frogs (reviewed by Fuller and Zouros, 1993). The order of genes appears conserved within taxonomic classes or phyla, but rearrangements have been detected between phyla (Meyer, 1994; Hoffmannn *et al.*, 1992; Moritz *et al.*, 1987). In *Mytilus edulis*, a novel mitochondrial genome organization has been discovered in a study determining the sequence of 13.9 kb of the 17.1 kb mtDNA (Hoffmann *et al.*, 1992): an additional tRNA was detected, and the arrangement of genes differed from that found so far in any other known mtDNA.

The two strands are named 'heavy strand' and 'light strand' due to the position they take up when centrifuged in a CsCl density gradient (its higher GT-content makes the 'heavy strand' occupy a position at higher density in the gradient). Most genes are located on the heavy strand. In contrast to the nuclear genome, the mitochondrial genome does not contain introns (except in yeast), and genes are usually separated by less than 10 kb; in some cases genes even overlap by several bases, making the mitochondrial genome extremely compact. The set of proteins coded for by mitochondrial genes is only a fraction of what is needed to sustain the complete mitochondrial metabolism. The majority of proteins involved in

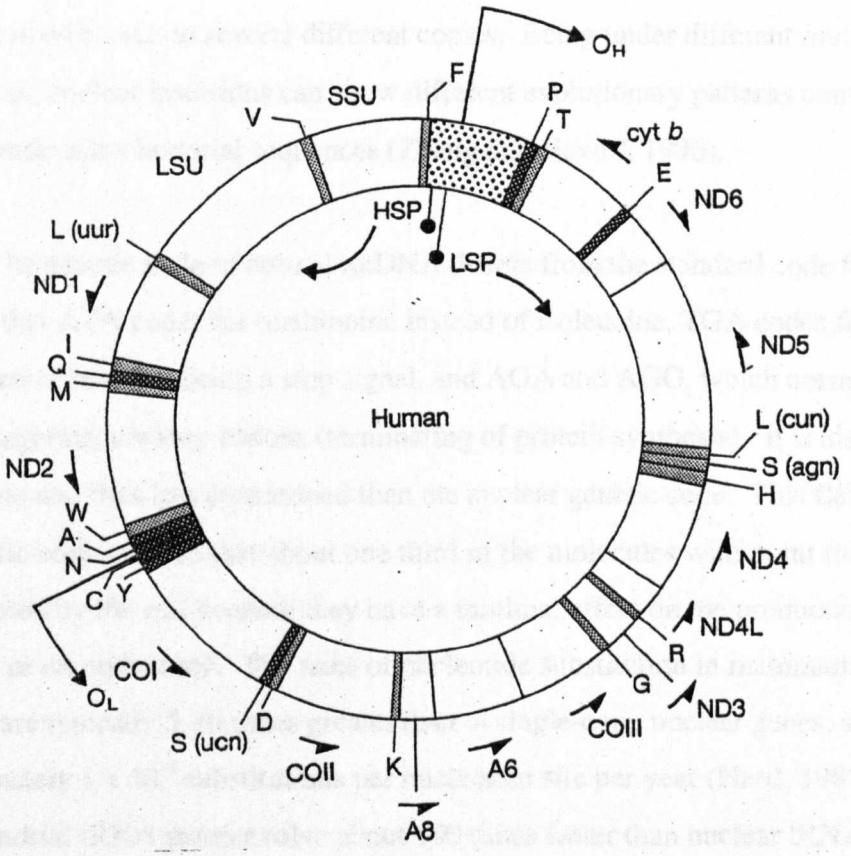


Figure 4.1. Organization of the human mitochondrial genome. Protein-coding and rRNA genes are shown as open boxes, tRNA genes are shown as hatched boxes; half-arrows denote transcriptional sense for the genes. The non-coding region (origin of leading-strand replication) is indicated by dotted shading (from: Kendrew, 1994).

mitochondrial metabolism is coded for by nuclear genes and transported from the cytoplasm into the mitochondria. It is believed that intergenomic transfer of genetic material is the reason for the incomplete set of mitochondrial genes. This poses a potential problem for PCR amplification of specific fragments, if the same genes are, in a transitional state, occurring both in the mitochondrial and the nuclear genome, in the latter maybe even in several different copies. Being under different mutational constraints, nuclear insertions can show different evolutionary patterns compared to the authentic mitochondrial sequences (Zhang and Hewitt, 1996).

The genetic code of animal mtDNA differs from the standard code for nuclear DNA in that ATA codes for methionine instead of isoleucine, TGA codes for tryptophan rather than being a stop signal, and AGA and AGG, which normally encode arginine, are stop codons (terminating of protein synthesis). It is also more degenerate and thus less constrained than the nuclear genetic code. This flexibility in the genetic code ensures that about one third of the molecules with point mutations are tolerated by the cell because they have a minimal effect on the production of gene products or on replication. The rates of nucleotide substitution in mammalian mtDNA are typically 5-10 times greater than in single-copy nuclear genes, averaging approximately 1×10^{-8} substitutions per nucleotide site per year (Hartl, 1987). Mitochondrial tRNA genes evolve about 100 times faster than nuclear tRNA genes (Brown, 1985; Hoeh *et al.*, 1991; Avise, 1994). The mean rate of divergence averaged over the whole mtDNA molecule is about 2 % per MA in different groups of species from which there is evidence from fossils, biogeography or proteins concerning divergence times (for references see Wilson *et al.*, 1985). Mutations are mainly length variants, and base changes due to transitional effects (changes from one purine to another or from one pyrimidine to another) (Brown, 1985). The cause of this high rate of sequence evolution could be a low efficiency of repair or the complete lack of repair mechanisms within the mitochondrial transcription system (Brown *et al.*, 1982; Clayton, 1982, 1984). In contrast to nuclear DNA polymerase, mitochondrial DNA polymerase, similarly to bacterial polymerase, has no proof-reading function. As the mismatch repair system of bacteria and yeast is mainly

designed to repair length mutations and transitions, which are the commonest replication errors, lack of that system in mitochondria would explain the observed mutational effects (Wilson *et al.*, 1985). The rate of mutation is highest in the non-coding control-region, lowest in rRNA genes, and intermediate in protein-coding genes (Meyer, 1994). Base substitutions in silent positions of protein-coding genes can conceivably be assumed neutral, and mutations in the non-transcribed control region could at most influence the transcription rate. Mutations in genes whose products are essential for energy metabolism are most likely under strict selection pressure. The obligatory collaboration of proteins coded by the mitochondrial genome with nucleus-coded proteins must impose a special dimension on selective processes.

4.1.2. MtDNA polymorphisms and population genetic processes

MtDNA has: small size; relatively rapid rate of sequence divergence; predominantly maternal-haploid inheritance (inherited through the egg cytoplasm); absence of recombination, making it a single heritable unit which is effectively a single locus with multiple alleles; and simple genetic structure lacking complicated features such as repetitive DNA, transposable elements, pseudogenes, and introns. These features make it suitable for examining population genetic processes among closely related species and even within populations, including colonisation (founder) effects and population bottlenecks (reviewed in Avise *et al.*, 1987; Moritz *et al.*, 1987; Wilson *et al.*, 1985; Avise, 1991). Divergence in mtDNA sequences occurs more rapidly between populations than does divergence in nuclear DNA, due to an increased rate of DNA mutation in mtDNA as well as to a smaller effective gene flow between subdivided populations for mitochondrial genes compared with nuclear genes (Ovenden, 1990). In cases where dispersal patterns differ between sexes, mtDNA structure may not be representative of the whole population (Jones *et al.*, 1995).

A population that goes through a bottleneck could lose all its mitochondrial variability, but retain a significant fraction of its nuclear variability, since a single breeding pair contains four nuclear genomes, but only one transmissable mtDNA

haplotype (Wilson *et al.*, 1985). A founder effect can have a great influence on genetic distances, especially if the founder bore a rare type of mtDNA.

There are numerous studies using mitochondrial markers for phylogenetic analysis. Data on mtDNA variation in human populations have been especially revealing. The amount of variation in human populations, assessed by restriction analysis of 147 humans, suggests that a common female ancestor of all modern humans lived about 200 000 years ago, assuming a rate of divergence of 1-2 % per lineage per million years; data further indicate an African origin of all modern humans and suggest that each non-African population is the result of multiple colonisation events (Cann *et al.*, 1987). Because the human population has an anomalously low level of mtDNA variability, the occurrence of a bottleneck during human evolution is thought possible (Wilson *et al.*, 1985).

In bivalves, restriction analysis of the whole mitochondrial genome distinguished two subpopulations of the American oyster (*Crassostrea virginica*) in a continuous north-south distribution from the Atlantic coast of Canada to Texas (Reeb and Avise, 1990). The two genetic arrays differed by 2.6 % in nucleotide sequence, whereas allozyme allele frequencies showed uniformity throughout the distribution. Restriction analysis of mtDNA of mussel populations from south west England and south Wales revealed evidence of a hybrid population of *Mytilus galloprovincialis* and *M. edulis* (Edwards and Skibinski, 1987). Restriction site variation of the mitochondrial DNA of *Argopecten irradians* indicated separate populations off the coasts of North Carolina and Florida. Nucleotide sequence divergence was 0.33 % between geographically distant populations (Blake and Graves, 1995). In the same study, similar analysis of *A. gibbus* mtDNA did not reveal significant population differentiation.

However, features which have made mtDNA a popular choice for evolutionary studies, like maternal-haploid inheritance, and conservation of size, of gene content and of gene order, might be more variable than previously believed. The

rate of nucleotide substitutions can vary greatly between taxa, questioning the use of molecular clocks, while biparental inheritance can also occur (see below). Length variation has been observed between species, within species and even within one individual.

4.1.3. Inheritance of mtDNA

In most metazoans mitochondrial DNA is inherited through the maternal line. Probably due to a high disparity in mtDNA copy number in the male and female gametes, male mtDNA becomes 'outreplicated' (Meland *et al.*, 1991) (A mature egg cell contains about 10^5 mtDNA molecules, whereas a sperm contains about 50 (Hecht *et al.*, 1984)). Alternatively, active degeneration of sperm mitochondria during fertilization has been suggested (Longo, 1997). Maternal inheritance has important consequences. For instance, if only half of the population passes on its DNA, and no recombination takes place, mtDNA is clonally inherited, reducing the effective population size for mtDNA to one-fourth of that for the nuclear genes of the same organism (Nei and Tajima, 1981).

Recently, however, some evidence has emerged for biparental / paternal transmission of mtDNA in mice, *Mus* spp. (Gyllensten *et al.*, 1991), *Drosophila* (Kondo *et al.*, 1990), and anchovies, *Engraulis encrasicolus* (Magoulas and Zouros, 1993). In the *Mytilus* species complex, mtDNA inheritance is coupled with gender (Zouros *et al.*, 1992; Skibinski *et al.*, 1994; Rawson and Hilbish, 1995; Stewart *et al.*, 1995). Females receive their mother's mtDNA and pass it on to both their daughters and sons. In addition, males receive mtDNA from their father and transmit this male mtDNA to their sons (Stewart *et al.*, 1995). This results in females being homoplasmic for a mitochondrial genome ('F') transmitted to eggs, and males being heteroplasmic, containing both the female mitochondrial genome and another form ('M' genome) found only in males. In the male gonad, the paternal mtDNA is preferentially amplified and transmitted to sperm. The male and female lineages are highly distinct and pre-date the divergence of the three species *Mytilus edulis, M. galloprovincialis* and *M. trossulus* (Rawson and Hilbish, 1995; Stewart *et al.*, 1995).

4.1.4. Size variation

The mtDNA of animals is, in many cases investigated to date, of a stable size; for instance it is 16.5 kb for humans (Anderson *et al.*, 1981), 17.4 kb for the blue mussel (Skibinski, 1985), and 15.7 kb for sea urchins (Brown, 1981). However, extensive within-species length variation has been documented for protozoa and higher plants, while smaller length variations have been found mainly in ectotherms (Rand, 1993), for example in lizards (Moritz and Brown, 1986) and in scallops (Rigaa *et al.*, 1993; Fuller and Zouros, 1993; Gjetvaj *et al.*, 1992; La Roche *et al.*, 1990; Snyder *et al.*, 1987). A study on seven species of scallops (*Argopecten irradians, Placopecten magellanicus, Pecten maximus, Crassodoma gigantea, Aequipecten opercularis, A. hastata* and *A. islandica*) revealed large-scale length variation of mtDNA between the different species (Gjetvaj *et al.*, 1992). The variation was found to be entirely attributable to tandemly repeated sequences. When cloned repeated sequences were used as hybridisation probes, no sequence similarity was seen between species. Only *A. irradians* showed a typical 16.2 kb invariant size and lacked repeated sequences. The largest mitochondrial genome found to date in animals is from *P. magellanicus;* it also shows large intraspecific size variation (Snyder *et al.*, 1987; Gjetvaj *et al.*, 1992). Usually a large-scale intraspecific size variation of the mitochondrial DNA corresponds to changing numbers of copies of direct tandem duplications (repeated elements) (Bermingham *et al.*, 1986; Harrison, 1989; Hoeh *et al.*, 1991; Broughton and Dowling, 1994) although minor size differences caused by additional deletions or insertions have also been observed (Gjetvaj *et al.*, 1992). In most studies the length polymorphism due to repeated elements has been found to be located in the control region, whereas in both a nematode species (Beck and Hyman, 1988) and two species of scallops (La Roche *et al.*, 1990; Gjetvaj *et al.*, 1992) length polymorphism (repeated elements) was found to occur in several dispersed portions of the genome.

4.1.5. Variation within individuals: heteroplasmy

Most individual organisms are homoplasmic, that is they contain only a single mtDNA genotype. However, there are reports of mitochondrial DNA molecules showing different genotypes *within* one individual, a phenomenon known as heteroplasmy (Bermingham *et al.*, 1986; Harrison, 1989; Fisher and Skibinski, 1990; Hoeh *et al.*, 1991; Zouros *et al.*, 1992; Skibinski *et al.*, 1994; Rawson and Hilbish, 1995; Stewart *et al.*, 1995). Avise *et al.* (1987) and Harrison (1989) regard heteroplasmy as the initial stage of a new mtDNA variant, after a mutation in a single molecule, whereas Hoeh *et al.* (1991) suggest limited biparental inheritance (paternal leakage of mtDNA via sperm). Heteroplasmy as a consequence of single base changes is rarely mentioned in the literature, possibly because of the difficulties of its detection. Heteroplasmy involving length variation is reported more often and is mainly caused by different numbers of copies of a repeated element (Densmore *et al.*, 1985; Snyder *et al.*, 1987; Harrison, 1989). Restriction-site-heteroplasmy and size-heteroplasmy due to repeated elements were observed in most species of scallops examined (Gjetvaj *et al.*, 1992). Rigaa *et al.* (1993) found one individual among 27 *P. maximus* which showed size heteroplasmy (20.0 and 21.7 kb, carrying 2 and 3 copies of the repeated element, respectively). It is unclear at present whether size heteroplasmy develops during the the lifetime of an individual and is therefore an acquired rather than inherited trait. In general, the heteroplasmic state is probably quite transitory, due to rapid sorting of mtDNA molecules in germ cell lineages. As heteroplasmic individuals are rare they should have little impact in routine surveys of populations, although one should be cautious in cases where biparental inheritance is known to take place.

4.1.6. The organisation of the mitochondrial molecule of *P. maximus*

The mitochondrial DNA of *P. maximus* ranges in size from 20.0 to 25.8 kb due to different numbers of a repeated element which occurs in the control region (Gjetvaj *et al.*, 1992; Rigaa *et al.*, 1993, 1995). Located next to the control region are the genes for $tRNA^{gly}$, $tRNA^{Asn}$, $tRNA^{Ile}$, $tRNA^{Lys1}$, $tRNA^{Ala}$, 12S rRNA, $tRNA^{Lys2}$, 16S rRNA, and COI (D. Sellos, pers. comm.). No further information on

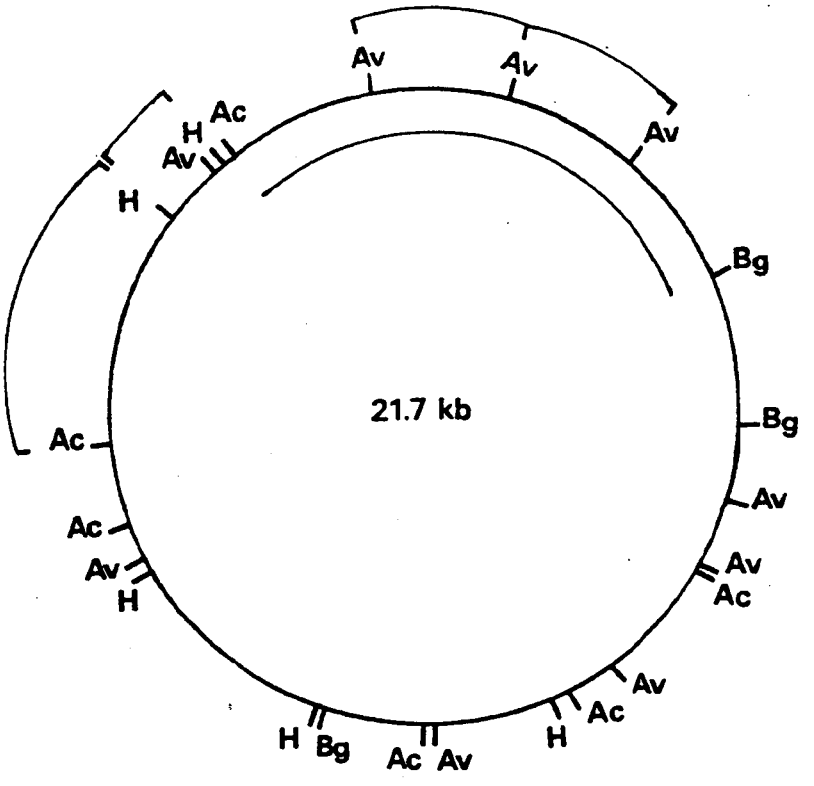


Figure 4.2. Circular map of restriction sites within the mtDNA of *P. maximus*. Restriction endonucleases: AccI (Ac), AvaI (Av), BglII (Bg), HindIII (H). The arc inside identifies the region containing repeated elements and the control region (from Rigaa *et al.*, 1993).

gene arrangements within the mitochondrial molecule of *P. maximus* is available to date. A restriction map of the mtDNA of *P. maximus* is shown in Figure 4.2.

4.1.7. Restriction analysis of PCR-amplified mtDNA fragments (PCR-RFLP)

The existence of unknown numbers of repeated elements within the mtDNA of one individual can considerably complicate the interpretation of restriction patterns derived from the whole mitochondrial molecule. Investigations of population structure using PCR-RFLP of mtDNA have been carried out on only few species, for example on honeybees (Garnery *et al.*, 1993), on *Daphnia* (Weider and Hobaek, 1994), and on tortoises (Osentoski and Lamb, 1995). Wilding *et al.* (in press) used PCR-RFLP to investigate population structure of *P. maximus* from the UK, Ireland and Brittany. No evidence of differentiation within and between the British and Brittany populations was found, but an Irish population (Mulroy Bay) was distinct from the others. In this Chapter, after initial confirmation of variability by restriction analysis of whole mtDNA, only non-size-variable parts of the mitochondrial genome were analysed for sequence differences. This was accomplished by PCR-amplification of mtDNA fragments not containing repeated elements, followed by digestion of these fragments with restriction enzymes (PCR-RFLP). This approach also renders unnecessary the time-consuming extraction of mitochondrial DNA. Other advantages of PCR-RFLP are that only small amounts of DNA are required (the mtDNA content obtained by a total DNA extraction is sufficient), and PCR-amplification can be repeated many times with the same sample, allowing screening with different enzymes.

4.2. Materials and Methods

4.2.1. Methods for isolation of mitochondrial DNA

Most studies using mtDNA have been performed with material isolated from total DNA extractions by CsCl-density-gradient ultracentrifugation (Sambrook *et al.*, 1982). This isolation procedure takes advantage of the different quaternary structures mtDNA shows (closed-circular, supercoiled and open-linear structure). The three forms intercalate different amounts of ethidium bromide and migrate to distinct positions after centrifugation in a density gradient (at ca. 40 000 rpm for 30 - 45 hours: White and Densmore, 1992). However, Skibinski (1985) found that mtDNA obtained by CsCl-gradient ultracentrifugation did not show an increased purity, due to a low yield of supercoiled mtDNA and a wide band of nuclear DNA in the gradient. As CsCl-gradient ultracentrifugation is very time-consuming and requires expensive equipment, short cuts for mtDNA isolation are desirable. Alternative methods for mtDNA isolation take advantage of the small size of the molecule compared to nuclear DNA, and its different solubility. To avoid contamination with nuclear DNA, intact mitochondria are isolated from homogenized tissue by differential centrifugation. The crude mitochondrial suspension obtained is purified on a sucrose-gradient (White and Densmore, 1992). This separates mitochondria from cell debris and nuclei, thus leading to relatively pure mitochondrial fractions. The mitochondrial membrane is then burst by alkaline lysis, modified from Birnboim and Doly (1979), or by means of a detergent like sodium dodecyl sulphate (SDS) (White and Densmore, 1992) or Nonidet. Hexadecyltrimethyl ammonium bromide (CTAB) (Fisher and Skibinski, 1990) can be added to remove polysaccharides. After lysis of the mitochondria, the solution containing mtDNA is further purified from proteins and lipids by phenol-chloroform extraction and mtDNA is precipitated with ethanol or isopropanol.

The following protocols for mtDNA extraction, all based on differential centrifugation, were tried in PEML (using Beckmann J2-HS centrifuge, maximal centrifugation speed 20 000 rpm, relative centrifugal field 31 000 x *g*):

a. White and Densmore (1992), SDS-phenol method and sucrose step gradient (Nonidet for lysis); b. Skibinski (1985), using MSB-EDTA, SDS; alternatively using Nonidet, CTAB and RNAse; c. Lansmann *et al.* (1981); d. Jones *et al.* (1988); e. Rigaa (1993) using 5 low-speed differential centrifugation steps, followed by a high speed centrifugation on a discontinuous sucrose gradient (SDS-lysis); f. Nucleobond-Kit (Macherey-Nagel, Dueren, Germany) for isolation of plasmids and cosmids (alkaline/SDS lysis based on the protocol of Birnboim and Doly (1979)); g. Tulva and Tulva (1993), using plasmid isolation kit; h. Boom *et al.*(1994), sucrose mini-gradient (protease *XX*III, Nonidet, CTAB); i. C. Wilding (pers. comm., May 1995) (protease *XX*III, Nonidet, CTAB, sucrose gradient).

For all protocols, fresh adductor muscle was used. Each protocol was repeated several times. Changes to the protocols were made one at a time - in particular the speed and the duration of centrifugation were altered. As the maximum speed of the centrifuge (Beckmann J2-HS) available at PEML was 20 000 rpm, it was not possible to carry out centrifugations at higher speeds as specified by most protocols. Similarly, all centrifugations had to be carried out with a fixed-angle rotor instead of the required swing-out rotor.

In summary, the protocols of Skibinski (1985) and C. Wilding (pers. comm., May 1995) yielded a small amount of mtDNA, but the other protocols failed to extract any DNA. It was concluded that the speed of the centrifuge available at PEML is not sufficient to pellet mitochondria, and the fixed-angle rotor is disadvantageous for a sucrose-gradient.

Using an ultracentrifuge with a swing-out rotor (30 000 rpm, 100 000 x *g*) at the MBA, Plymouth, extractions of mtDNA could be carried out successfully.

4.2.2. Protocol for extraction of mtDNA from *P. maximus*

The following protocol for extraction of mtDNA from *P. maximus* was finally applied to all subsequent mtDNA extractions. It is based on the technique of Snyder *et al.* (1987) and includes modifications of Boulding *et al.* (1993), a CTAB incubation to remove polysaccharides (Fisher and Skibinski, 1990) and other minor adjustments.

4.2.2.1. Isolation of intact mitochondria

Approximately 5g of adductor muscle tissue was minced, transferred to ice cold 50 ml centrifugation tubes containing 10 ml isotonic buffer (see Appendix) and homogenised with an electric homogenizer (Citenco) until no tissue flakes were visible in the solution (highest setting for ca. 1 min). 2 ml of 200 mg/ml protease type XXIII (Sigma) was added to each tube. After incubation in a shaker at 37°C for at least three hours, the solution had changed from a pale-brown viscous 'soup' to a dark-brown watery liquid. 10-15 ml isotonic buffer was added and the tubes centrifuged (Heraeus Megafuge 1.0R, swing-out rotor) for 10 min at 4300 rpm and 4°C to pellet residual debris and intact nuclei. The supernatant was poured into new tubes and centrifuged (Beckman L8-70M Ultracentrifuge, swing-out rotor) for 35 min at 19000 rpm and 4°C to pellet the mitochondria. If the resulting pellet was loose, this step was repeated after resuspending the pellet in 10-5 ml isotonic buffer. The supernatant was aspirated off and the pellet was resuspended in 3 ml isotonic buffer. A sucrose gradient (Lansman *et al.*, 1981) was prepared by underlaying 5 ml 1 M sucrose (see Appendix) with 2.5 ml 1.5 M sucrose (see Appendix) in a 10 ml centrifuge tube. The resuspended mitochondrial pellet was carefully pipetted on top of the sucrose layers. Centrifugation for 50 min at 30000 rpm and 4°C was carried out. The band at the 1M/1.5M interface of the gradient contained the mitochondria and was taken off with a Pasteur pipette. 10-15 ml isotonic buffer was added and the mitochondria were pelleted for 30 min at 20000 rpm and 4°C. The resulting pellet containing the intact mitochondria was resuspended in STE (see Appendix) to a final volume of 600 µl and transferred to a microfuge tube on ice.

4.2.2.2. Extraction of mitochondrial DNA

To lyse the mitochondria, 80 µl 10 % (v/v in H_2O) Nonidet P-40 (Sigma) was added, and the tubes left on ice for 15 min. Subsequently mitochondrial debris was pelleted by centrifugation at 12000 *g* for 3 min in a microcentrifuge (Eppendorf 5415C). 600 µl of the supernatant was pipetted into a new microcentrifuge tube. 100 µl 5M NaCl and 80 µl prewarmed CTAB (see Appendix) were added to precipitate polysaccharides and the tubes placed in a water bath at 37°C for 30 min. After cooling the tubes for a few minutes, one volume of chloroform / isoamyl alcohol (24:1) was added, the solution mixed on a rotator and centrifuged in a microfuge at 12000 *g* for 3 min. Two phenol/chloroform extractions and a final chloroform / isoamyl alcohol extraction were carried out. To the final aqueous phase 1/10th vol of 3 M sodium acetate (pH 5.2) and 0.6 vol of isopropanol were added and the samples kept at -20°C overnight. After spinning at 13000 rpm for 30 min, the pellet was washed with 70 % ethanol and air-dryed until translucent, then re-dissolved in 50 µl molecular-grade water.

4.2.3. General protocol for restriction digests of (whole) mtDNA

Restriction digests were performed in a total volume of 20 µl, including ca. 0.5 - 1.0 µg DNA, 2 µl 10 x buffer, 2-3 units restriction enzyme, and water *ad* 20 µl. The tubes were incubated in a PCR-machine (Perkin Elmer TC1) for 1-3 hours at the appropriate temperature for the restriction enzyme. Subsequently, the samples were cooled to 4°C and 4 µl bromophenol blue mix (BPB, see Appendix) was added before loading the samples on a 1% or 1.4% agarose gel in TBE buffer. Electrophoresis took place over 6 h at 80 V.

MtDNA from *P. maximus* originating from the Isle of Man and Plymouth was digested with the restriction endonucleases HindIII, EcoRI, ClaI, PvuII, BamHI, AvaI, DraI, HpaI, and AsnI (see Chapter 2, Tables 2.3. and 2.4., for features of restriction enzymes and buffer concentration).

4.2.4. Design of primers for amplification of part of the mitochondrial genome by PCR

To amplify only parts of mitochondrial DNA by PCR and circumvent the need for mtDNA extraction, specific primers have to be designed, based on sequence information either obtained directly by sequencing of cloned mtDNA fragments or from publications. As many regions among mitochondrial genes are conserved, the possibility exists that primers, originally designed for one species, work across species ('universal' primers) (Kocher *et al.*, 1989). In this study, three different approaches were used to obtain primers for amplification of *P. maximus* mtDNA: (1) use of 'universal primers'; (2) design of primers from published mtDNA sequences of *P. maximus*; (3) cloning and sequencing of mtDNA fragments with the intention of developing new primers (for cloning see Chapter 5). In addition, a primer pair (PMA, designed by C. Wilding, University of Wales, Bangor) was obtained from G. Dahle, Institute of Marine Research, Bergen, Norway.

4.2.4.1. Primers for amplification of the cytochrome *b* / 12S rRNA genes

At the beginning of this study, amplification of as large as possible a fragment of mtDNA was attempted, based on the protocol of Barnes (1994a). DNA sequences of various species published in databases of the EMBL were searched for conserved regions of the mitochondrial genome to enable the design of 'universal' primers. Two primers, one for a 37-base region of the 12S rRNA-gene (primer cyt*b*-f) and one for a 34-base region of the adjacent cytochrome *b*-gene (primer cyt*b*-r) were constructed this way:

cyt*b*-f 5' GGC GCT GAC GAC CGT GGA CCA ACC TGA GGA GAT TAT T
37 bases, forward primer
cyt*b*-r 5' AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A
34 bases, reverse primer

4.2.4.2. PCR conditions for attempted 'long PCR'

The reaction mixture of 1µl total DNA, 100 pM of each primer cyt*b*-f and cyt*b*-r (synthesised by the Department of Biochemistry, University of Liverpool), 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Sigma), PCR-buffer (50mM Tris-HCl, pH 9.2; 3.5 mM $MgCl_2$; 16 mM $(NH_4)_2SO_4$, 150 µg/ml BSA), and water *ad* 100 µl was overlaid with mineral oil. After a hot start of 3 min at 94°C, 25 units Mobi*Taq*-L-polymerase (a *Taq*-polymerase with proof-reading function; MoBiTec, Goettingen, Germany) were added and the samples were subjected to 25 cycles of 20 sec 94°C, 11 min 70°C.

The cyt*b* primer pair gave a range of different bands after PCR, and further adjustment of PCR-conditions would be necessary to obtain a usable PCR product. This was not pursued further.

4.2.4.3. Primers for the 12S rRNA and 16S rRNA genes ('DPM')

Based on the sequences of parts of the 12S and 16S rRNA genes of the mitochondrial genome of *P. maximus*, published by D.Y. Sellos (1995) in the EMBL/GenBank/DDBJ databases (see Appendix), a primer pair was designed for amplification of these genes, referred to below as DPM. Primers were checked for their suitability with the program PRIMER (The Whitehead Institute for Biomedical Research, 1991).

primer 12S 5' AGA TCG ACC CGA GGA AGT AGG G
forward primer, 22 b, Tm = 58.4 C
primer 16S 5' CAC AGT ACA GGC CAT CAC CTG G
reverse primer, 22 b, Tm = 58.4 C

4.2.4.4. PCR-conditions for DPM

The DPM primer pair amplified a fragment of ca. 3 kb under the following PCR-conditions: total genomic or mtDNA 1µl (the optimal concentration of template DNA was determined in preliminary trials), 25 pmol of each primer (synthesized by Perkin Elmer, Warrington), 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Sigma), 5 µl 10x PCR-buffer (100mM Tris-HCl, pH 9.0; 500 mM KCl; 1.0 % Triton X-100; Promega Ltd., Southampton, UK), 3.75 mM $MgCl_2$ (Promega), and water *ad* 50 µl (molecular biology grade, BDH, Poole, UK). The reaction mixture was overlaid with mineral oil (Sigma, Poole, UK). After a hot start of 3 min at 94°C, 1.5 units *Taq*-polymerase (Promega) was added and the samples were subjected to 25 cycles of 30 sec 94°C, 1 min 60°C, 3 min 72°C.

4.2.4.5. Primers for an anonymous 2 kb fragment (PMA)

According to the restriction mapping carried out by Rigaa *et al.* (1993), the 2kb HindIII/EcoRI fragment does not contain repeated elements. In the following, the primer pair for the 2 kb fragment is referred to as PMA. There is some indication of sequence similarity to the cytochrome *b* gene (Wilding, 1996).

PMA TTT TAA GGA GGT AAT CGC TAT TCG forward primer, 24 bp
PMA CAG CAA TCT GTA TGG GTA GAA CC reverse primer, 23 bp

4.2.4.6. PCR-conditions for PMA

Best results (a single fragment, high yield) were obtained under the following conditions: total DNA 1µl (the optimal concentration of template DNA was determined in preliminary trials), 0.25 µmol of each primer (synthesised by Cruachem, Glasgow), 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Sigma), PCR-buffer (100mM Tris-HCl, pH 9.0; 500 mM KCl; 1.0 % Triton X-100; Promega Ltd., Southampton, UK), 3.75 mM $MgCl_2$ (Promega), and water *ad* 50 µl (molecular biology grade, BDH, Poole, UK). The reaction mixture was overlaid with mineral oil (Sigma, Poole, UK). After a hot start of 3 min at 94°C, 1.5

units *Taq*-polymerase (Promega) was added and the samples were subjected to 25 cycles of 45 sec 94°C, 30 sec 52°C, 2 min 72°C.

4.2.5. Restriction digests

In a preliminary experiment, both the 3 kb (DPM) and the 2 kb (PMA) PCR-fragments were digested with a range of restriction enzymes (AluI, RsaI, MvaI, DraI, PvuII, AvaI, BamHI, HindIII, HpaI, EcoRV, ClaI, EcoRI, CfoI, HaeIII, HinfI, TaqI, Tru9I) and combinations of these. Tru9I created fragments too small for analysis on agarose gels (< 300 bp). The following restriction enzymes produced polymorphic bands in 6-12 individuals and were chosen for population analysis: HaeIII, MspI, RsaI, TaqI, and the combination DraI/PvuII. The amplified DPM (3 kb) and PMA (2kb) fragments of two year-classes of 24 individuals from each of the locations Bradda Inshore, Targets, Ramsey, East Douglas and Chicken Rock, plus Peel (only one year-class), were subjected to restriction analysis. Samples from Mulroy Bay and Plymouth (24 individuals each) were included as outgroups. For each fragment, a total number of 336 individuals was analysed with the six restriction enzymes.

The digest was carried out in 0.5 ml microfuge tubes, containing 4-6 µl PCR-product, 3 units restriction enzyme, 2µl 10x buffer and water *ad* 20 µl. The samples were incubated for 3 hours at the appropriate temperature in the Perkin Elmer DNA thermal cycler (model TC1, Perkin Elmer, Warrington, UK). The reaction was stopped by adding 4 µl BPB and samples were electrophoresed through 1% or 1.4% agarose gels (MP-agarose, Boehringer-Mannheim, Lewes, UK), containing ethidium bromide (BDH), in 1x Tris-borate-EDTA-buffer (pH 8.3, Sigma), for 12-14 hours at 60 V. After de-staining the gel for several hours in distilled water, it was photographed with a polaroid camera (DS34, Polaroid, Herts, UK) and monochrome negative film type 665 (Polaroid).

4.2.6. Data analysis

Restriction sites were scored as present or absent, and haplotypes for each restriction enzyme were designated A, B, C, etc., whereby A was the commonest. A composite haplotype then described the haplotype composition of each individual for all enzymes employed. Both fragments and both year-classes were analysed separately. Data for both fragments were also combined and analysed separately for both year-classes and also with the two year-classes together. Only individuals for which complete banding information was available were included in the analysis.

If populations are genetically discrete, then the amount of diversity within each population will be less than the amount of diversity between populations. This can be assessed by correcting the magnitude of inter-population diversity for intra-population 'noise'. An overall weighted estimate of divergence ('*d*') between each pair of composite haplotypes was generated from a binary character state matrix of restriction site data according to Nei and Tajima (1981) and Nei and Miller (1990, eq. 4)(D program in the REAP package). Weighting followed Nei and Tajima (1983) and was based on the proportion of sites generated by each class of enzyme. Based on the dissimilarity matrix of *d* values, and a frequency distribution matrix (number of each haplotype in each population), haplotype and nucleotide diversity within populations, and nucleotide divergence between populations, were calculated following Nei and Tajima (1981) and Nei (1987) (DA program in REAP). Total nucleotide sequence diversity between two populations was corrected for within-population polymorphisms by subtracting the average of within-sample diversities. The resulting nucleotide divergence among populations was visualized by UPGMA cluster diagrams (NTSYS-pc). UPGMA cluster diagrams were also constructed for nucleotide divergence with negative values set to zero. This resulted in a number of possible trees (ties), which were combined in a majority-rule consensus tree (NTSYS-pc).

The high number of haplotypes found, resulting in low sample numbers per cell, can invalidate X^2 analysis. This problem can be overcome by comparing the magnitude of the X^2 values obtained with the magnitude of many pseudo-X^2 values

obtained from random rearrangements of the data table. Monte Carlo simulation (Roff and Bentzen, 1989) was carried out to assess the significance of haplotype frequency variation between all locations and between Manx locations only (MONTE in REAP). A mean X^2 value was determined by randomizing the matrix of haplotype frequency data ten thousand times, and the probability of encountering a X^2 value as large as that calculated for the original matrix was thereby determined.

Based on a matrix of squared Euclidian distances (Nei and Tajima, 1981) among haplotypes (NTSYS-pc), molecular analysis of variance (AMOVA: Excoffier *et al.*, 1992) was used to partition variance between and within populations and regions, and to provide pairwise estimates of population differentiation in the form of phi_{ST} values. Phi_{ST} is an F_{ST}-analog, developed to adjust the statistics for haplotype data (Excoffier *et al.*, 1992). (F_{ST} may be interpreted as the variance of allele frequencies among populations, standardized relative to the maximum value possible given the observed mean allele frequency (Weir and Cockerham, 1984).) One thousand permutations of the original data matrix were performed to test for significance of the variance components and phi_{ST} values, avoiding the assumption of normality. Data were also subjected to a Bartlett's test for heteroscedasticity (Stewart and Excoffier, 1996) to test for unequal genetic variability between populations.

For the combined fragments, a Mantel matrix comparison (NTSYS-pc) was applied to the matrices of nucleotide divergence for the 1995 and 1996 samples at the five Manx locations, testing for possible correlation between the year-classes. The significance of Mantel's normalized Z was tested by 1000 random permutations.

4.3. Results

4.3.1. Restriction digests of the whole mtDNA molecule

The pattern and length of the fragments obtained after restriction digestion of the whole mtDNA molecule was in most cases consistent with those published by Rigaa *et al.* (1993) (Plate 4.1.A.); due to a large amount of RNA on the agarose gel, fragments smaller than 0.80 kb could not be detected. BamHI produced two fragments of 11.9 and 9.65 kb. DraI produced a range of fragments difficult to distinguish from each other, between 3 and 1.5 kb. AvaI gave fragments of 4.45, 3.45, 3.40, 2.70, 2.15, 1.60 and 1.50 kb, and the 1.60 kb fragment was assumed to occur in several copies. EcoRI produced four fragments of 9.90, 6.30, 2.95, and 2.0 kb. HindIII showed three fragments of 11.8, 3.85, 2.6 kb. Digestion with PvuII resulted in three fragments of ca. 7, 10 and 3.5 kb. Four fragments of ca. 9, 5.3, 3.4, and 1.0 kb could be seen for AsnI. ClaI produced three fragments of ca. 12, 6.2, and 2.3 kb. HpaI gave four fragments of 8.35, 5.75, 3.65 and 2.3 kb; the 0.8, 0.7 and 0.1 kb fragments could not be seen.

Plate 4.1.B. shows the banding pattern resulting from a digest with EcoRI and AccI. Two haplotypes can clearly be distinguished, with individuals 1, 4 and 5 differing from individuals 2,3 and 6. HpaI digests of mtDNA from six individuals is shown in Plate 4.1.C. Again, two haplotypes can be distinguished: in individuals 1, 4 and 5 the largest band (present in individuals 2, 3 and 6) is replaced by two bands of ca. 5.5 and 2.8 kb, indicating an additional restriction site.

4.3.2. Restriction analysis of PCR-products

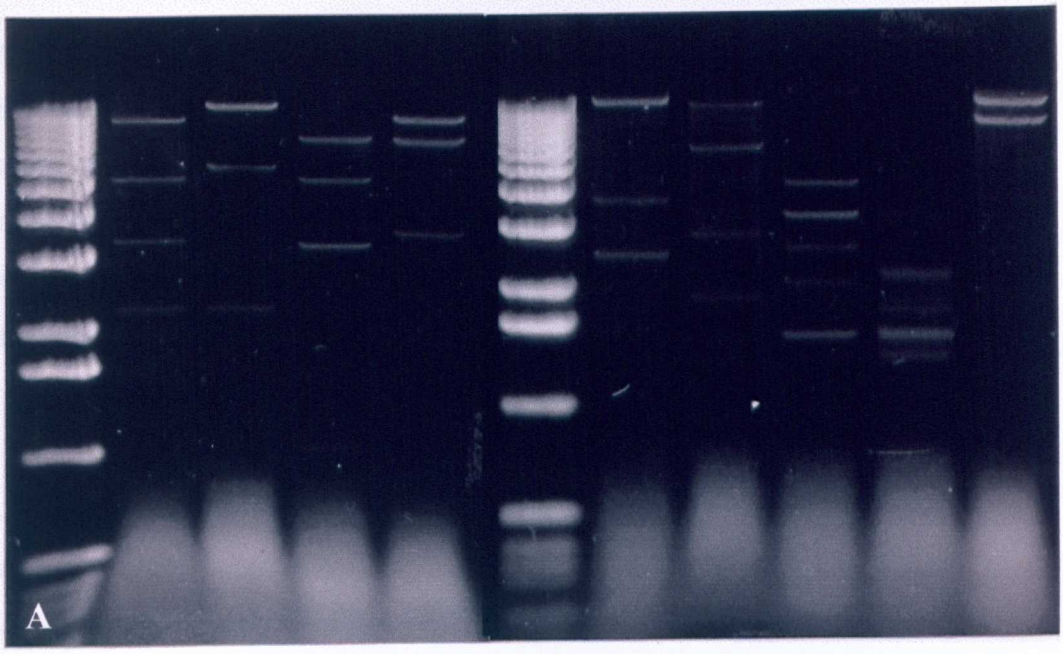
PCR products did not differ in size between individuals. All PCRs gave a high yield of product, which made it possible to see most bands, even small ones, after electrophoresis of restriction digests. In most cases, the restriction sites could be easily worked out by comparing the different banding patterns obtained. However, in a few cases, the presence of smaller bands had to be assumed to make up the total length of the fragment. For data on presence/absence of restriction sites see

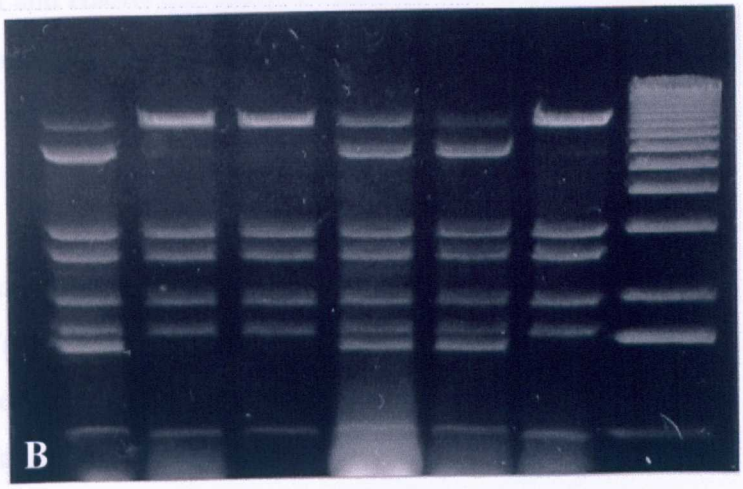
Plate 4.1.

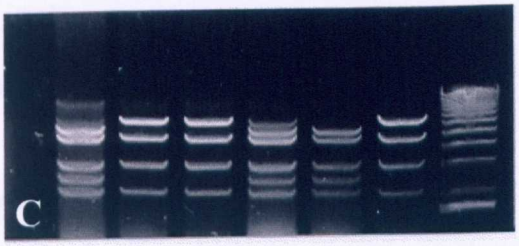
A. 0.7 % agarose gel, stained with ethidium bromide; fragments resulting from restriction digestion of *P. maximus* (whole) mtDNA (20 µl on gel). Restriction endonucleases: Lane 2 HpaI, Lane 3 ClaI, Lane 4 AsnI, Lane 5 PvuII, Lane 7 HindIII, Lane 8 EcoRI, Lane 9 AvaI, Lane 10 DraI, Lane 11 BamHI (Lanes 1 and 6: 1 kb-marker).

B. EcoRI and AccI double-digests of whole mtDNA from six individuals. Lane 7: 1kb-marker. (The 2.9 kb fragment was gel-purified and used for cloning with the pBluescript.)

C. HpaI digests of whole mtDNA from six individuals. Lanes 1-6: mt-digests of different individuals; Lane 7: 1kb-marker (The 2.4, 3.7 and 5.8 kb fragments were gel-purified and used for cloning with the pCR-script-kit.).


A


B


C

Appendix. The combined length of the two fragments was ca. 5.0 kb, thus 25 % of the total mtDNA was screened, assuming a mitochondrial genome size of 20.0 kb (including two repeated elements). The recognition sequences of the six enzymes accounted for approximately 1.22 % of all nucleotides. One heteroplasmic individual was encountered in the Bradda 96 population (Plate 4.2.) and was not included in the data analyses.

4.3.3. Separate analyses of both fragments DPM and PMA and of both year-classes

4.3.3.1. Restriction analysis of the DPM fragment

The fragments obtained after restriction analysis with the enzymes DraI/PvuII, RsaI, TaqI, MspI and HaeIII are listed in Table 4.1. Plate 4.2. shows the corresponding banding patterns on the agarose gel, Plate 4.4. shows examples of the banding patterns obtained after restriction digests of individuals from different populations.

The overall number of haplotypes was 25 for the 1995 samples and 22 for the 1996 samples (Tables 4.2. and 4.3.). In the 1995 samples, Targets and Mulroy had the lowest number of haplotypes (6), Ramsey and Chickens the highest (9). In the 1996 samples, Targets and Ramsey had the lowest number of haplotypes (5), Douglas the highest (8). In both year-classes, the main haplotypes were AAAAA (43.98% and 57.07% of individuals) and AABBA (27.11% and 25.13%), which differed in two restriction sites. 15 (9.3 % of individuals) and 11 (5.76 %) unique haplotypes occurred in the samples from 1995 and 1996, respectively.

Haplotype diversity was lowest for Mulroy (0.50) and high for Plymouth (0.73). Amongst the Manx samples, it was highest in 1995 for Ramsey and Chickens (0.80). Mean nucleotide sequence diversity was low in Mulroy (0.55), and in the 1995 samples lowest in Douglas (0.54), and highest in Ramsey (0.89). In the 1996

samples, Douglas (0.48) had the lowest value, Chickens the highest (0.69) (Table 4.4. and 4.5.).

The X^2 tests including all populations gave similar values in both year-classes (144.66 and 142.51), as did X^2 tests including the Manx populations only (76.67 and 78.95). However, the Monte Carlo procedure indicated that none of the values were significant (Table 4.20.).

A UPGMA cluster analysis of the nucleotide divergence values for DPM95 showed a cluster consisting of samples from Bradda and Ramsey, with Chickens associated, plus a cluster of Targets and Plymouth, and a more distant one of Douglas and Mulroy (Figure 4.3.). This was generally the same for the DPM96 fragment, except that the additional population Peel clustered with Chickens (Figure 4.4.). The consensus trees for DPM95 and DPM96, with the negative values set to zero, both showed Douglas and Mulroy clustering together and a separate cluster consisting of all other populations (Figures 4.5. and 4.6.).

Plate 4.2.

Banding patterns obtained after restriction digests of the DPM-fragment with various enzymes:

A. RsaI, HaeIII

Lane 1: 1 kb-marker, Lanes 2 - 6: RsaI, haplotypes: A, E, C, B, A; Lane 7: 100 bp-marker; Lane 8: 1 kb-marker; Lanes 9 -13: HaeIII, haplotypes: B, A, C, B, A; Lane 14: 100 bp-marker, Lane 15: 1 kb-marker.

B. MspI

Lane1: 1kb-marker; Lane 2: 100 bp-marker; Lanes 3 - 7: haplotypes E, A, C, D, D; Lane 8: 100 bp-marker.

C. TaqI

Lane 1: 100 bp-marker; Lanes 2 - 9: haplotypes A, A, D, E, D, C, B, A; Lane 10: 1 kb-marker.

D. DraI/PvuII

Lane 1: 1 kb-marker; Lanes 2 - 10: haplotypes A, D, D, undigested, B, B, A/B, A, A; Lane 11: 100 bp-marker; Lane 12: LW-marker. Arrow marks heteroplasmic individual A/B.

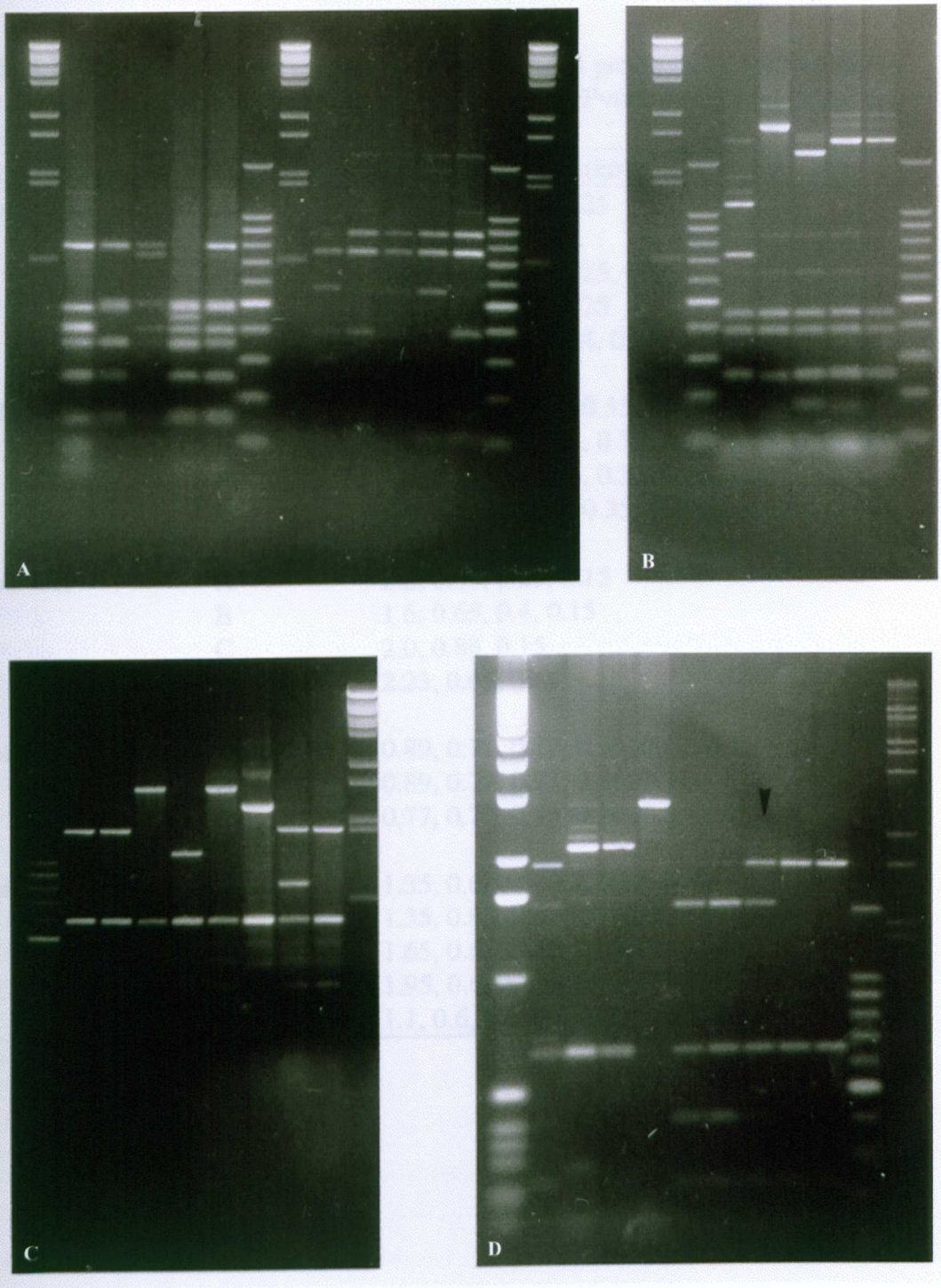

A
B
C
D

Table 4.1. Number of haplotypes and size of fragments produced by restriction digestion of the DPM fragment with the enzymes DraI/PvuII, RsaI, TaqI, MspI and HaeIII.

Restriction enzyme	Haplotypes	Size of fragments (kb)
MspI	A	1.85, 0.45, 0.4, 0.25
	B	2.3, 0.4, 0.25
	C	1.65, 0.45, 0.4, 0.25, 0.2
	D	1.73, 0.45, 0.4, 0.25
	E	1.1, 0.75, 0.45, 0.4, 0.25
RsaI	A	0.8, 0.5, 0.5, 0.4, 0.35, 0.25, 0.15, 0.05
	B	0.5, 0.5, 0.45, 0.4, 0.35, 0.35, 0.25, 0.15, 0.05
	C	0.8, 0.75, 0.5, 0.4, 0.35, 0.15, 0.05
	E	0.8, 0.5, 0.4, 0.4, 0.35, 0.25, 0.15, 0.1, 0.05
Dra/PvuII	A	2.0, 0.65, 0.23, 0.15
	B	1.6, 0.65, 0.4, 0.15
	C	2.0, 0.88, 0.15
	D	2.23, 0.65, 0.15
HaeIII	A	0.89, 0.77, 0.77, 0.39, 0.17, 0.11
	B	0.89, 0.77, 0.77, 0.56, 0.11
	C	0.77, 0.77, 0.59, 0.39, 0.3, 0.17, 0.11
TaqI	A	1.35, 0.6, 0.6, 0.3, 0.23, 0.17
	B	1.35, 0.83, 0.6, 0.3, 0.23, 0.17
	C	1.65, 0.6, 0.6, 0.23, 0.17
	D	1.95, 0.6, 0.3, 0.23, 0.17
	E	1.1, 0.6, 0.6, 0.3, 0.25, 0.23, 0.17

Table 4.2. Numbers of each composite haplotype observed in DPM, 1995; order of restriction enzymes: MspI, RsaI, DraI/PvuII, HaeIII, TaqI. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M	P
AAAAA	8	8	14	5	11	17	10
AABBA	9	7	3	9	6	3	8
ADBBC	1						
AABBB	2	2		2	1		
CAAAA	1						
AADAA	1		2		1		1
ABBBA	1	1					
AAABA		1			1		2
AABBD		1					
AAACA		1					
AACAA		1	1	1			
ACAAA		1					1
DAAAA			1				
AEAAA			1				
AAAAB			1	1			1
AAAAD			1				
BAABA				1			
ABAAA				1			
EAAAA				1			1
CABBA				1			
BABBA					1		
ADBBB						1	
AABCB						1	
BAAAA						1	
AABAA						1	

Table 4.3. Numbers of each composite haplotype observed in DPM, 1996; order of restriction enzymes: MspI, RsaI, DraI/PvuII, HaeIII, TaqI. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M	P
AAAAA	14	12	15	14	13	13	17	10
AABBA	5	7	3	7	9	6	3	8
BABBC	1							
ABAAA	1							
DAAAA	1		1					
AABBD	1							
AAACA		1			1			
AADAA		2				1		1
AABBB		1			1	1		
AACAA			1					
ADAAA		1	1		1	1		
ACAAA			1	1				1
CAAAA			1					
AAABA			1					2
AAAAE				1				
ABBBA				1				
ABBAA						1	1	
ABAAB						1		1
ADBBB							1	
AABCB							1	
BAAAA							1	
EAAAA								1

Table 4.4. DPM95, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M	P
no. individuals	23	23	24	22	21	24	24
no. haplotypes	7	9	8	9	6	6	7
haplotype diversity	0.74	0.80	0.66	0.80	0.67	0.50	0.73
% mean nucleotide sequence diversity	0.83	0.84	0.54	0.89	0.67	0.55	0.67

Table 4.5. DPM96, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M	P
no. individuals	23	24	24	24	24	24	24	24
no. haplotypes	6	6	8	5	5	7	6	7
haplotype diversity	0.60	0.68	0.61	0.59	0.59	0.66	0.50	0.73
% mean nucleotide sequence diversity	0.68	0.69	0.48	0.59	0.60	0.63	0.55	0.67

In the AMOVA, DPM95 showed significant variance components between the Manx locations and between individuals within locations (Table 4.6.). After Bonferroni correction, pairwise phi_{ST} values remained significant for Bradda and Douglas, Ramsey and Douglas, and Chickens and Douglas (Table 4.7.). No significant heterogeneity of variances between populations was revealed for DPM95 by the Bartlett's test (probability value $p = 0.10$). No significant variance components and phi_{ST} values were found for DPM96, nor was significant heterocedasticity present ($p = 0.91$) (Table 4.8. and 4.9.).

Table 4.6. Analysis of molecular variance for DPM95

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	2.38	1.19	-0.02	-2.47	0.57
Among locations within regions (Isle of Man region only)	4	7.18	1.80	0.05	6.76	0.01
Among individuals within locations	154	106.51	0.69	0.69	95.71	0.01

Table 4.7. Phi_{ST} values for DPM95; *, ** significance after Bonferroni correction (+, ++, +++ significance before Bonferroni correction). *, + $p < 0.05$; **, ++ $p < 0.01$; +++ $p < 0.001$. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	-0.0293					
D	0.2088**, ++	0.1547*,++				
R	-0.0366	-0.0314	0.2106**, +++			
T	-00020	-0.0233	0.0917+	0.0008		
M	0.1352+	0.0880+	-0.0057	0.1369++	0.0325	
P	0.0179	-0.0120	0.0729	0.0198	-0.0398	0.0258

Table 4.8. Analysis of molecular variance for DPM96

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	1.09	0.55	0.001	0.01	0.32
Among locations within regions (Isle of Man region only)	5	2.72	0.54	-0.002	-0.38	0.49
Among individuals within locations	183	109.36	0.60	0.58	100.37	0.51

Table 4.9. Phi_{ST} values for DPM96 (no significant values). Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M
C	-0.0257						
D	0.0156	0.0350					
R	-0.0335	-0.0287	0.0344				
T	-0.0122	-0.0243	0.0985	-0.0230			
E	-0.0268	-0.0376	0.0269	-0.0280	-0.0174		
M	-0.0095	-0.0045	-0.0092	0.0042	0.0042	-0.0179	
P	-0.0202	-0.0308	0.0600	-0.0299	-0.0314	-0.0244	0.0258

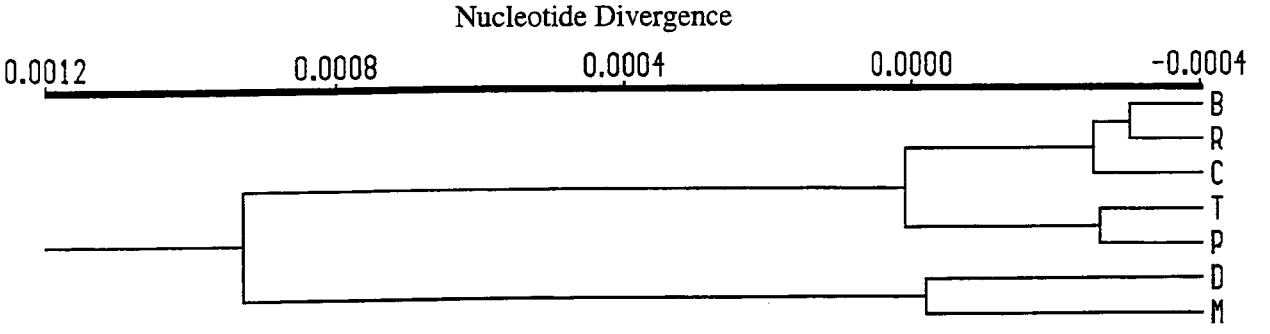


Figure 4.3. UPGMA cluster diagram based on nucleotide divergence for DPM95. Abbreviations as in Figure 2.2.

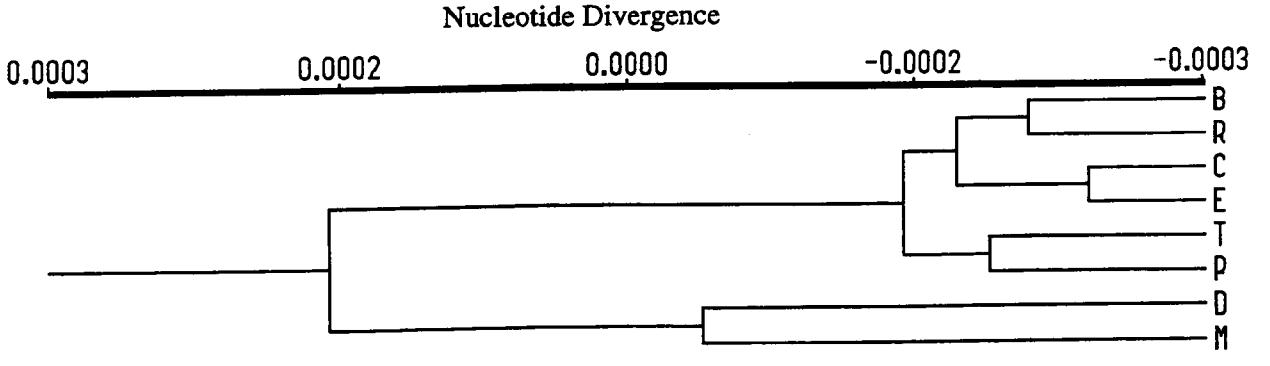


Figure 4.4. UPGMA cluster diagram based on nucleotide divergence for DPM96. Abbreviations as in Figure 2.2.

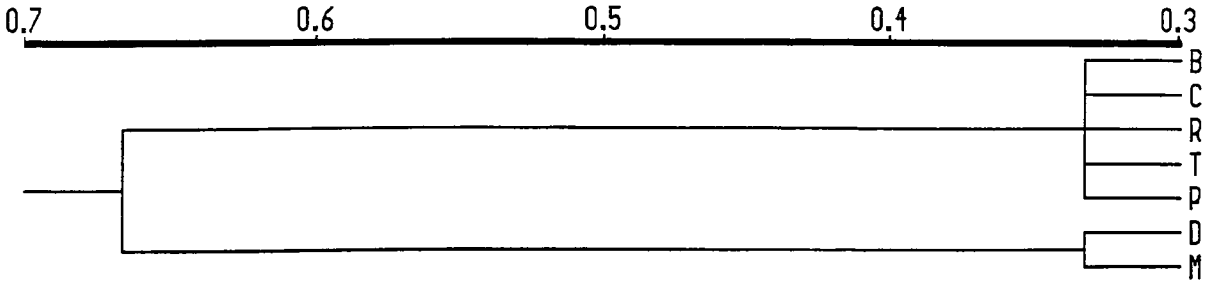


Figure 4.5. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for DPM95. Abbreviations as in Figure 2.2.

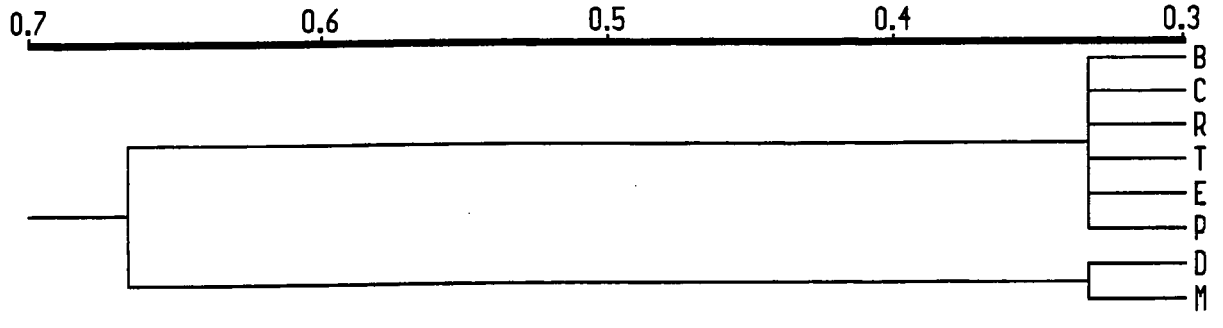


Figure 4.6. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for DPM96. Abbreviations as in Figure 2.2.

4.3.3.2. Restriction analysis of the PMA fragment

Contrary to Wilding *et al.* (in press), CfoI-digests on 48 individuals and HinfI digests on 24 individuals from different locations did not show any polymorphisms, and both enzymes were therefore excluded from the study. The result of the restriction digests of the PMA fragment with the enzymes DraI/PvuII, RsaI, TaqI, MspI and HaeIII is shown in Plate 4.3. The size of the fragments produced is summarized in Table 4.10.

The overall number of haplotypes was 28 for PMA95 and 27 for PMA96. For PMA95, eight different haplotypes occurred in Chickens, nine in Mulroy and Targets, and ten in Bradda, Douglas, Ramsey and Plymouth (Table 4.11. and 4.12.). For PMA96, Ramsey had the lowest number of haplotypes (7), Chickens the highest (12). In both year-classes, the main haplotypes were AAAAA (37.96% and 45.03% of individuals) and BBAAB (15.66% and 13.08%), which differed in three restriction sites. The percentage of unique haplotypes was 6.63% for PMA95 and 6.28% for PMA96. Haplotype diversity was lowest in the Mulroy Bay sample (0.66), highest in Plymouth (0.87). Within the Manx PMA95, Douglas and Ramsey had low values (0.67, 0.69), and Bradda the highest (0.80) (Table 4.13.). PMA96 showed lowest haplotype diversity in Douglas (0.66), and the highest in Chickens and Peel (0.84, 0.82) (Table 4.14.). Nucleotide sequence diversity was again lowest in Mulroy, and highest in both year-classes in Chickens. All X^2 results were non-significant (Table 4.20.).

Whereas the UPGMA cluster analysis for PMA95 was very similar to both DPM95 and DPM96, PMA96 gave a different result, with Douglas clustering with Bradda and Ramsey, and Mulroy being separate from all other populations (Figure 4.7. and 4.8.). When the negative values were set to zero, the consensus tree for PMA95 showed a cluster of Bradda, Ramsey, Targets and Plymouth; Chickens was associated with this group and Douglas and Mulroy formed a separate cluster (Figure 4.9.) However, the consensus tree for PMA96 showed a cluster of Bradda, Chickens, Douglas and Ramsey. Targets and Plymouth clustered separately, as did Peel; Mulroy did not cluster with any other population (Figure 4.10.).

Plate 4.3.

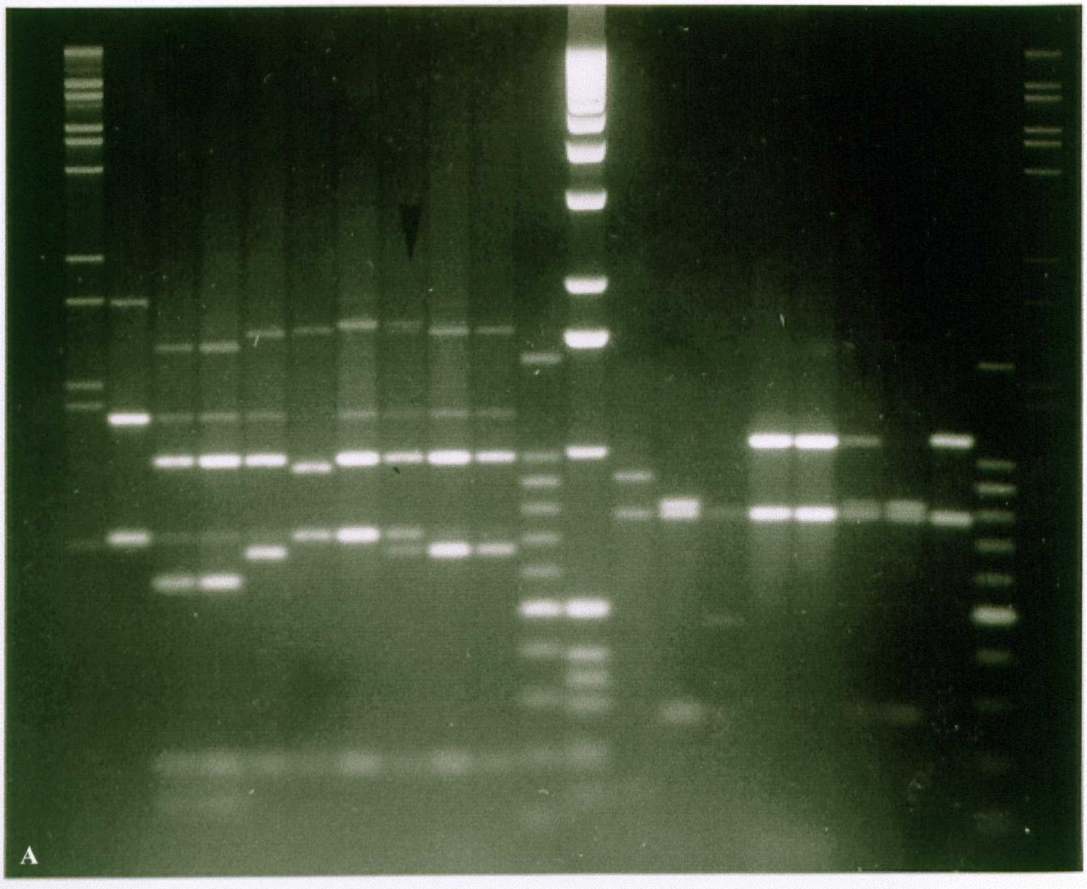
Banding patterns obtained after restriction digest of the PMA fragment.

A. MspI and TaqI

Lane 1: 1 kb-marker; Lanes 2-10: MspI, haplotypes F, D, D, A, C, B, A/B, A, A; arrow marks heteroplasmic individual A/B; Lane 11: 100 bp-marker; Lane 12: 1 kb-marker; Lane 13: incomplete digest; Lanes 14-20: TaqI, haplotypes B, C, A, A, A/B, B, A; Lane 21: 100 bp-marker; Lane 22: 1 kb-marker.

B. DraI/PvuII, RsaI, HaeIII

Lane 1: 100 bp-marker; Lanes 2 - 5: DraI/PvuII, haplotypes: D, C, B, A; Lane 6: 100 bp-marker; Lanes 7- 12: RsaI, haplotypes: I, G, F, C, B, A. Lane 13: 100 bp-marker; Lanes 14 - 21: HaeIII, haplotypes: A, B, A, B, D, C, B, A; Lane 22: 100 bp-marker.


A

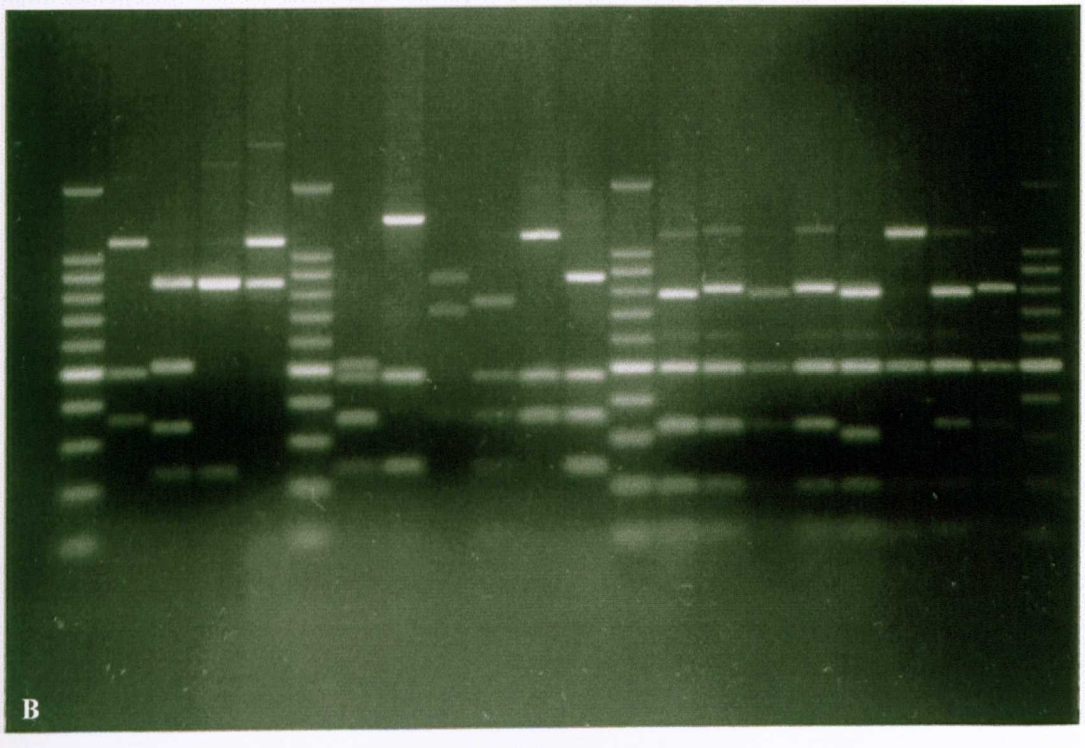

B

Plate 4.4.

A, B. Examples of the banding patterns obtained after restriction digests of the **DPM** fragment of individuals originating from the same population

A. DraI/PvuII, Peel 96

B. TaqI, Chickens 95

C, D. Examples of the banding patterns obtained after restriction digests of the PMA fragment of individuals originating from the same populations

C. DraI/PvuII, Chickens 95;

D. RsaI, Ramsey 95.

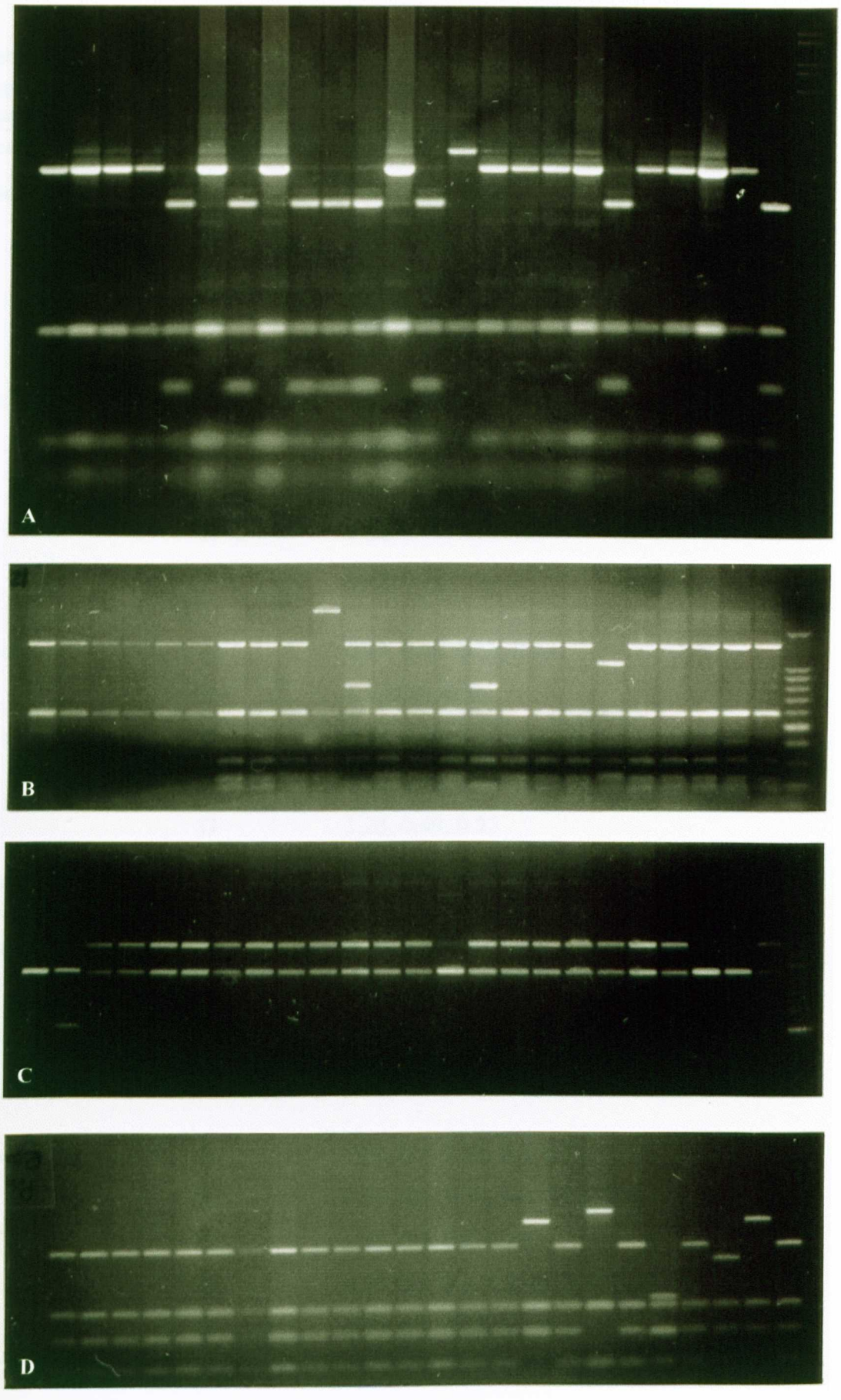

A
B
C
D

Table 4.10. Number of haplotypes and sizes of fragments produced by restriction digestion of the PMA fragment with the enzymes DraI/PvuII, RsaI, TaqI, MspI and HaeIII.

Restriction enzyme	Haplotypes	Size of fragments (kb)
TaqI	A	0.82, 0.79, 0.29
	B	1.11, 0.79
	C	0.79, 0.47, 0.35, 0.29
	D	0.93, 0.79, 0.18
MspI	A	1.08, 0.68, 0.2, 0.04
	B	1.08, 0.72, 0.2
	C	0.98, 0.72, 0.2, 0.1
	D	1.08, 0.57, 0.2, 0.15
	F	1.28, 0.72
RsaI	A	0.87, 0.49, 0.36, 0.23
	B	1.1, 0.49, 0.36
	C	0.77, 0.49, 0.36, 0.23, 0.1
	F	0.87, 0.72, 0.36
	G	1.23, 0.49, 0.23
	I	0.51, 0.49, 0.36, 0.36, 0.23
Dra/PvuII	A	1.15, 0.85
	B	0.85, 0.85, 0.25
	C	0.85, 0.55, 0.32, 0.25
	D	1.15, 0.5, 0.35
HaeIII	A	0.82, 0.5, 0.33, 0.2, 0.12
	B	0.79, 0.5, 0.33, 0.2, 0.12, 0.04
	C	1.15, 0.5, 0.2, 0.12
	D	0.79, 0.5, 0.3, 0.2, 0.12, 0.04, 0.03
	E	0.72, 0.5, 0.33, 0.2, 0.12, 0.1

Table 4.11. Numbers of each composite haplotype observed in PMA, 1995; order of restriction enzymes: TaqI, MspI, RsaI, DraI/PvuII, HaeIII. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M	P
AAAAA	10	10	14	9	10	14	8
BBAAB	5	5		7	5	1	3
BBBBB	1	3	1	1			
ABAAC	2						
ADAAB	1	1		1	1	2	2
ACAAB	1			1	1		
BBABB	1	1					
ABAAB	1			1			1
AFAAB	1						
BAAAA	1		1			1	
AABAA		2	1				
BBBAB		1		1	1		
BBBCB		1					
BBIBB			1				
AAAAB			2		1		2
AAIAA			1			1	
AAGAA			1	1		1	
BAAAB			1				1
ABAAA			1		1	2	3
BACAA				1			
AAICA				1			
AAADA					1		
BBAAC					1		
ACAAA						1	
DBAAB						1	
ABBAA							1
BBAAA							2
ABBAB							1

Table 4.12. Numbers of each composite haplotype observed in PMA, 1996; order of restriction enzymes: TaqI, MspI, RsaI, DraI/PvuII, HaeIII. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M	P
AAAAA	11	9	14	10	12	8	14	8
BBAAB	3	4	3	4	3	4	1	3
ABAAB	2	1	1	2	2	2		1
BAAAA	1			3		6	1	
ABAAA	1	2		1			2	3
AAIAA	1	1	1				1	
AAGAA	1						1	
BBAAC	1							
AAAAB	1	1			1	1		2
BBBAB	1		1					
ACAAA		1	1		1	1	1	
AAABA		1						
CAIAA		1						
AAAAC		1		2				
BBBAB		2			2			
ADAAB		1			1		2	2
AAAAE			1					
BAAAB			1			1		1
CAAAA					1			
ADBAB				1				
BBIBB					1			
ABIAB					1			
AFAAA						1		
DBAAB							1	
ABBAA								1
BBAAA								2
ABBAB								1

Table 4.13. PMA95, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M	P
no. individuals	24	24	24	24	22	24	24
no. haplotypes	10	8	10	10	9	9	10
haplotype diversity	0.80	0.79	0.67	0.69	0.76	0.66	0.87
% mean nucleotide sequence diversity	2.16	2.62	1.41	2.41	1.97	1.33	1.74

Table 4.14. PMA96, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M	P
no. individuals	23	24	24	24	24	24	24	24
no. haplotypes	10	12	8	7	10	8	9	10
haplotype diversity	0.77	0.84	0.66	0.79	0.74	0.82	0.66	0.87
% mean nucleotide sequence diversity	1.73	2.27	1.51	1.75	2.13	1.64	1.33	1.74

AMOVA on PMA95 gave significant variance components ($p = 0.02$) between individuals within locations, and almost significant values ($p = 0.06$) between the Manx locations (Table 4.15.); phi_{ST} values were non-significant after Bonferroni correction Table 4.16.). A Bartlett's test revealed heteroscedasticity between the locations ($p = 0.048$), but values were non-significant after Bonferroni correction (Table 4.17.). For PMA96, no significant variance was detected (Table 4.18.), and none of the phi_{ST} values were significant (Table 4.19.). No significant heterocedasticity was detected by the Bartlett's test for the PMA96 fragment ($p = 0.41$).

Table 4.15. Analysis of molecular variance for PMA95

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	4.01	2.00	0.005	0.45	0.45
Among locations within regions (Isle of Man region only)	4	7.28	1.82	0.036	3.63	0.06
Among individuals within locations	159	152.85	0.96	0.961	95.92	0.02

Table 4.16. Phi_{ST} values for PMA95; no significant values after Bonferroni correction; +: $p < 0.05$ *before* Bonferroni correction. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	0.0053					
D	0.1128+	0.1212+				
R	-0.0303	-0.0140	0.0904+			
T	-0.0216	0.0200	0.0551	-0.0257		
M	0.1115+	0.1512+	-0.0157	0.0979+	0.0460	
P	-0.0188	0.0241	0.0696+	-0.0165	-0.0359	0.0551

Table 4.17. Pairwise Bartlett's test values for PMA95; no significant values after Bonferroni correction; +: $p < 0.05$ *before* Bonferroni correction. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	1.2137					
D	4.7607+	5.7933+				
R	0.3363	0.6661	4.6335+			
T	0.5712	1.7809	2.7114	0.6440		
M	4.9666+	6.9657+	0.6260	5.1233+	2.6686	
P	0.8058	2.2789	2.8765	1.1309	0.2548	2.6332

Table 4.18. Analysis of molecular variance for PMA96

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions		2.59	1.23	0.017	1.97	0.13
Among locations within regions (Isle of Man region only)		3.09	0.61	-0.011	-1.22	0.82
Among individuals within locations		159.93	0.87	0.874	99.24	0.54

Table 4.19. Phi_{ST} values for PMA96 (no values significant). Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E - Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M
C	-0.0272						
D	-0.0310	-0.0112					
R	-0.0357	-0.0193	-0.0262				
T	-0.0141	-0.0341	0.0023	-0.0067			
E	-0.0116	0.0034	0.0085	-0.0149	0.0111		
M	0.0045	0.0243	-0.0151	0.0110	0.0548	0.0670	
P	-0.0200	-0.0230	0.0045	-0.0138	-0.0271	0.0081	0.0551

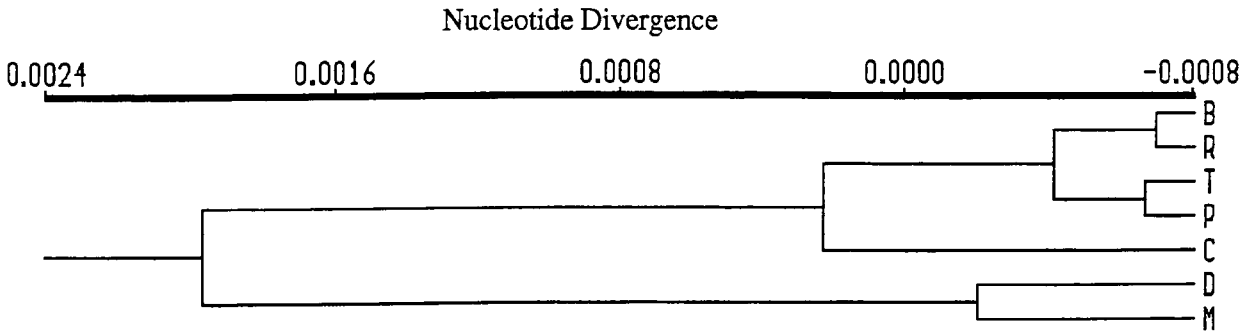


Figure 4.7. UPGMA cluster diagram based on nucleotide divergence for PMA95. Abbreviations as in Figure 2.2.

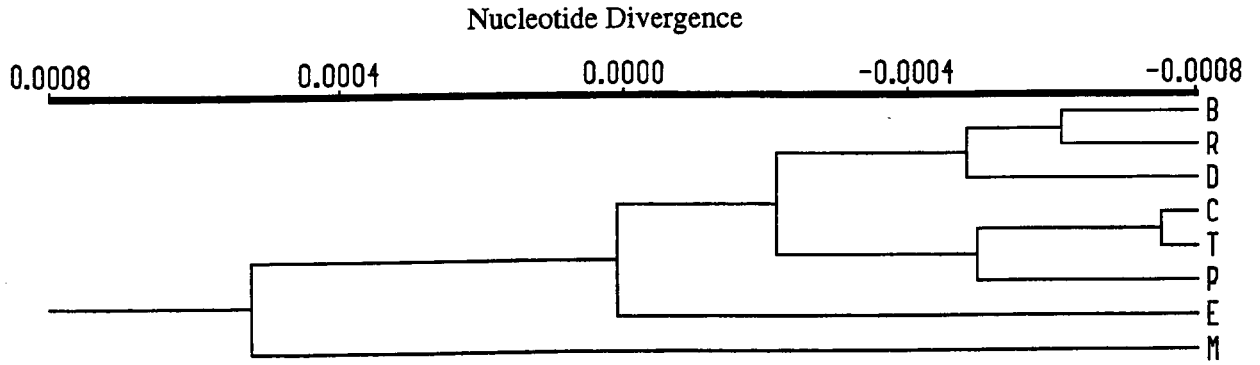


Figure 4.8. UPGMA cluster diagram based on nucleotide divergence for PMA96. Abbreviations as in Figure 2.2.

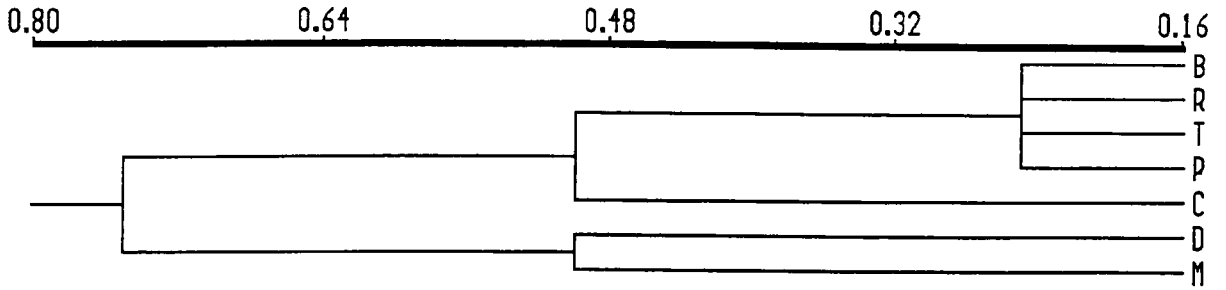


Figure 4.9. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for PMA95. Abbreviations as in Figure 2.2.

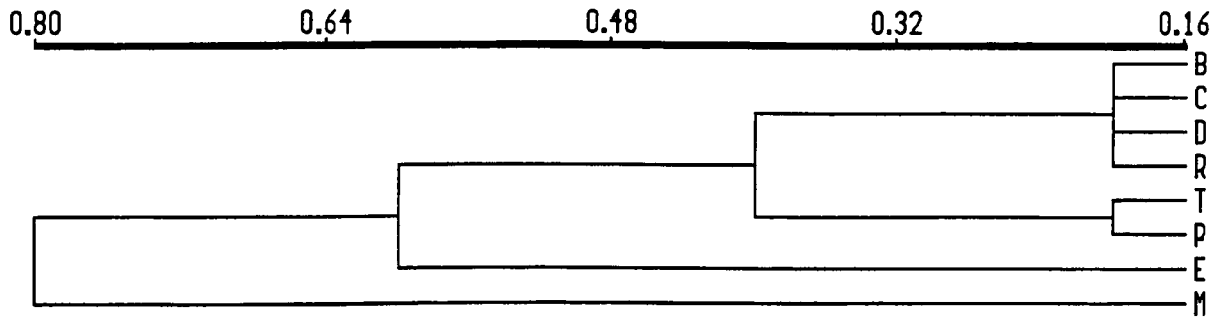


Figure 4.10. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for PMA96. Abbreviations as in Figure 2.2.

Table 4.20. Summary statistics of separate analyses of both fragments (DPM and PMA) and both samples (1995 and 1996).

	DPM95	DPM96	PMA95	PMA96
no. individuals	161	191	166	191
no. populations	7	8	7	8
no. haplotypes	25	22	28	27
main haplotypes	AAAAA	AAAAA	AAAAA	AAAAA
	73, 43.98 %,	109, 57.07 %,	62, 37.96%,	86, 45.03%,
	AABBA	AABBA	BBAAB	BBAAB
	45, 27.11 %	48, 25.13 %	26, 15.66 %	25, 13.08 %
no. of unique haplotypes	15, 9.30 %	11, 5.76 %	11, 6.63 %	12, 6.28 %
X^2 all populations	144.66	142.51	172.34	199.18
probability value	0.462	0.688	0.181	0.108
X^2 Manx populations only	76.76	78.95	85.91	112.59
probability value	0.7010	0.8280	0.6080	0.4130

4.3.4. Comparison between DPM and PMA fragments

Both fragments showed a similar distribution of the commonest haplotypes between the two year-classes, although PMA generated a few more haplotypes than DPM. X^2 values for frequencies of haplotypes were similar between year-classes, but corresponding *p*-values were closer to significance for the PMA fragment. PMA96 gave the higher X^2 value and lower *p*-value for both the analyses, i.e. those including all individuals, and Manx individuals only. Whereas the haplotype diversity is of similar magnitude in both fragments and both year-classes, the values for nucleotide sequence diversity are up to three times higher in PMA95/96 than in DPM95/96. AMOVA revealed more variation in the 1995 than in the 1996 samples in both fragments.

4.3.5. Analysis of combined fragments

Data for both mitochondrial fragments DPM and PMA were also subjected to a combined analysis. In the following, the combined fragments DPM95 and PMA95 are referred to as com95, DPM96 and PMA96 as com96. It must be borne in mind that com96 includes one additional population, Peel, making a total of 191 individuals, compared to 161 individuals for com95.

The sample from Plymouth had the highest number of haplotypes (16), while Mulroy had relatively few haplotypes (11). Amongst the Manx samples, in com95, Douglas had the highest number of haplotypes (16), Targets the lowest (12) (Table 4.21). In com96, Ramsey and Targets showed the lowest number of haplotypes (10), and Douglas had again the highest number (16) (Table 4.22). The main composite haplotypes were very similarly distributed in both year-classes (see Appendix): AAAAAAAAAA occurred in 34.16% (com95) and 36.65% (com96) of all individuals, AABBABBAAB in 12.42% (com95) and 11.52% (com96), while unique haplotypes had a frequency of 26.70% and 22.51% for com95 and com96, respectively. The overall number of haplotypes was 63 for com95 and 61 for com96 (Table 4.23).

Haplotype diversity for the combined fragment was lower in Mulroy (0.71) than in any other sample, while the value for Plymouth was high (0.93). Amongst the Manx samples, Targets showed the lowest value in both year-classes (0.85; 0.78), and Ramsey the highest (0.92; 0.96).

The lowest nucleotide diversity for the combined fragments was again exhibited by Mulroy (0.80). For com95, Douglas also showed low nucleotide diversity (0.81). Chickens had the highest values with 1.41 (com95) and 1.17 (com96). Nucleotide diversity for all populations was lower in 1996 than in 1995, except for Targets, but here the values were very similar.

The X^2 analysis for frequencies of haplotypes resulted in a higher value (439.90) for com96 than for com95 (374.46) (Table 4.23). Testing for significance by means of Monte Carlo permutations gave non-significant values for either year-class, although the *p*-value for com96 (0.08) was lower than for com95 (0.47). As the same analysis for only the Manx populations showed much lower X^2 values in both year-classes, most of the potential population differentiation would be due to the Mulroy and Plymouth populations.

UPGMA cluster analysis of nucleotide divergence, including negative values, drew a similar picture for both com95 and com96; Bradda and Ramsey clustered together, as did Douglas and Mulroy. Targets and Plymouth were close in both year-classes (Figure 4.11. and 4.12.). The consensus trees gave a similar picture for both year-classes: Bradda, Chickens, Ramsey and Plymouth formed a cluster, Targets clustered separately in 1995, but was included in 1995. Peel was different from all other Manx populations. Douglas and Mulroy formed a cluster in both year-classes (Figure 4.13. and 4.14.).

AMOVA on com95

A Bartlett's test revealed significant heterogeneity of variances (Table 4.25.) between populations, with a probability value $p = 0.0130$. Lowest within population sums of squares occurred in Douglas and Mulroy, highest in Chickens and Ramsey. The variance component was negative (non significant) for the analysis among regions, low, but significant among the Manx populations, and significant variance was present among individuals within locations (Table 4.24.). Many of the phi_{ST} values were negative, and none remained significant after Bonferroni correction (Table 4.27.).

AMOVA on com96

In 1996, there was a more even spread of sums of squares than in 1995, consequently no heterogeneity of variances was present ($p = 0.60$). As for com95, com96 showed lowest within population sums of squares for Douglas and Mulroy, and the highest value for Chickens. None of the variance components were significant, nor were the phi_{ST} values (Table 4.26. and 4.28.).

The Mantel test on Manx com 95 and com96 did not show any significant correlation between pairwise nucleotide divergence values in the two year-classes ($p = 0.24$) (Figure 4.15.).

Table 4.21. com95, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M	P
no. individuals	23	23	24	22	21	24	24
no. haplotypes	14	14	16	15	12	11	16
haplotype diversity	0.89	0.90	0.89	0.92	0.85	0.71	0.93
% mean nucleotide sequence diversity	1.27	1.41	0.85	1.36	1.03	0.80	1.03

Table 4.22. com96, summary statistics. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E- Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M	P
no. individuals	23	24	24	24	24	24	24	24
no. haplotypes	14	12	16	10	10	12	11	16
haplotype diversity	0.85	0.89	0.87	0.96	0.78	0.88	0.71	0.93
% mean nucleotide sequence diversity	1.01	1.17	0.81	0.97	1.09	0.95	0.80	1.03

Table 4.23. Summary statistics for com95 and com96

	com95	com96
no. individuals	161	191
no. populations	7	8
no. haplotypes	63	61
main haplotypes	AAAAAAAAAA 52, 34.16%	AAAAAAAAAA 70, 36.65%
	AABBABBAAB 20, 12.42%	AABBABBAAB 22, 11.52%
	single haploypes 43, 26.70%	single haplotypes 43, 22.51%
X^2 all populations	374.46	439.90
probability value	0.4722	0.0827
X^2 Manx only	170.65	222.50
probability value	0.9340	0.4320

Table 4.24. Analysis of molecular variance for com95

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	6.13	3.07	-0.01	-0.71	0.56
Among locations within regions (Isle of Man region only)	4	13.71	3.43	0.08	4.59	0.03
Among individuals within locations	154	253.82	1.65	1.65	96.12	0.01

Table 4.25. Pairwise Bartlett's test values for com95; no significant values after Bonferroni correction; +: $p < 0.05$ *before* Bonferroni correction. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	0.7011					
D	6.2440+	4.9651+				
R	0.3035	0.4390	5.9562+			
T	1.0280	1.0325	2.6690	1.0855		
M	5.3125+	4.7270+	0.7099	5.1928+	1.9678	
P	0.8826	1.0394	3.5138+	1.1122	0.1864	2.7540

Table 4.26. Analysis of molecular variance for com96

Source of variation	df	SSD	MSD	Variance component	% of total variance	P-value
All regions, nested analysis						
Among regions	2	3.70	1.85	0.02	1.21	0.12
Among locations within regions (Isle of Man region only)	5	5.80	1.16	-0.01	-0.88	0.71
Among individuals within locations	183	268.59	1.47	1.47	99.67	0.55

Table 4.27. Phi_{ST} values for com95; (+: $p < 0.05$, ++: $p < 0.01$ significance *before* Bonferroni) no significant values after Bonferroni. Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	M
C	-0.0124					
D	0.1643	0.1133+				
R	-0.0336	-0.0258	0.1422++			
T	-0.0049	-0.0076	0.0600	-0.0116		
M	0.1308++	0.1035+	-0.0113	0.1129++	0.0289	
P	-0.0124	-0.0083	0.0886+	-0.0118	-0.0375	0.0586+

Table 4.28. Phi_{ST} values for com96 (no significant values). Abbreviations: B - Bradda Inshore, C - Chickens, D - East Douglas, R - Ramsey, T - Targets, E- Peel, M - Mulroy Bay, P - Plymouth.

	B	C	D	R	T	E	M
C	-0.0295						
D	-0.0087	0.0080					
R	-0.0323	-0.0232	-0.0014				
T	-0.0159	-0.0309	0.0400	-0.0128			
E	-0.0184	-0.0116	0.0161	-0.0203	0.0001		
M	0.0033	0.0142	-0.0127	0.0082	0.0493	0.0316	
P	-0.0228	-0.0264	0.0279	-0.0279	-0.0288	-0.0056	0.0425

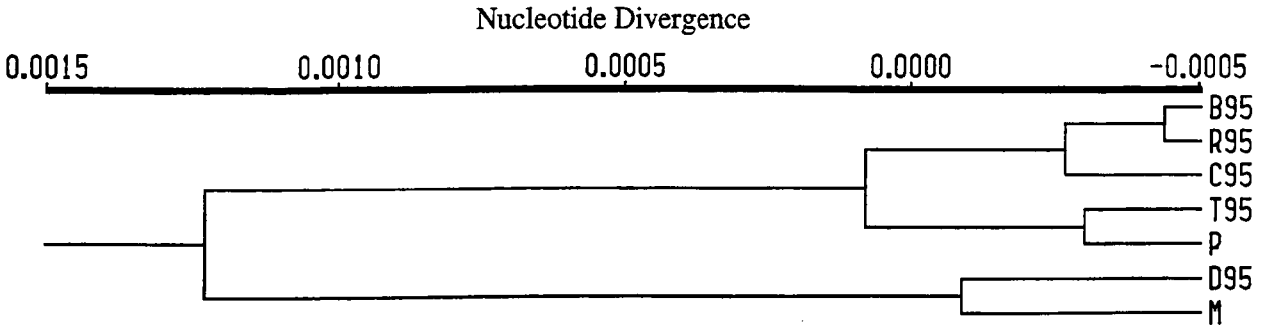


Figure 4.11. UPGMA cluster diagram based on nucleotide divergence for com95

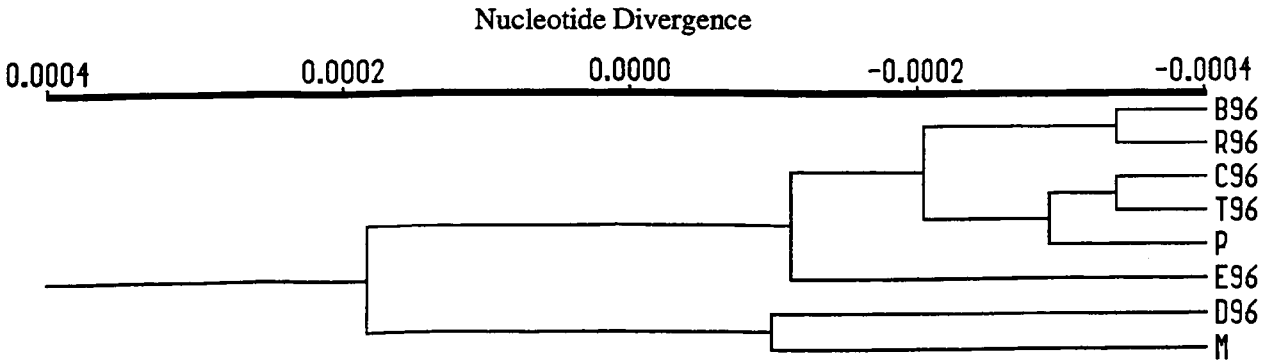


Figure 4.12. UPGMA cluster diagram based on nucleotide divergence for com96

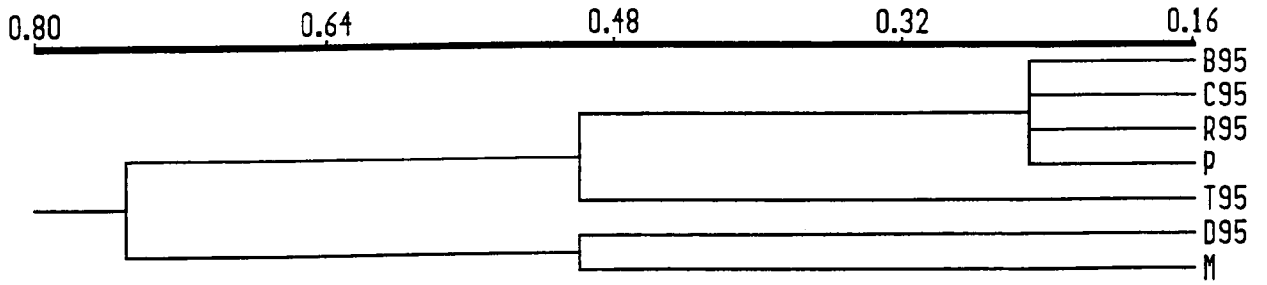


Figure 4.13. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for com95

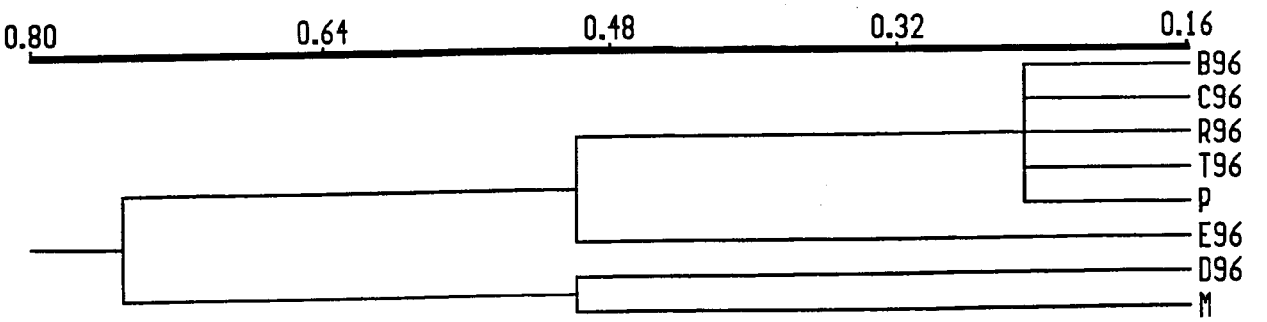


Figure 4.14. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for com96.

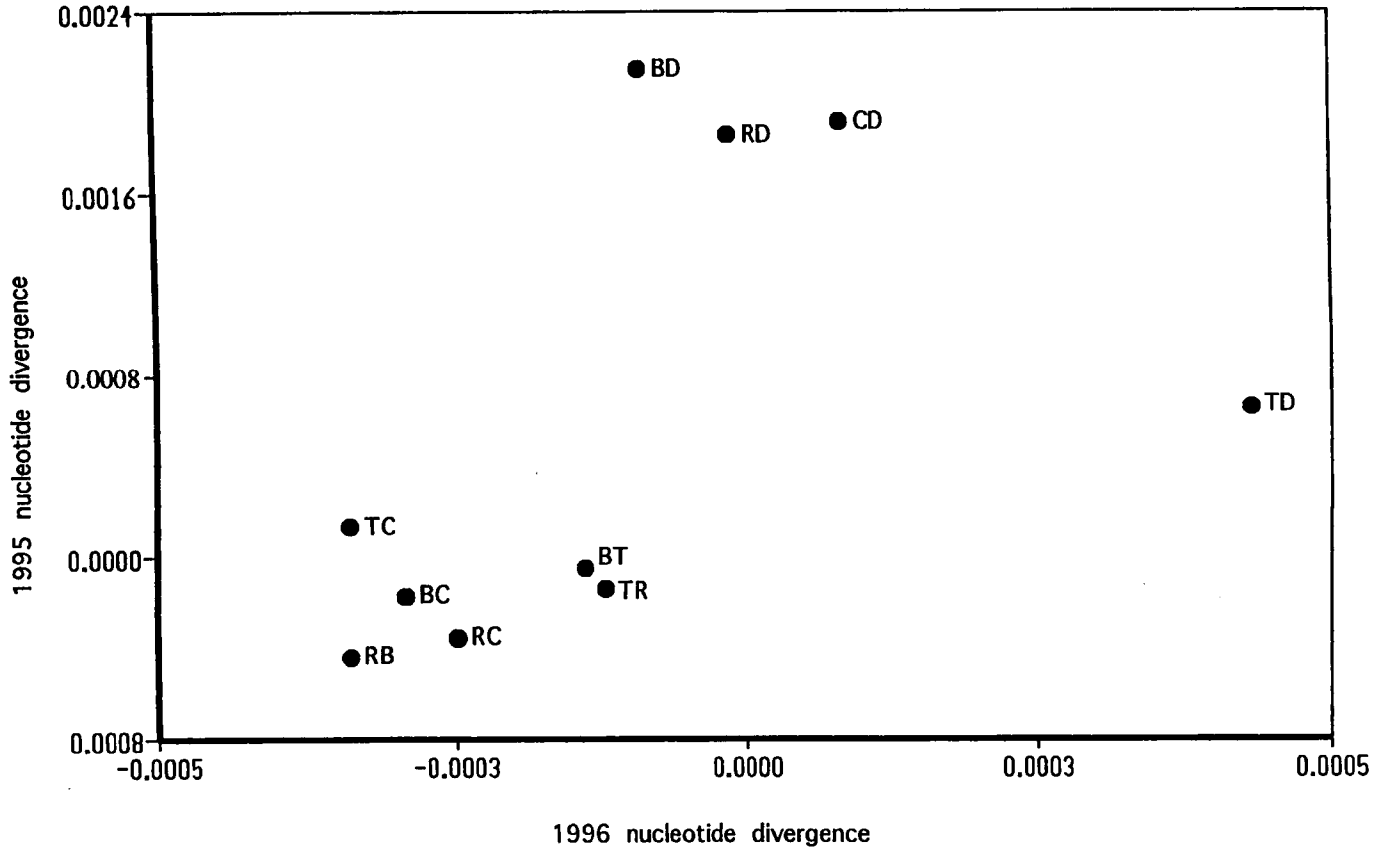


Figure 4.15. Scatter-plot showing correlation of nucleotide divergence for the Isle of Man locations in the two year-classes.

4.3.6. Analysis of combined data

The data for both fragments and both year-classes were also analysed together in one matrix, in the following referred to as com9596. A total of 85 haplotypes were identified (see Appendix). In the UPGMA cluster diagram of nucleotide divergence values, Bradda, Ramsey, and Chickens of both year-classes clustered separately, with Plymouth in between but more closely associated with the 1996 cluster (Figure 4.16.). Both year-classes from Targets were in the 1996 cluster, while both samples from Douglas formed a cluster with Mulroy. However, in the consensus tree, Targets 96 clustered with the 1995 populations, whereas Targets 95 was in the 1996 cluster. Plymouth was part of the 1996 cluster. A separate cluster consisted of both Douglas year-classes and Mulroy (Figure 4.17.).

Table 4.29. Summary statistics for com9596

	com9596
no. individuals	357
no. populations	13
no. haplotypes	85
main haplotypes	AAAAAAAAAA 103, 28.9 %
	AABBABBAAB 38, 10.6 %
	single haploypes 52, 14.6 %
X^2 all populations	1026.48
probability value	0.2337

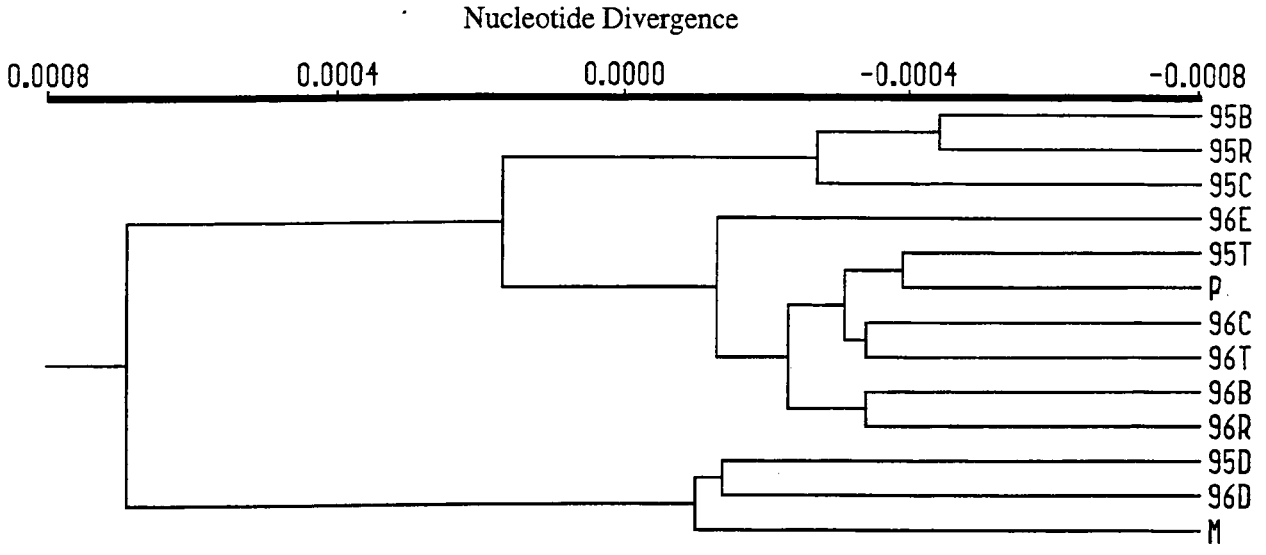


Figure 4.16. UPGMA cluster diagram based on nucleotide divergence for com9596.

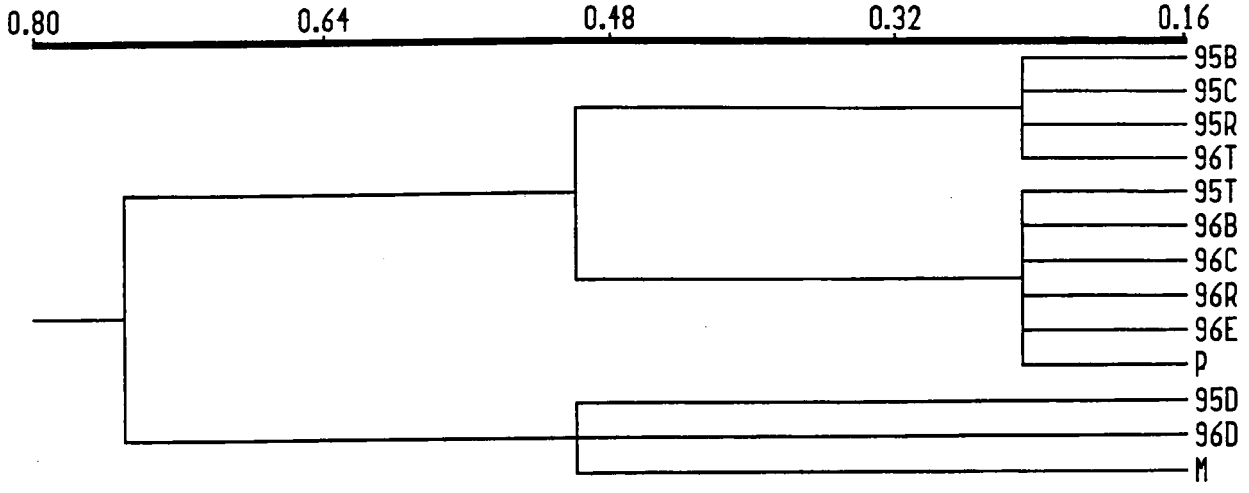


Figure 4.17. Consensus tree of UPGMA dendrograms based on nucleotide divergence, with negative values set to zero, for com9596.

4.4. Discussion

4.4.1. Heteroplasmy

The fundamental drawbacks of using mtDNA for population studies include possible biparental inheritance and heteroplasmy. To date, no investigation concerning inheritance of mtDNA in *P. maximus* has been carried out; for the statistical analysis of this study maternal inheritance was assumed. Heteroplasmy did not pose a problem, as only one heteroplasmic individual amongst 336 specimens was found, and was excluded from the analyses. Generally, heteroplasmy is not very frequently detected within coding regions of the mtDNA. No heteroplasmic individual was reported by Wilding *et al.* (in press) and only one heteroplasmic individual amongst 159 oysters was recorded by Boom *et al.* (1994). The relatively high frequency of heteroplasmic individuals reported by Rigaa *et al.* (1993) in *P. maximus* was due to varying numbers of repeated elements. The present study only analysed regions of mtDNA not containing repeated elements, and the one detected case of heteroplasmy is probably due to single base changes or insertions or deletions of short sequences unrelated to repeated elements.

A more mundane practical difficulty in studies of mtDNA is the relatively small yield of whole mtDNA after laborious extraction procedures. The application of PCR-based technology proved to be very successful in providing high yields of amplified mtDNA and enabling all digests to be carried out on the same PCR product, thus overcoming problems with the small amount of mtDNA obtained after extraction. To date, few publications using the same technique are available for comparison. Therefore, the data of this study are interpreted in relation to studies of RFLPs of the whole mitochondrial genome, although this comparison might not be strictly valid. However, the levels of diversity encountered are of a similar magnitude to those reported in other marine species.

4.4.2. Comparison of the PCR-fragments DPM and PMA

Contrary to findings by Wilding *et al.* (in press), the restriction enzymes HinfI and CfoI did not reveal any polymorphism in the PMA fragment. Restriction analysis of the DPM fragment with the enzymes TaqI, MspI, HaeIII, DraI/PvuII and RsaI resulted in a slightly higher number of individuals displaying the main haplotype and fewer haplotypes overall in both year-classes than the analysis of the PMA fragment with the same enzymes. This is particularly notable because DPM is the longer fragment. Furthermore, the values for mean nucleotide sequence diversity were generally much higher for PMA than for DPM. Both trends are probably due to the DPM fragment including rRNA-genes, which are known to exhibit lower mutation rates than other mtDNA genes (Meyer, 1994). Hence, any potential population differentiation is more likely to show up in the PMA fragment; this was indeed the case for the X^2 analysis of PMA fragment haplotype frequencies, which gave values closer to significance than for the DPM fragment.

The number of haplotypes is directly dependent of the number of restriction sites sampled; accordingly, the values for haplotype diversity are higher for the combined fragments than for the single fragments. X^2 analysis involving a large number of haplotypes is unlikely to give a significant result, so combining fragments may conceal population differentiation under this test.

4.4.3. Differences in haplotype and nucleotide diversity

The two most common haplotypes were found in all populations, suggesting that these haplotypes are more ancient in origin (Neigel and Avise, 1993), and that there has been, and maybe still is, extensive gene flow between all locations. Within the analyses of both fragments the frequency of the most common composite haplotype (AAAAA) varied between 37.96 % and 57.07 %, indicating a high overall diversity of haplotypes in *P. maximus* from all locations. This is also reflected in the relatively large number of individuals showing unique or rare haplotypes. Wilding *et al.* (in press) reported very similar values: 38.80 % of all specimens possessed the most frequent haplotype, while 11.45 % had unique haplotypes. Blake and Graves

(1995), in a survey of haplotypes among five populations of *Argopecten irradians,* found even higher levels of genetic variation: a lower percentage (25.93 %) of animals showed the main haplotype and a higher number of unique haplotypes (25.19 %) was present within and among populations, which were found to be genetically differentiated. Compared to the present study, this greater variation could be due, in part, to the higher number of restriction enzymes (8) used by Blake and Graves (1995), and the longer sequence analysed (data based on restriction analysis of the whole mt genome), or indicate more diversity in *A. irradians*. Similar distributions of haplotypes were found in Pacific oysters, *Crassostrea gigas*. Whereas about 60 % of the oysters fell into the two common haplotypes, almost 40 % of individuals carried rare, often unique, variants (Boom *et al.*, 1994); comparable observations have been made for American oysters by Reeb and Avise (1990). A study on population differentiation of Pacific starfish, *Linckia leavigata*, reported haplotype diversity values of similar magnitude to those found in this study (Williams and Benzie, 1997).

No population-specific haplotypes were found, and unique haplotypes were present at all locations. If populations were truly isolated, then mutations within haplotype lineages could result in 'private' haplotypes characteristic of a particular population (Slatkin, 1985). Since the unique haplotypes were, by definition, only encountered once in all samples, no statement can be made about their 'private' character.

Blake and Graves' (1995) values for the mean nucleotide sequence diversity ranged from 0.22 % to 0.53 % in the genetically differentiated *A. irradians* populations, and 0.54 % and 0.69 % in the *A. gibbus* populations, which were not significantly heterogenous. Thus both *Argopecten* species proved to be less variable than the *P. maximus* populations, which exhibited mainly high figures, ranging from 0.50 % (Mulroy, DPM) to 2.62 % for the Chickens population (PMA95). Values of similar magnitude (2.6 %) were found by Reeb and Avise (1990) for populations of American oysters (*Crassostrea virginica*). Up to 1.84 % mean nucleotide sequence diversity was reported for populations of the spiny lobster, *Panulirus argus*, by

Silberman *et al.* (1994), which was considered to be amongst the highest values reported for a marine species. No population structure was revealed for the spiny lobster. Surveys of mtDNA variation in tortoise populations found values of mean nucleotide sequence diversity between 0.0 % and 5.3 % (Osentoski and Lamb, 1995). Populations of Pacific starfish showed nucleotide diversities up to 3.1 % (Williams and Benzie, 1997).

4.4.4. AMOVA-Results

In the present study, AMOVA revealed that almost all genetic variation (> 95 % of total variance) was distributed within populations. Accordingly, most pairwise phi_{ST} values were close to zero or even negative (except for the analysis of DPM95). Comparable results have been reported by Silberman *et al.* (1994) for the spiny lobster, which was found to exhibit high levels of gene flow between the populations examined. Furthermore, X^2 values for heterogeneity of *P. maximus* haplotype frequencies proved non-significant, and high nucleotide sequence diversity, characteristic of extensive gene flow, suggested little population differentiation. However, there are some notable trends in the data, opposing the conclusion that *P. maximus* represents a panmictic population.

4.4.5. Inter-population differences

For both fragments and year-classes, samples from Mulroy and Douglas showed low values of haplotype diversity, and even more clearly, of mean nucleotide sequence diversity, indicating greater homogeneity within these populations than in the others. AMOVA supported this by calculating the lowest values of within-population sums of squares for these two populations. Mulroy and Douglas formed a cluster distinct from the other Manx populations and from Plymouth in all UPGMA diagrams except PMA96. The Mulroy population originated from an enclosed environment, animals from which have indeed been found to be genetically different from samples around the UK in a previous study (Wilding *et al.*, in press); however, the reason for the Douglas population showing more homogeneity than the other Manx populations is not apparent, but could be due to circular current systems or

gyres leading to some retention of larvae in Douglas Bay, possibly making the Douglas population at least partially self-sustaining, especially in the 1995 samples (see Chapter 7). Although Mulroy and Douglas are distinct from the other populations, having similar (lower) proportions of the two commonest haplotypes compared to the other locations, they are also dissimilar to each other. This is reflected by the different unique/rare haplotypes found in the two populations. The lowest nucleotide diversity was exhibited by Mulroy. Chickens had the highest diversity values, which is of possible significance, since Mulroy is regarded as a truly enclosed site, while Chickens as an open-water population.

Another surprising feature is the consistent grouping of Bradda and Ramsey, and of Targets and Plymouth, in UPGMA clustering of nucleotide divergence including negative values. There is no obvious geographical reason for this, as the former pair of populations are located on opposite sites on the Isle of Man and the latter are very distant. The analysis of the combined fragments (com95 and com96) largely confirmed the trends revealed by the separate analyses of the fragments: Bradda and Ramsey, and Targets and Plymouth plot in close proximity; Chickens is either associated with Bradda and Ramsey or with Targets and Plymouth. It is, however, debatable how much weight should be given to these findings, which are largely redundant when negative values are set to zero and a consensus tree is constructed. Although true values of nucleotide sequence divergence can not be negative, the error terms associated with marginally positive and marginally negative values will be similar, and applying a cut-off at zero may thus be regarded as an arbitrary convention. It does not follow that negative values carry no information. The true value associated with a large negative value will tend to be smaller than that associated with a small negative value. It is apparent from the consistent clustering in the sub-zero regions of dendrograms presented here that a signal does exist in the relative magnitude of negative values of nucleotide divergence.

Nevertheless, the consensus trees broadly confirmed the results of the UPGMA's with negative values included. The Manx populations Bradda, Ramsey, Chickens, and Targets, plus Plymouth cluster close together; Peel is more distant; Douglas and Mulroy form a separate cluster, but at some distance from each other.

4.4.6. Comparison of year-classes

There are several signs pointing to different recruitment processes for the year-classes present in the populations as 3-year-olds in 1995 and 1996. In particular the 1995 3-year-olds (1992 year-class) showed some evidence of genetic population structure: AMOVA variance components were significant and phi_{ST} values approached significance. The Bartlett's test revealed significant heteroscedasticity between populations for PMA95 and com95. In contrast, the 1996 3-year-olds (1993 year-class) generally appeared more homogenous, consisting of fewer haplotypes and more individuals with the main (AAAAA) haplotype, and lower mean nucleotide sequence diversity for Bradda, Chickens and Ramsey for both fragments (Douglas and Targets were lower in 1996 for PMA, but higher in 1995 for DPM).

The construction of an UPGMA cluster diagram based on nucleotide divergence including all data (both year-classes and both fragments, com9596) resulted in a remarkable separation of the year-classes; Bradda, Ramsey and Chickens of the 1995 samples clustered separately from Bradda, Ramsey, Chickens and Targets 1996. Both Targets year-classes and Plymouth were closely associated with the Manx 1996 samples. In the consensus tree, Targets 1995 formed a cluster with the Manx 1996 populations, and Targets 1996 clustered with the Manx 1995 populations. One possible reason could be that the second commonest haplotype (AABBA) is commoner for Targets in DPM96 than in DPM95, thus reversing the trend shown by the other populations. Data for the population from Peel are only available for the 1996 sample. As Peel clustered separately from other Manx samples in the analyses of PMA96, com96 and com9596, this population might be somehow distinct from the nearby populations.

Both Douglas year-classes clustered together in com9596, indicating more consistent recruitment to the Douglas populations than to any other Manx population. As with all previous analyses, Mulroy is closest to the Douglas population. In contrast, the analysis of the PMA96 data placed Douglas 1996 in the Manx cluster, possibly partially due to relative lower numbers of the second commonest haplotype at the other Manx locations compared to PMA95 and the presence of this haplotype in Douglas 1996 (absent in 1995); this would tend to indicate a different recruitment source in the 1993 year-class, affecting all Manx populations including Douglas, whereas in 1992 Douglas might have been supplied with larvae from elsewhere, or was partly self-sustaining.

Different recruitment sources for the two year-classes could explain the result of the Mantel test, which showed no significant correlation between pairwise nucleotide divergence values of the Manx population samples from 1995 and 1996. The 1996 sample is genetically more homogenous, pointing towards a more even supply of larvae, possibly due to more extensive mixing of water layers during the time of larval drift. The 1995 sample, in showing more signs of population differentiation, could be the product of recruitment from independent sources. Some evidence that recruitment sources of Manx *A. opercularis* populations can differ from year to year was provided earlier by Macleod *et al.* (1985). One possible picture to be drawn is that of spatially fragmented populations, but with exchange of larvae at a rate high enough to prevent accumulation of marked genetic differentiation. Depending on the weather conditions, this exchange of larvae could be more or less pronounced between years.

It can be concluded that, although the mitochondrial genome of *P. maximus* displayed high nucleotide sequence diversity, little evidence exists for genetic heterogeneity among geographically separated populations. AMOVA indicated only slight population subdivision for the 1995 sample. Cluster analysis based on sequence divergence provided consistent indication of population structure, but only Mulroy and Douglas remained distinct when negative values for nucleotide sequence

divergence were corrected to zero. In contrast, the year-classes remained dissimilar. A number of factors could be responsible for the temporal inconsistency of recruitment and act to hinder more pronounced genetic subdivision of populations in the Irish Sea. These will be discussed in Chapter 7.

CHAPTER FIVE: CLONING OF MITOCHONDRIAL DNA FRAGMENTS

5.1. Introduction

Due to the possession of different numbers of copies of repeated elements, the mtDNA of *P. maximus* varies in size between individuals, considerably complicating restriction fragment analysis of the complete mtDNA (see Chapter 6). Alternatively, restriction analysis of size-invariable PCR-amplified fragments of mtDNA can be carried out, which also circumvents the laborious extraction of mtDNA and provides enough product for several digests. To PCR-amplify a fragment, primers first have to be designed, and therefore the sequence at both ends of the desired fragment has to be known. This can be achieved by 'cloning' the fragment and using the sequencing primers of the cloning vector to determine the first few hundred bases of both ends of the inserted fragment. During the process of cloning, the mtDNA fragment is inserted into a carrier molecule, known as a vector, plasmid or phage ('ligation'), which is then introduced into a bacterium ('transformation'). The transformed bacteria are grown on agar plates to amplify the inserted plasmid. Several different methods allow for selection of bacterial colonies ('clones') containing the plasmid. Such positive clones are selectively grown and the plasmid extracted, now in copy numbers sufficient to allow further procedures, such as sequencing or restriction digests, to be carried out.

In the work reported in this Chapter, cloning of several mitochondrial DNA restriction fragments was attempted, with a view to sequencing the cloned fragments and to designing PCR-primers. This would enable the fragments to be amplified by PCR and subsequently subjected to restriction analysis, without the need to extract whole mtDNA, and at the same time circumventing the problem of length-variability due to repeated elements.

5.1.1. Plasmids

Plasmids are extrachromosomal genetic elements, mainly circular molecules, that are found in both prokaryotic and eukaryotic cells, encoding between ten and several hundred genes. The main difference from viruses is that plasmids do not form

infectious particles. They can carry a wide variety of different genes, e.g. for proteins or toxins (increasing the virulence of bacteria), catabolic enzymes, antibacterial proteins, antibiotic resistance, tumor-inducing substances etc. Many of these genes are located on transposons, genetic elements which can transfer copies of themselves to other replicons. As circular molecules, plasmids occur in different quarterny stuctures: linear, closed circular, open circular and supercoiled. Supercoiled forms have additional twists in their double helices, introduced by the enzyme gyrase. A nick in one of the two strands of a covalently closed circular plasmid produces an open circular ("relaxed") molecule, while nicks in both strands result in the linear form.

The first plasmid to be discovered was the fertility factor (F plasmid) in the *Escherichia coli* strain K-12, which confers the ability to conjugate. Plasmids used as vectors in molecular biology are artificially constructed by joining DNA from a variety of replicons (plasmids, bacteriophage chromosomes and bacterial chromosomes) together, to form a molecule which has an origin of replication, a selectable marker (usually an antibiotic-resistance gene) and a promoter for expressing genes of interest in the host cell. They are available in a great range of sizes. The pUC plasmids (UC = University of California) are extremely versatile vectors consisting of a 2297 bp EcoRI/PvuII fragment from pBR322, plus a 433 bp HaeII fragment of the lac-operon of *E.coli* (Figure 5.1.). The pBR fragment contains the origin of replication and the modified gene for ß-lactamase, conferring ampicillin resistance. The HaeII fragment codes for control elements of the operon (promotor and operator) and the α-terminal part of ß-galactosidase (*lacZ* gene of *E.coli*, Vieira and Messing, 1982). Inserted in the *lacZ* gene is a multiple cloning site with recognition sites for several restriction endonucleases. Deletion of the *rop*-gene, which otherwise regulates the number of plasmid copies inside the host cell, leads to an increased production of plasmids.

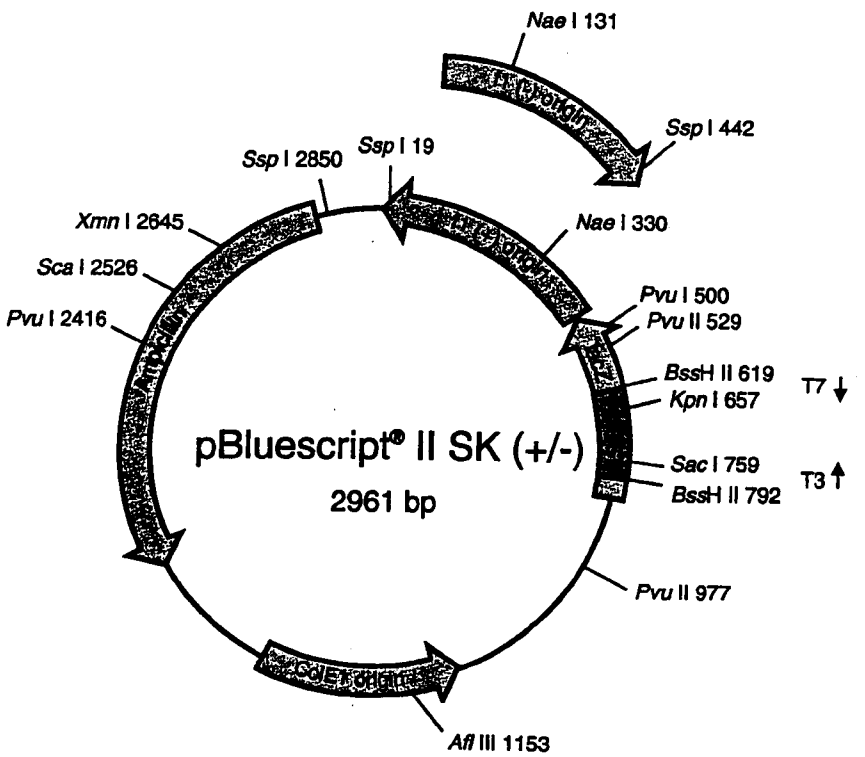


Figure 5.1. Map of the pBluescript II SK phagemid as derived from pUC19 (from Stratagene catalogue).

5.1.2. α-complementation

Colonies of recombinant clones of bacterial strains with a *lacZ* M15 deletion can be identified by blue/white colour screening by expression of the *lacZ* α-peptide. The pUC-series of vectors and its derivatives carry a short segment of *E.coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the *β*-galactosidase gene (*lacZ*). The multiple cloning site is located in this gene and introduces a small number of additional amino acids at the amino-terminal fragment of the *β*-galactosidase gene without disrupting the reading-frame. Together with the host-encoded carboxy-terminal fragment it forms a functional unit, the active enzyme *β*-galactosidase: α-complementation (Ulmann *et al.*, 1967). An active enzyme enables the bacteria to form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (x-gal) which becomes metabolised into the indoxyl derivate, which then oxidises in air into 5,5-dibromo-4,4-dichloroindigo. Isopropyl-*β*-D-thiogalactopyranoside (IPTG) is a non-metabolizable inducer of the *lac* operon. It binds to the *lacI* repressor without an initial requirement for functional *β*-galactosidase (Horwitz *et al.*, 1964; Langley *et al.*, 1975). Insertion of a fragment of foreign DNA into the plasmid disrupts the gene, α-complementation is no longer possible, the colonies are white and can be easily selected. The ampicillin-resistence genes on the plasmid allows the selection of transformed bacteria containing the plasmid by addition of ampicillin to the agar medium.

5.1.3. Ligation

For ligation, the ideal ratio of insert:plasmid is variable. However, a reasonable starting point is 2:1, measured in 'available picomole ends'. This is calculated as follows: picomole ends/micrograms DNA = (2×10^6) / (number of base pairs x 660). The overlapping DNA strands of the fragment and the plasmid are joined by DNA ligase, which catalyses the formation of phosphodiester bonds between the 3'-OH and 5'-phosphate groups at double-helical DNA molecules. Ligases are either of bacterial origin (*E. coli*) or isolated from T4 phages. T4-ligase offers the advantage of joining DNA with blunt (equal) ends as well as sticky

(unequal) ends. An energy source and cofactor, usually ATP, is required for the reaction. The bond between the three or four bases of the two overlapping fragments becomes unstable with increasing temperature. For this reason, a compromise has to be made between the stability of base-pairing and the optimal temperature for the enzyme ligase. In general, ligation can take place over either 12 - 16 hours at 4 °C, or 4 hours at room temperature.

5.1.4. Transformation

To take up the recombinant plasmids, cells must be in a 'competent' state, which can be artificially induced in *E.coli* either by treatment with ice-cold $CaCl_2$ (Mandel and Higa, 1970), or electroporation (Neumann *et al.*, 1982). (The details of the process of transformation are still largely unknown; it probably involves binding of double-stranded DNA to receptors on the cell surface, followed by irreversible uptake of the DNA into the cell.)

5.2. Materials and Methods

All cloning procedures were carried out at the laboratory of Dr. J.D.D. Bishop, Marine Biological Association, Plymouth, and at the laboratory of Prof. A. Cossins, School of Biological Sciences, University of Liverpool. Cycle sequencing was attempted in the Donnan Laboratories, Department of Genetics and Microbiology, University of Liverpool.

5.2.1. Cloning of blunt-ended fragments using the pCR-Script SK (+) plasmid and *Epicurian coli* XL1-Blue MRF´Kan supercompetent cells

5.2.1.1. Restriction digests of (whole) mitochondrial DNA

Mitochondrial DNA was digested with HpaI, the resulting blunt-ended restriction fragments separated on a 1 % agarose gel and the 5.8, 3.7 and 2.4 kb bands excised and gel-purified, following the instructions given by the manufacturer

(Quiaquick-kit, Qiagen, Crawley, UK). Plate 4.1.C. (Chapter 4) shows the result of digesting the whole mitochondrial DNA molecule with HpaI.

Blunt-end cloning was first attempted using the pCR-Script SK (+) cloning kit (Stratagene), which contained predigested pCR-Script SK (+) vector, *Epicurian coli* XL1-Blue MRF´Kan supercompetent cells (efficiency 5 x10^9 cfu/µg of pUC 18 DNA), T4 DNA ligase (4 U/µl), 10 x reaction buffer, rATP (10mM), control insert, pUC 18 control plasmid (0.1 ng/µl), and 1.44 M ß-mercaptoethanol. This kit is especially designed for cloning of blunted (polished) PCR-fragments. The pCR-Script SK (+) vector, a 2961-bp plasmid, has a SrfI site incorporated into the multiple cloning site (Fig 5.2.). SrfI is a rare-cleavage restriction enzyme with the octanucleotide recognition sequence GCCC GGGC, producing blunt ends, and thus enabling the insertion of a blunt-ended HpaI-fragment. According to the manufacturer, including the restriction enzyme SrfI in the ligation reaction maintains a high steady-state concentration of digested vector DNA and thereby increases the ligation efficiency of blunt-ended fragments by the simultaneous, opposite reactions of the SrfI restriction enzyme and T4 ligase. Cloning was carried out according to the protocol supplied with the pCR-Script kit.

As a control for efficiency of the cloning kit, two RAPD-fragments (0.8 kb fragment of the ascidian *Diplosoma listerianum*, primer Y13, and 1.6 kb of *P. maximus*, individual R12/95, primer R06) were gel-purified and, after 'polishing' (blunting) the ends with *Pfu* polymerase, cloned into the pCR-Script SK (+) vector.

Polishing of purified PCR products was carried out as follows: 10 µl of purified PCR product, 1µl of dNTP (2 mM), 1µl of 10 x *Pfu* polymerase buffer, 1µl of *Pfu* polymerase (2.5 U, Stratagene) were added to a microfuge tube, gently mixed, overlaid with mineral oil and incubated for 30 min at 72 °C.

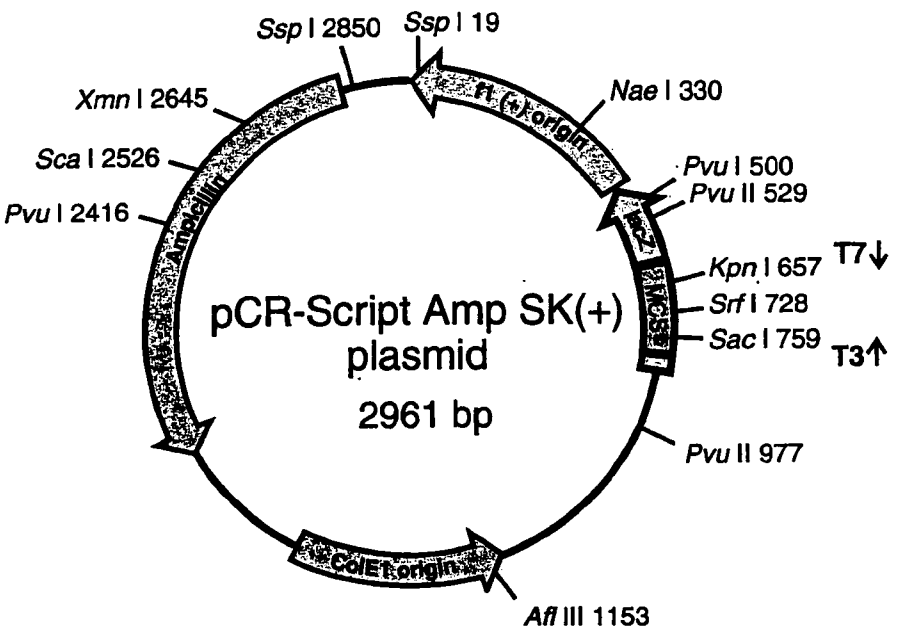


Figure 5.2. Map of the pCR-Script SK (+) plasmid. The SK designation indicates that the polylinker is oriented such that *lacZ* transcription proceeds from SacI to KpnI. MCS: 102 bp multiple cloning site, flanked by T3 and T7 RNA promotors (from Stratagene catalogue).

5.2.1.2. Ligation

For each ligation reaction, the following components were added to a 0.5 ml microcentrifuge tube:

1µl of pCR-Script cloning vector (10 ng/µl)
1µl of pCR-Script 10 x reaction buffer
0.5 µl of 10 mM rATP
4 µl of insert DNA (or control insert)
1 µl of SrfI restriction enzyme (5 U/µl)
1µl of T4 DNA ligase (4 U/µl)
1.5 µl distilled H_2O

The ligation took place over 1 hour at room temperature. Subsequent heating for 10 minutes at 65°C destroyed the enzymes. The samples were kept on ice until transformation.

5.2.1.3. Transformation

The supercompetent cells (*Epicurian coli* XL1-Blue MRF´Kan, Stratagene) were thawed on ice. 40 µl aliquots were pipetted into prechilled 15-ml Falcon 2059 polypropylene tubes. After 0.7 µl ß-mercaptoethanol was added into the contents of each tube (final concentration 24 mM), the samples were placed on ice for 10 minutes, and swirled gently every 2 minutes. 2 µl of ligation mixture (pUC 18 plasmid for the control) was added, and the tubes were kept on ice for 30 minutes. The samples were heat-pulsed in a 42°C waterbath for 45 seconds, and placed on ice for 2 minutes. 0.45 ml of preheated (42°C) SOC-medium (see Appendix) was added and the tubes were shaken at 37°C for 1 hour. On LB-ampicillin agar plates containing X-gal and IPTG (see Appendix), 50, 100, 150 and 190 µl of the transformation mixture was plated using a sterile glass spreader. (To increase the volume of the 50 µl transformation mixture, 150 µl SOC-medium was added on the plate before spreading.) The plates were incubated upside-down overnight at 37°C. White colonies were transferred into 10 ml liquid LB-medium (see Appendix) and

grown at 37°C in a shaker (225 rpm) overnight. A glycerol-stock was prepared from each liquid culture (0.75 ml liquid culture plus 0.75 ml glycerol) and stored at -20°C. Plasmid isolation was carried out on the remaining liquid culture with the Promega Wizard miniprep DNA purification system according to the manufacturer's instructions (Promega, Southampton, UK). 5 µl of plasmid DNA was cut with EcoRV (which has one restriction site within the plasmid), and run on a 1% agarose gel to check the sizes of plasmid and insert.

5.2.2. Blunt-sticky and sticky-sticky cloning in pBluescript II SK (+) vector and *E. coli* XL1-Blue MRF´Kan supercompetent cells

5.2.2.1. Restriction digests of (whole) mitochondrial DNA

The approximate size of fragments obtained after double-digestion with two restriction enzymes could be estimated by means of a map of the restriction sites in the mitochondrial DNA of *P. maximus* from Rigaa *et al.* (1993) (Figure 4.2.).

MtDNA was cut with

a) HpaI and EcoRI (2, 2.8, 2.5 and 3.3 kb fragments, blunt-sticky),

b) EcoRI and AccI (2.9 kb fragment, sticky-sticky),

c) HindIII (2.6 and 2.5 kb, sticky-sticky),

d) HpaI (3.7 and 2.4 kb, blunt-blunt).

The plasmid was cut with

a) SmaI and EcoRI (blunt-sticky),

b) EcoRI and AccI (sticky-sticky),

c) HindIII (sticky-sticky),

d) SmaI (blunt-blunt).

All digests were run on a 1% agarose gel. The fragments of the EcoRI/HpaI digest, for which cloning in the pBluescript vector was attempted, are shown in Plate 5.1.A. Fragments of the other restriction digests were of a similar yield. The fragments between 1.9 and 3.7 kb and the plasmid were excised from the agarose gel and purified (Quiaquick-kit, Qiagen). An aliquot of each sample was gel-electrophoresed to estimate the relative concentrations of insert and plasmid (by eye) for ligation.

5.2.2.2. Ligation

The ligation was carried out in a total volume of 10 µl, including gel-purified plasmid (pBluescript II SK (+), Stratagene), gel-purified mtDNA-fragment, 1µl 10 x reaction buffer (for Promega ligase: 300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP; for Pharmacia ligase:'one-phor-all plus'), and T4 DNA ligase (Promega or Pharmacia), plus separate rATP (Stratagene), H_2O *ad* 10 µl, in concentrations as shown in Table 5.1. Trials included different combinations of the ligation conditions in Table 5.1.

Table 5.1. Ligation conditions for pBluescript

	blunt-blunt	blunt-sticky	sticky-sticky
ratio vector:insert	1:3	1:3	1:2
rATP	0.5 mM	0.5 / 1 mM	0.5 / 1 mM
Ligase (Promega or Pharmacia)	10 U / 3 U	3 U / 1 U	0.5 U / 1 U
reaction conditions	14 °C overnight / room temperature 6 hours	14 °C overnight / room temperature 6 hours	room temperature 3 hours / 4 °C overnight

5.2.2.3. Transformation

5- 7 µl ligation mix (for all trials) were used to transform 100 µl *E. coli* XL1-Blue MRF´Kan supercompetent cells (efficiency 5 x 10^9 cfu/µg of pUC 18 DNA; Stratagene), according to the protocol described above (section 5.2.1.3.), except that 1.7 µl ß-mercaptoethanol was applied (final concentration 25 mM), the samples were heat-pulsed in a 42°C waterbath for 30 seconds, and 0.9 ml of preheated (42°C) SOC-medium was added. Cells were plated on agar plates containing 12.5 µg/ml tetracycline (Sigma), 50 µg/ml ampicillin (Sigma), X-gal and IPTG (see Appendix).

5.2.3. Blunt-sticky and sticky-sticky cloning with pBluescript II SK (+) vector and *E. coli* SURE 2 supercompetent cells

Transformation was also attempted using the same ligation mixtures as above, but 100 µl *E. coli* SURE 2 supercompetent cells (efficiency > 1 x 10^9 cfu/µg of pUC 18 DNA; Stratagene). These are cells developed to clone inserts capable of forming secondary and tertiary structures. Two structures that occur frequently in eukaryotic DNA, and which are known to be highly unstable, are cruciforms (caused by inverted repeats) and Z-DNA (formed with alternating purine-pyrimidine stretches). Inserts forming these structures are normally deleted or rearranged by *E. coli*. In *E. coli* SURE cells the primary bacterial pathways catalysing these events are inactivated.

For all trials, cells from twelve white colonies were picked and simultaneously 1) transferred into liquid medium, 2) spread out on a grid-labelled agar-plate, and 3) used to set up a PCR using T3 and T7 primers. Cells in liquid medium were shaken at 37°C and 225 rpm overnight, agar plates were incubated overnight at 37°C. Additionally, 24 - 48 white colonies per trial were subjected to T3/T7 PCR only. Blue colonies were analysed correspondingly as controls.

5.2.4. T3 / T7 PCR

The multiple cloning site of the pBluescript II SK (+) is flanked by priming sites for T3 and T7 primers, enclosing a sequence of 166 bp. When an insert is present in the multiple cloning site, amplification should result in PCR products the size of the insert plus 166 bp.

Primer T3 5' AATTAACCCTCACTAAAGGG 3'
Primer T7 5' GTAATACGACTCACTATAGGGC 3'

5.2.4.1. Conditions for T3 / T7 PCR

1.5 µl $MgCl_2$ (25 mM, Promega Ltd., Southampton, UK), 1µl dNTPs (2 mM, Sigma, Poole, UK), 1 µl 10 x buffer (100mM Tris-HCl, pH 9.0; 500 mM KCl; 1.0 % Triton X-100; Promega), 1µl of each primer T3 and T7 (20 ng/µl; Cruachem, Glasgow, UK), 0.5 U *Taq* (Promega) and H_2O *ad* 10 µl were pipetted into a PCR-tube. Selected white colonies were touched with a pipette tip which was then dipped into the PCR mix. The reaction mix was overlaid with mineral oil. A Perkin Elmer DNA thermal cycler (model TC1, Perkin Elmer, Warrington, UK) was programmed to carry out 30 cycles at 1 min 94°C, 30 s 55°C, 3 min 72°C. The PCR product was electrophoresed through a 1.4 % agarose gel.

For cloned RAPD fragments, a PCR using the original RAPD-primer on the purified plasmid was also carried out, applying the RAPD protocol (see Chapter 3).

5.3. Results

5.3.1. Cloning with the pCR-Script SK (+) plasmid and *E. coli* XL1-Blue MRF´Kan cells

Cloning of the 2.4, 3.7 and 5.8 kb HpaI fragments into the pCR-Script cloning vector resulted in only a few white colonies after ongrowing on agar plates. Restriction fragments of the purified plasmids added up to approximately the expected size of plasmid plus insert (Plate 5.1.B), although plasmids which should have the same size insert showed variation in size. Sequencing of the plasmid samples 2, 3, 4, 9 was intended, but could not be carried out, as the cycle-sequencing reaction failed.

The T3/T7 PCRs of clones containing the RAPD-fragments produced bands of the expected size. A PCR using the original RAPD primer and the plasmid containing the 0.8 kb RAPD band also gave a PCR-product of the right size.

5.3.2. Cloning with pBluescript II SK (+) vector and *E. coli* XL1-Blue MRF´Kan cells

Each attempt produced numerous white colonies. Most white colonies remained white after repeated transfer and incubation on new agar plates. However, the T3/T7 PCR on 24 - 48 white clones per cloning attempt produced only bands of ca. 160 bp, and a T3/T7 PCR on the vector only (untreated, no insert) showed bands of corresponding size (160 bp), thus confirming the absence of any insert; diffuse bands of ca. 3 kb occurred in some cases (Plate 5.1.C). When plasmids, extracted from the same clones (after ongrowing in liquid medium overnight), were cut with the restriction enzyme EcoRI, no insert of larger size could be detected (Plate 5.1. D and E). Several repetitions of ligation, transformation, T3/T7 PCR and plasmid digests did not result in any insert of the desired size.

5.3.3. Cloning with pBluescript II SK (+) vector and *E. coli* SURE cells

Very few white colonies could be detected, which were much smaller than the white colonies obtained with the *E. coli* XL1-Blue MRF´Kan cells. Neither T3/T7 PCRs, nor restriction digests of plasmids isolated from the white colonies, revealed any large inserts.

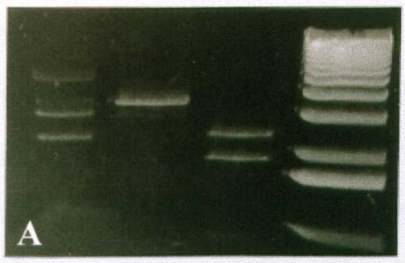
Plate 5.1.

A. EcoRI-digest of HpaI-fragments; all fragments were used for cloning after gel-purification. Lane 4: size marker (1 Kb ladder, GibcoBRL).

B. Result of pCR-script blunt cloning: purified recombinant plasmids were cut with EcoRV. Lane 2 - 12: plasmids isolated from different bacterial clones; lane 1: 1 Kb-ladder.

C. Result of the T3/T7 PCR of white clones containing the pBluescript vector. Lanes 1 and 10: low-weight-marker.

D. and E. Plasmids (pBluescript) isolated from white clones and digested with EcoRI (Lane 8 in each: 1 kb ladder).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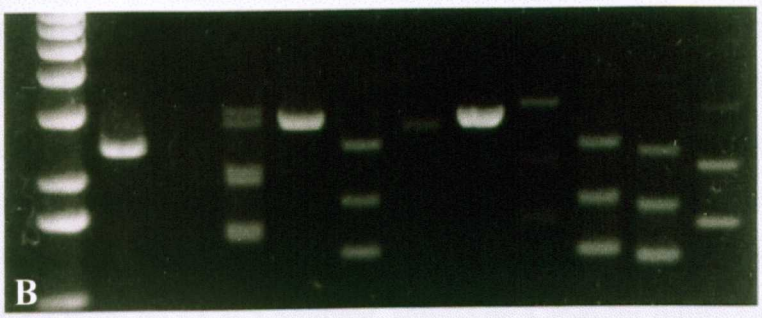
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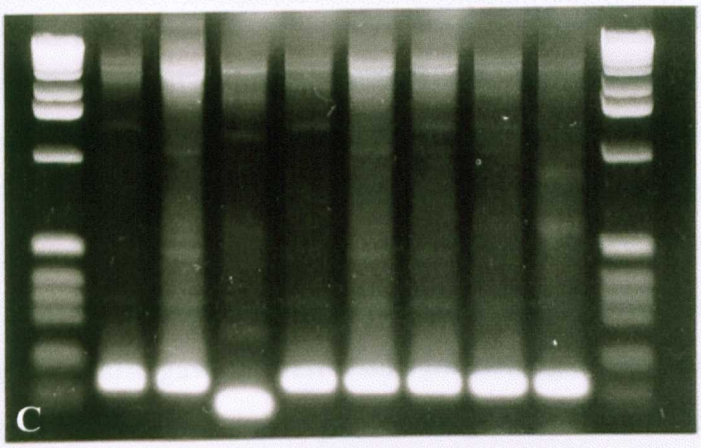
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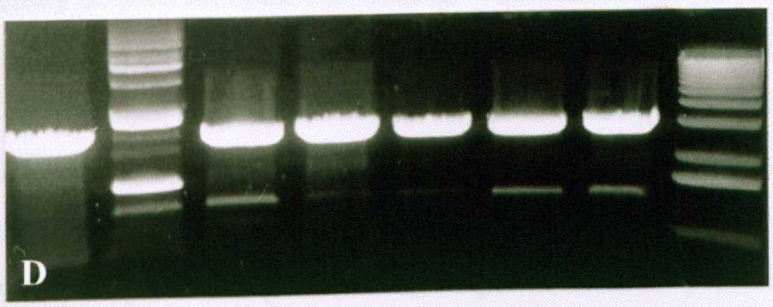
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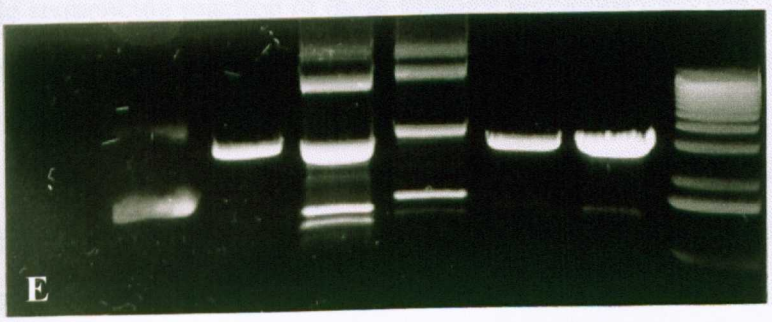
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5.4. Discussion

The different protocols employed for the cloning of mitochondrial DNA fragments generally were successful inasmuch as bacterial colonies, including white ones, were present in all trials and repetitions, and the controls showed the expected results. The use of ultracompetent cells, and exact adherence to the manufacturer's protocols are likely to have contributed to this. Uncertainties arose from estimation of concentrations of insert and plasmid in the ligation reaction. The low starting concentration of mtDNA, which decreased even more with gel purification of restriction fragments, made it difficult to adjust concentrations of plasmid and insert accurately. Increasing the concentration of mtDNA, by combining many samples and purifying them on a CsCl-gradient, should be a goal for future attempts. Although several variations and repetitions were tried, the ligation conditions might not have been optimised in any of these. Furthermore, the fragments chosen were relatively large, which makes cloning potentially more difficult. However, smaller fragments would not generate enough information for population studies.

The pCR-script cloning kit proved to be successful for cloning of RAPD fragments. It also gave the best results for cloning of blunt-ended mitochondrial DNA fragments. Although it was originally designed to clone PCR products, it can generally be used for blunt-end cloning. The inclusion of the restriction enzyme SrfI in the ligation reaction may have helped to increase ligation success. However, very few recombinant clones could be isolated with mtDNA. The restriction fragments of the recombinant plasmids added up to approximately the right size, but there was size variation between plasmids extracted from different clones. The T3/T7 PCR did not show any amplification product of the expected size, and problems occurring with the cycle sequencing reaction of these samples precluded their use for the design of PCR primers. It is not known what caused the failure of these PCR reactions.

Cloning of *P. maximus* mtDNA has proved before to be difficult (D. Sellos, pers. comm.). Wilding (1996) also reported a very low yield in several attempts to clone a 2 kb fragment: only 2.5 % of white colonies contained a 2 kb insert, 1.7 % a

1 kb insert, 46 % had inserts smaller than 0.5 kb and 50 % had none, or a very small insert. This raises the question of why there were so many false positive colonies in both Wilding's investigation and the present study. Wilding (1996) suggests one reason to be ligase-inhibiting compounds extracted along with the DNA during gel purification, which he accomplished by centrifugation through glass-wool. However, the gel purification kit used in the present study should have excluded such compounds.

The overall low yield of recombinant plasmids and the unsuccessful attempts at cloning with different plasmids and cells, applying a wide range of different conditions, suggest a more general problem. MtDNA is known to produce secondary structures, which the bacterial host cuts out of recombinant plasmids (Stratagene, pers. comm.). This could explain the occurrence of inserts of different sizes in the case of cloning by means of the pCR-script. It could also be a possible reason for the large number of false white colonies: after excision of inserted sequences by the bacterial host, plasmids could still have small inserts left, which would interrupt the reading frame for the *lacZ*-gene. The use of SURE cells was expected to solve this problem, but was not successful; the low efficiency of SURE cells certainly did not help.

White colonies were abundant when employing the pBluescript, but the T3/T7 PCR detected no recombinant plasmids. One possible reason could be that the conditions for the T3/T7 PCR were not optimal for amplification of large fragments. In some cases, diffuse bands of a size around 3 kb occurred, which could have been the result of an amplification of whole plasmids without insert, or potentially amplified inserts. Improving PCR conditions for long-PCR should be a priority in future attempts. In view of the low yield of true white colonies, more white colonies should be screened by restriction digestion of isolated plasmids. A higher efficiency compared with the pCR-script has been reported for a new plasmid (pCR-blunt, Invitrogen; Invitrogen application note), which could be employed in future trials.

CHAPTER SIX: REPEATED ELEMENTS

6.1. Introduction

6.1.1. Occurrence of repeated elements

Mitochondria are believed to have descended from free-living purple bacteria (Cedergren *et al.*, 1988; Gray, 1989). Along this lineage there has been a dramatic reduction in genome size: the chromosome of a purple bacterium (*E. coli*, ca. 4.5 million bp) is more than 270 times larger than the mitochondrial genome of vertebrates. There is evidence that selection for small size, possibly mediated by a 'race for replication', is an important force in the evolution of mitochondrial DNA (reviewed by Rand, 1993). This view is challenged by the occurrence of comparatively large and variable mitochondrial genomes in some species. Length variation of the mitochondrial genome, due to variation in copy number of presumably non-coding repeated elements, has been reported from a variety of animal taxa (Harrison *et al.*, 1985, Rand and Harrison, 1989, crickets; Boyce *et al.*, 1989, bark weevils; Solignac *et al.*, 1986, *Drosophila*; Powers *et al.*, 1986, nematodes; Gjetvaj *et al.*, 1992, scallops; Wallis, 1987, newts; Densmore *et al.*, 1985, Moritz and Brown, 1986, 1987, lizards; Bentzen *et al.*, 1988, sturgeon; Arnason and Rand, 1992, cod; Wilkinson and Chapman, 1991, bats; see also Moritz *et al.*, 1987, Brown *et al.*, 1992). Although the repeated elements of various species vary widely in size, structure and sequence, they generally share the feature of close association with the control region (Moritz *et al.*, 1987), which contains signal elements controlling transcription and replication. Exceptions were reported for a nematode species (Beck and Hyman, 1988) and two species of scallops (La Roche *et al.*, 1990; Gjetvaj *et al.*, 1992; Fuller and Zouros, 1993), where length polymorphisms were shown to occur in several dispersed portions of the genome. In most studies repeated elements were typically tandem and direct and ranged in size from less than 50 bp to 9 kb, causing length-variation of the mitochondrial genome within and between species. As such variation can confound studies on population and species relationships, understanding the origin and processes of length variation is important for population genetic investigations based on mtDNA.

The largest metazoan mitochondrial genome found so far was from *Placopecten magellanicus;* it also showed large intraspecific size variation (Snyder *et al.*, 1987; Gjetvaj *et al.*, 1992; Fuller and Zouros, 1993). The molecule was up to 42 kb, about twice the length required to encode the standard set of mitochondrial genes, and differed among individuals collected at the same location by as much as 30%. Three regions exhibiting length polymorphism were identified, which could account for the size-range of 31-42 kb, but not for the large basic size (about 28 kb) of the mitochondrial genome. The three variable regions were located in a generally variable part of the mitochondrial genome, showing other minor mutations, which could be distinguished from an invariant 16 kb region (Fuller and Zouros, 1993).

A study of seven species of scallops (*Placopecten magellanicus, Pecten maximus, Chlamys gigantea, Aequipecten opercularis, A. irradians, A. hastata* and *A. islandica*) by Gjetvaj *et al.* (1992) revealed large-scale size variation of mtDNA between the different species. The variation was found to be entirely attributable to different sizes and numbers of copies of repeated elements. The size of these elements ranged from 400 bp (*A. opercularis*) to 1442 bp (*P. magellanicus*) and 1586 bp (*P. maximus*) (Table 6.1.). The sequence of the repeated element was different for each species.

Restriction-site-heteroplasmy and size-heteroplasmy due to repeated elements was observed within individuals of most species of scallops (Gjetvaj *et al.*, 1992). Rigaa *et al.* (1993) found one individual among 27 *P. maximus* which showed size heteroplasmy (genome sizes 20.0 and 21.7 kb, carrying two and three copies of the repeated element, respectively).

The repeated elements of *P. maximus* are arranged in direct tandem within the control region of the mtDNA molecule. Digesting the whole mtDNA with the restriction enzyme AvaI produced fragments of 3.45 kb and 2.15 kb, each containing part of the repeated element, and a 1.6 kb fragment, consisting of the repeated

Table 6.1 Presence of repeated sequences, size range, and index of genotype diversity in seven species of scallops (after Gjetvaj *et al.*, 1992; Fuller and Zouros, 1993; Rigaa *et al.*, 1993).

	Pecten maximus	***Aequipecten opercularis***	***Placopecten magellanicus***	***Argopecten irradians***	***Chlamys hastata***	***Chlamys islandica***	***Crassadoma gigantea***
Size of the repeated sequence (kb)	1.6	> 0.4 (accurate length not established)	1.4	no repeated element present	0.6 (accurate length not established)	1.2	1.0
Copy no. per molecule	2 - 5	?	2 - 8	-	?	1 - 3	3 - 5
most frequent size of whole mt molecule (Kb)	21.5	24.6	35.5	16.2	25.5	23.6	23.8
Size range (Kb)	19.9 - 26.3	21.0 - 28.2	31.0 - 42.0	16.2	23.9 - 27.2	22.2 - 25.0	22.8 - 24.8
Haplotype diversity*	0.967	0.978	0.990	0.380	0.894	0.796	0.879

* from Nei and Tajima (1981)

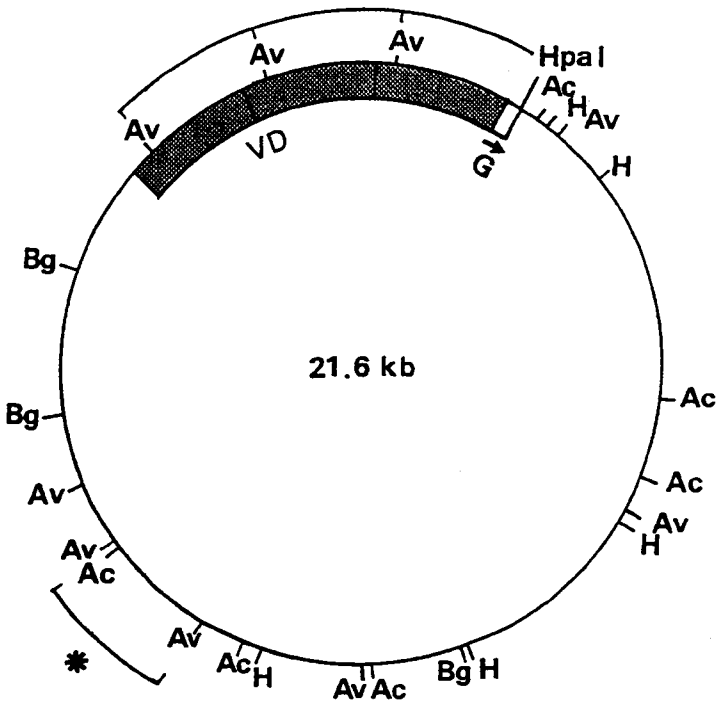


Figure 6.1. Circular restriction map of *P. maximus* mtDNA molecule for the four endonucleases AvaI (Av), AccI (Ac), BglII (Bg), HindIII (H). Note the 1.6 kb AvaI fragment outside the region of repeated elements (*) (from Rigaa *et al.*, 1995).

element only (Rigaa *et al.*, 1995) (Figure 6.1.). This pattern of fragments was consistent for two and more copies of the repeated element. The presence of more than two copies led to the accumulation of the 1.6 kb fragment, due to a single restriction site for AvaI within the repeated element. AvaI also produced a 1.6 kb fragment unrelated to the repeated element (Figure 6.1.).

Within the 27 individuals of *P. maximus*, Rigaa *et al.* (1993) found six size classes of mtDNA covering a range of 20 to 25.8 kb, the most frequent being 21.7 kb. This variation was caused by repeated elements occurring in two to five copies, with 86 % of the individuals carrying three or four copies. In contrast, Gjetvaj *et al.* (1992) reported up to seven copies of the repeated element in the same species. The 1.6 kb AvaI fragment outside the region with the repeated elements could have lead Gjetvaj *et al.* (1992) to an overestimation. Within the repeated elements, nucleotide substitutions were frequent and created additional restriction sites and variation within and between individuals (Rigaa *et al.*,1993).

6.1.2. Molecular structure and possible origin of repeated elements

In *P. maximus*, the 1586 bp long repeated element ends two bp upstream relative to the beginning of the $tRNA^{gly}$ gene (Rigaa *et al.*, 1995). The middle part is characterised by an A+T-rich domain, in which stretches of 4-11 nucleotides of A and T are present, giving a 75 % A+T content over 784 nucleotides. G+C rich stretches are abundant in the last quarter of the unit, giving a 54 % G content over 174 nucleotides. Rigaa *et al.* (1995) found that two 84 bp sequences, from nucleotide 201 to 284 and from 845 to 928, showed a high level of complementarity (94%), and were therefore likely to form a stable secondary structure, a long stem. Two other potential secondary structures were identified, which were predicted to fold into a hairpin (stem and loop). The structure and the relative organization of the different domains is very similar between *P. maximus* and *P. magellanicus* (Figure 6.2.), but sequence homology was only present for one of the hairpin structures (La Roche *et al.*, 1990; Rigaa *et al.*, 1995). The repeated element of *P. maximus* can thus be

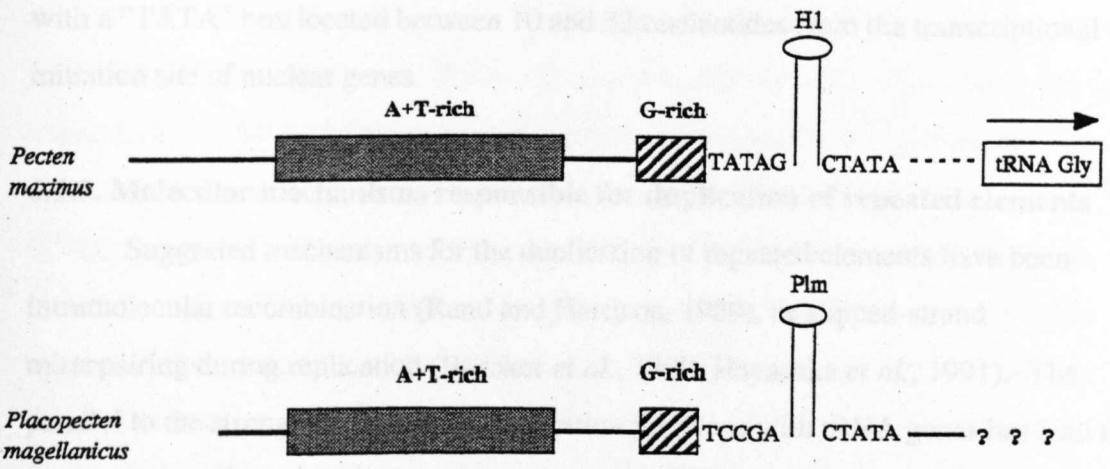


Figure 6.2. Schematic organisation of the 1.6 kb and 1.4 kb repeated units of *P. maximus* and *P. magellanicus*. The arrow indicates direction of transcription. The question marks indicate the unknown sequence flanking the repeated unit of the *P. magellanicus* mtDNA (from Rigaa *et al.*, 1995).

divided into a central domain of low stability (A+T-rich) flanked by two more stable, G+C-rich regions, potentially folding into hairpin structures. Interestingly, the control region of the mtDNA of *Mytilus edulis* showed similar features (Hoffman *et al.*, 1992). This led Rigaa *et al.* (1995) to the hypothesis that the length-variable domains of the *Pecten* species originated from the amplification of the noncoding region of mtDNA of a common ancestor of *Mytilus* and *Pecten* and that this could have a regulatory role in transcription. In this context, Rigaa *et al.* (1995) see a parallel between a "TATA" motif located exactly 33 nucleotides upstream of the beginning of the $tRNA^{gly}$ gene in *P. maximus* and the prokaryotic and eukaryotic gene promotors with a "TATA" box located between 10 and 33 nucleotides from the transcriptional initiation site of nuclear genes.

6.1.3. Molecular mechanisms responsible for duplication of repeated elements

Suggested mechanisms for the duplication of repeated elements have been intramolecular recombination (Rand and Harrison, 1989), or slipped-strand misrepairing during replication (Buroker *et al.*, 1990; Hayasaka *et al.*, 1991). The parallel to the strong association of duplication junctions with tRNA genes has lead to the suggestion that secondary structures in repeated elements influence duplicate formation (Brown, 1985; Moritz and Brown, 1987; Rand and Harrison, 1989; Moritz, 1991). Stem and loop structures are known to act as signals for initiation of light strand replication (Tapper and Clayton, 1981) and transcript editing (Battey and Clayton, 1980; Ojala *et al.*, 1981); therefore such structures could produce abnormalities in the replication process, perhaps resulting in the generation of additional copies of repeated elements.

6.1.4. Repeated elements as population markers

To date, few studies have employed repeated elements as markers to investigate population differentiation. In a study of several subspecies of the honeybee, *Apis mellifera*, Garnery *et al.* (1993) made use of the variable number of copies of a repeated element in the COI-COII intergenic region to distinguish between populations and subspecies from different locations. Length variation in the

mitochondrial genome of the minnow, *Cyprinella spiloptera*, could not be associated with geographic population structure (Broughton and Dowling, 1994). Rigaa *et al.* (1993) found no evidence of subdivision of *P. maximus* stocks from the western coast of Brittany, France, with respect to their copy number of repeated elements. However, their samples were very small (two populations with four individuals each, one population with eight individuals) and from a limited geographical range.

Nucleotide substitutions and deletions within repeated elements are frequent (Rigaa *et al.*, 1993). Similar findings have been reported for *Drosophila* (Monnerot *et al.*, 1990), in which a similarly A+T-rich region has been shown to evolve through a high rate of point mutations. Since repeated elements are non-coding, these loci are expected to exhibit high DNA sequence polymorphism and the variants are predicted to be selectively neutral. It would be interesting to see whether populations of different geographical origin can be distinguished by polymorphism within repeated elements. After PCR amplification of repeated elements, the PCR product could be subjected to restriction analysis. In view of this possibility, an attempt was made to design primers for PCR amplification of *P. maximus* repeated elements and to screen for restriction site polymorphisms.

6.2. Materials, Methods and Results

6.2.1. PCR amplification of repeated elements

6.2.1.1. Primer design

Two primers (length 31 and 32 bases, respectively) for amplification of repeated elements were designed, based on a sequence for the repeated element of *P. maximus* from Concarneau, Bretagne, published by Sellos (1993) in the EMBL/GenBank/DDBJ database. Primers of longer sequence (> 30 bp) than usual for conventional PCR were expected to assist long-PCR (see Chapter 2) in potentially amplifying all copies of repeated elements present in a mitochondrial genome at once. The unusual annealing and extension time employed in protocol 2 was recommended for long-PCR by Barnes (1994a). As the repeated elements are tandemly arranged in

the mitochondrial genome, it was hoped that the primers would anneal to different copies of repeated elements, thus resulting in a PCR-product consisting of fragments of different length, the longest ideally representing the total number of copies present in an individual mitochondrial genome, the smallest consisting of one copy only.

Primer P1: GGT TTG GGG GAA GAG CAT GGC TTC AAA ATT
31 bases, forward primer Tm = 78.0 C
Primer P2: GCG GGG CAT GAA CCC AAC GTA CTA CAC ATA TA
32 bases, reverse primer Tm = 80.5 C

6.2.1.2. PCR conditions

The following protocols were developed in a series of trials with different annealing and extension temperatures and times, and different *Taq* polymerase, $MgCl_2$ and DNA concentrations.

a. Protocol 1: using Promega-*Taq* polymerase

The reaction mixture included 1 µl total DNA or mtDNA, 100 pM of each primer P1 + P2 (synthesised by the Department of Biochemistry, University of Liverpool), 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Sigma), 10 µl 10 x PCR-buffer (100mM Tris-HCl, pH 9.0; 500 mM KCl; 1.0 % Triton X-100; Promega Ltd., Southampton, UK), 3.75 mM $MgCl_2$ (Promega), and water *ad* 100 µl (molecular biology grade, BDH, Poole, UK). The reaction mixture was overlaid with mineral oil (Sigma, Poole, UK). After a hot start of 3 min at 94°C, 5 units *Taq*-polymerase (Promega) were added and the samples were subjected to 35 cycles of 1 min at 94°C, 1 min at 45°C, 4 min at 70°C.

b. Protocol 2: using *MobiTaq*-polymerase

The reaction mixture included 1 µl total DNA or mtDNA, 100 pM of each primer P1 + P2, 0.25 mM of each deoxynucleotide triphosphate, PCR-buffer (50mM Tris-HCl, pH 9.2; 3.5 mM $MgCl_2$; 16 mM $(NH_4)_2SO_4$, 150 µg/ml BSA), and water *ad* 100 µl. The reaction mixture was overlaid with mineral oil. After a hot start of 3 min

at 94°C, 25 units *MobiTaq*-L-polymerase (a *Taq*-polymerase with proofreading function; MoBiTec, Goettingen, Germany) were added and the samples were subjected to 25 cycles of 20 sec at 94°C, 11 min at 70°C.

In a series of experiments, both polymerases were employed under reciprocal conditions, e.g., PCR under *'MobiTaq'* conditions was tested with Promega *Taq* and *vice versa*. The optimal amount of polymerase was determined for both protocols with trials of 1, 2.5, 5, 10, and 25 units. In addition, both protocols were tested with different amounts (1, 5 and 25 units) of Boehringer-Mannheim *Taq* (Boehringer-Mannheim, Lewes, UK).

6.2.1.3. Results obtained with the different PCR protocols

Primers P1 + P2 consistently amplified a 1370-bp-fragment under both protocols. In no case was a longer amplification product obtained. The use of either total DNA or mtDNA made no difference to PCR-product quantity or quality. The yield of PCR-product was much higher with *MobiTaq*-L polymerase (Plate 6.1.B.) than with Promega *Taq* (Plate 6.1.A.). Because of the high cost of *MobiTaq*-polymerase, it was necessary to switch to a less expensive polymerase, as reducing the amount of *MobiTaq* below 2.5 units gave much less product. Promega *Taq* amplified a fragment of the right size under the conditions of protocol 1, but the PCR failed when using protocol 2 or amounts of enzyme other than 5 units. Boehringer-Mannheim *Taq* (1 and 5 units) produced PCR-fragments of the correct size when utilized with protocol 2 (Plate 6.1.C.). Both Promega *Taq* and Boehringer-Mannheim *Taq* were tested on a number of individuals from locations around the Isle of Man. Unfortunately, both Boehringer and Promega *Taq* polymerase amplified additional smaller bands for some individuals and gave much less product than *MobiTaq*. Plate 6.1.D. shows the amplification products, obtained by means of protocol 2 and Boehringer-Mannheim *Taq*, of *P. maximus* individuals from Ramsey and Bradda (1995 samples). In some individuals a smaller band appeared close to the 1.37 kb band. The primers failed to amplify DNA from *Aequipecten opercularis*.

6.2.2. Re-amplification of the PCR product

Additional bands in the PCR product complicated the analysis of restriction patterns. To overcome this problem, the PCR products were electrophoresed on an 1% agarose long-gel at 50 V, 30 mA overnight, and the larger band was purified from the agarose gel (Qiaquick gel-extraction kit, Quiagen, Crawley, UK). The yield after gel-extraction was relatively low. Since radiolabelling of DNA was not possible at PEML and only ethidium bromide staining of agarose gels could be undertaken, at most one restriction digest could be carried out on the purified product. To obtain more product for the restriction analysis, the gel-extracted fragments were subjected to a PCR re-amplification.

6.2.2.1. PCR conditions for re-amplification

Different protocols were tested for the re-amplification and it was found that the best results were obtained with Boehringer-Mannheim *Taq* under the following PCR conditions:

1µl gel-purified PCR-product, 100 pM of each primer P1 + P2, 0.25 mM of each deoxynucleotide triphosphate, 10 µl 10x PCR-buffer (100mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 500 mM KCl, Boehringer-Mannheim) and water *ad* 100 µl. The reaction mixture was overlaid with mineral oil. After a hot start of 3 min at 94°C, 5 units Boehringer-Mannheim *Taq*-polymerase were added and the samples were subjected to 35 cycles of 1 min at 94°C, 1 min at 45°C, 4 min at 70°C.

Although a PCR-product of the right size was obtained, unfortunately, in a few samples, additional smaller bands were again present in the re-amplified product.

6.2.3. Sequencing of the PCR product

To ensure that the PCR product amplified by means of protocol 2 (samples with a single band only) was indeed the repeated element, corresponding to the sequence obtained from the EMBL database, it was purified with microspin columns (Pharmacia) and both ends were cycle-sequenced (for method see Chapter 2) in the Department of Genetics and Microbiology, University of Liverpool.

Plate 6.1.

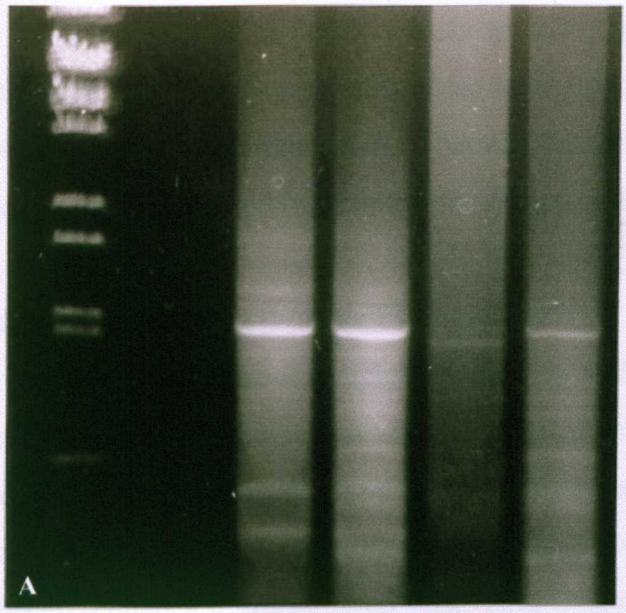
A. PCR product (ca. 1.37 kb) of protocol 1 (Promega *Taq*). 20 µl on 0.7 % agarose gel. Lane 1: low-weight-marker (Amresco), Lane 2: control (no DNA), Lane 3 and 4: total DNA; Lane 5 and 6: mtDNA; Lanes 2, 4, 6: 2.5 U *Taq*, Lane 3 and 5: 5 U *Taq*.

B. PCR product (ca. 1.37 kb) of protocol 2 (*Mobitaq*). 10 µl on 0.7 % agarose gel. Lane 1: 1Kb-marker (GibcoBRL); Lane 2: control, Lanes 3 and 4: PCR product (total DNA, 25 U *Mobitaq*).

C. PCR product (ca. 1.37 kb) of protocol 2, but with Boehringer-Mannheim *Taq*. 10 µl on 0.7 % agarose gel.; Lane 1: control (no DNA, 5 U *Taq*); Lane 2: 25 U *Taq*; Lane 3: 5 U *Taq;* Lane 4: 2.5 U *Taq*. All carried out on total DNA. Lane 5: low-weight-marker (Amresco).

D. PCR product (20 µl on 1 % agarose long-gel) of 12 individuals originating from Ramsey (Lanes 1-12) and 12 individuals originating from Bradda (Lane 13-24) using protocol 2 and 5 U Boehringer-Mannheim *Taq*. Lane 25: 1Kb-ladder. Note the additional smaller bands in some of the individuals.

E. The PCR-amplified repeated element (individuals without additional smaller amplification products) digested with restriction endonucleases (Lanes 2 and 3: HindIII; Lanes 4 and 5: EcoRI; Lanes 6 and 7: AvaI; Lanes 8 and 9: DraI; Lanes 10 and 11: BamHI). As predicted from the published sequence, only DraI (Lanes 8 and 9) has a restriction site within the fragment, resulting in two fragments of ca. 300 and 1070 bp. Lane 1: 1Kb-marker (GibcoBRL).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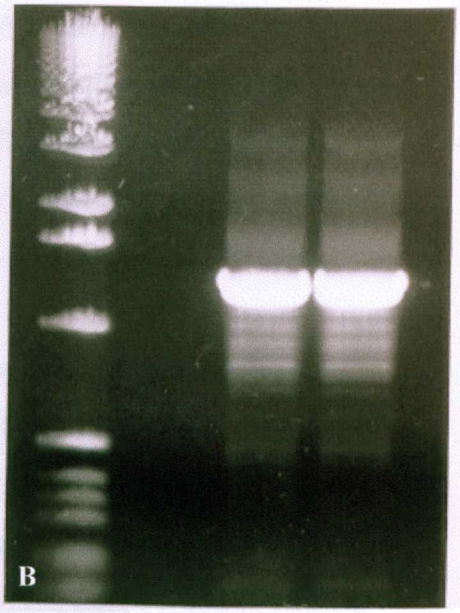
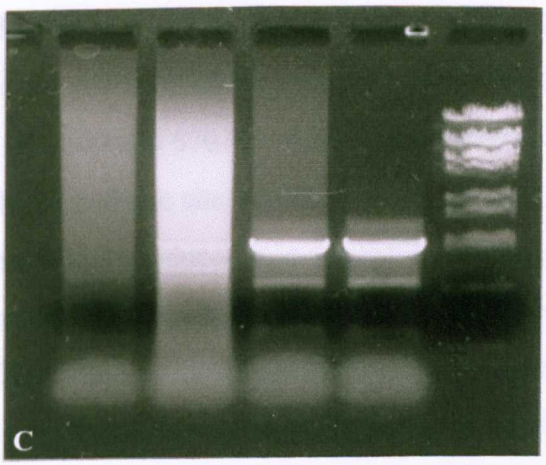
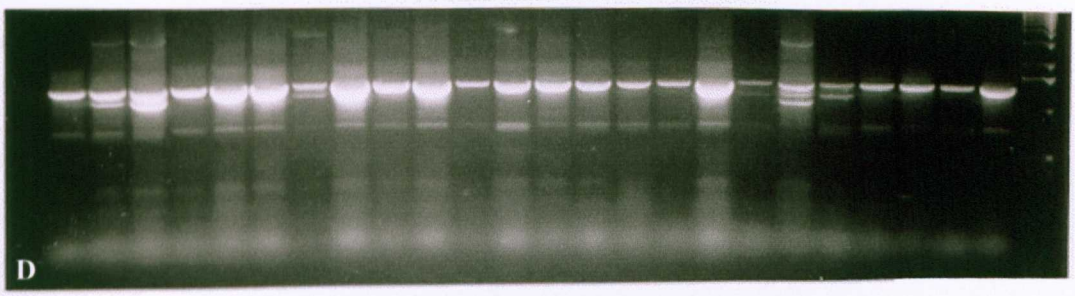
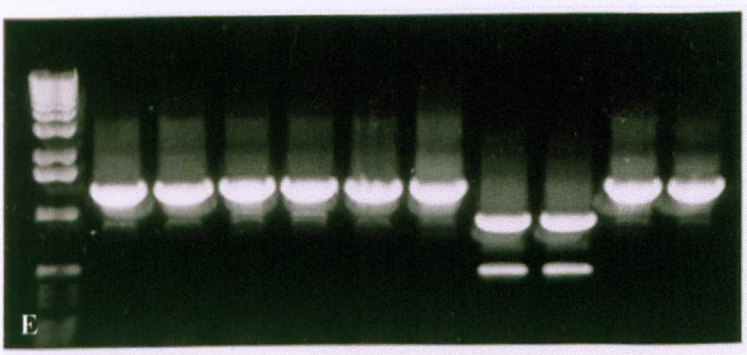

B


C


D


E

The result of the sequencing is shown in Figure 6.3. (see Appendix for automatic sequencing output); most of the bases determined by sequencing are identical to the published sequence. This was the case for both primers and two separately PCR-amplified fragments from different individuals.

6.2.4. Restriction digests of the PCR product

The PCR product of samples not showing additional smaller bands was cut with the restriction endonucleases BamHI, DraI, AvaI, EcoRI, HindIII, Tru9I, AluI, MvaI, RsaI, EcoRV to screen for differences in restriction sites. The number of fragments obtained was in agreement with predictions made from searching the published sequence for recognition sites. DraI (Plate 6.1.E., Lanes 8 and 9) had one restriction site within the fragment, giving two fragments of ca. 300 and 1070 bp, while BamHI, AvaI, EcoRI and HindIII did not cut. EcoRV had two sites, yielding three fragments of ca. 150, 500 and 670 bp. AluI produced three fragments of ca. 350, 460 and 560 bp. MvaI had one site, yielding two fragments of ca. 370 and 1000 bp. Most of the enzymes tested produced monomorphic banding patterns, while in a few cases it was not certain if the digest was complete. Tru9I produced fragments between ca. 80 and 450 bp, and presumably some smaller fragments, which could not be detected on agarose gels; three different haplotypes could be identified (Plate 6.2.A.). RsaI generated at least five clearly different banding patterns for the seven individuals examined (Plate 6.2.B.).

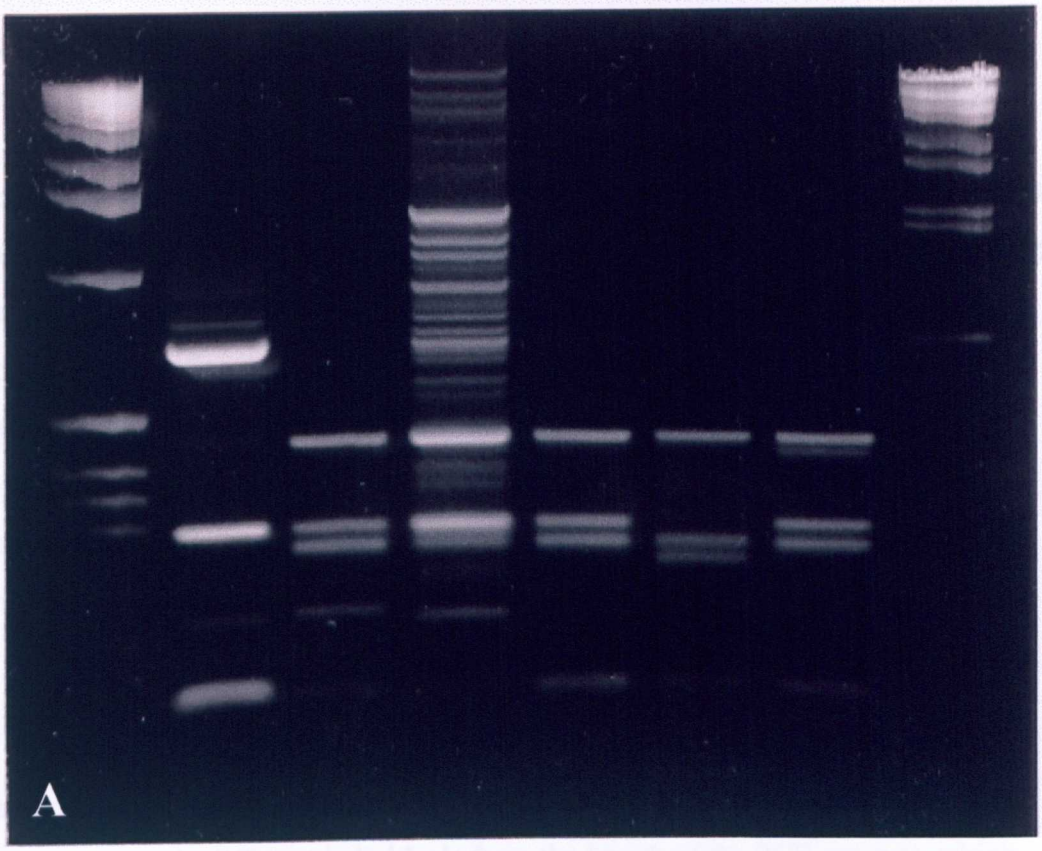
5'cccggggtgtttaataagtgggtttataggtaggtcagtggagcctctttagagctagcgaagtcgtaactcgtagttgg
gtttgggggaagagcatggcttcaaaattcgtaagtca**gtgcgtgtttttcatgcc**acaggaatttactcactaagtaca
aaatatatttttttgtaaaaaaaagtttttttttccaaaaaaagtcgggttttttttgaaaatttctcttattttgtgcaaaatg
ggac**gtacaaaaaaggcaaaaaaaacgcaaaaaatgttgaaaaaacgt**tgaaaaaaggggtgtttttggaacct
gtaagtcaaaagtctttttaggagtaaaatcgcttttaacaggaatttacgtagtaaaatgggggggttttttccaaaaaa
gttgcaaaaaatcggttttttgatggtttttataaaaattaaggaaaataagtgttttttttataaaattttataaaatttttacttag
tgcggggttttgttagggtttagttctgtttgttttttaaaaaaataagaaatttttgtaaaaaaaagtgtcatttttttaggagttt
ttcgaggtctttcaagaggttcaggggaactatctttctagcttaaaatttttttatttttttttgtaaaaaaatttttttttttaaggg
ggagtaaatttattgcattgtaagtggggcacaaaaacttggttttaggggtataaaaaatctaagaattgggcaattttgc
ctattaagaagttttgtggaatatcttgagaaacagggttttttgttgttttttttgccaaaaaaaaaaaattttttttttttttggctttt
tttgtacgtgtcattttgcacaaaataagagaaattttcaaaaaaaaccctactttctaggaaaaaaaatctcgtgggagaa
aggggacatttaccgaaaataatttttcggtgaatggccccttttgttgggggatttttggggcatggccgaaaatagctccg
actagcaacactgtaaagggtaaacccccctccccccaaaaaaataaattcccctataatccttcctttatctccccaggaattt
tattccagagggagggagagggaggtaggggctccagttctaggaaggggggaggggataggggtgggggaaga
agaagtggttcggggtgtggagggagagggagggtttgtaaaaagaggtgggggggttctaggggggaggagaatttt
ctcatagcccccctaggatggggtgaggggttata**gggggaagggatatccatcccctgcttcccccctatacgactg
gtgaacatgaacgattagtggtttagtagaaattggcaagttggcagatggatgtgtttgacttaggatcaaaaga
gggggttatagtagtgccccacttgtttgggcagggtag**tatatgtgtagtacgttgggttcatgccccgctagtggga
agatgtttcttccctcctgcctaggttgtctagtagtttagagattgaagtctggagtgtaaaaaacgtccatagatgtacttc
aagggttgttcacccggg-3'

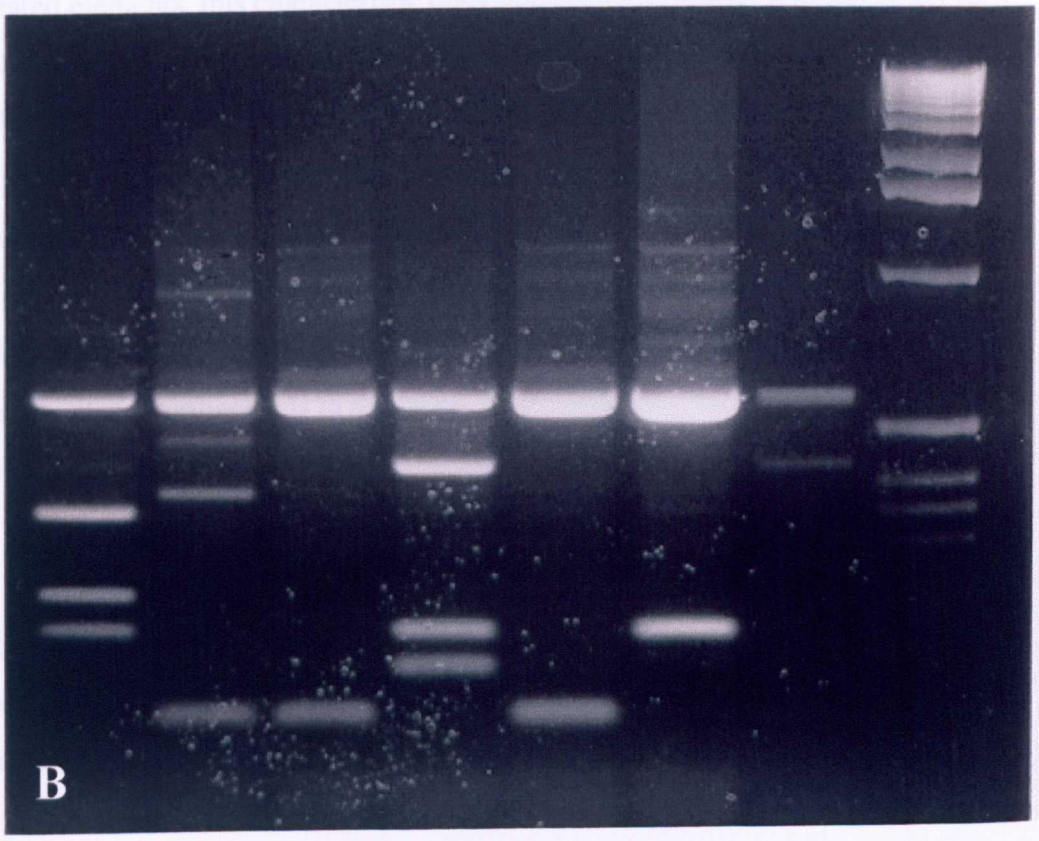
Figure 6.3. Sequence of a repeated element of a *P. maximus* individual from Concarneau (Sellos (1993) in EMBL /GenBank / DDBJ database). The alignment (100 % match) of the sequence of the PCR-amplified fragment of a *P. maximus* individual originating from Ramsey is shown in bold (primers underlined).

Plate 6.2.

A. Restriction digests of the PCR-amplified repeated element of 6 individuals with Tru9I (lanes 2 -7); lane 1: 1Kb-marker, lane 8: low-weight marker.

B. Restriction digests of the PCR-amplified repeated element of 7 individuals with RsaI (lanes 1-7); lane 8: 1Kb-marker.


A


B

6.3. Discussion

The primers designed for PCR amplification of the repeated elements successfully amplified a 1370 bp fragment, the sequence of which proved to be almost identical to the published sequence of repeated elements from *P. maximus* originating from Brittany, particularly at the 3'end. This is remarkable, assuming a non-coding function and selective neutrality of the repeated element, as one would expect relatively high mutation rates and therefore sequence divergence between these samples from geographically distant locations. However, the sequence was only determined for both 'ends' of the repeated element, and it is possible that the middle section exhibited more sequence variability (see below).

No amplification products exceeding the length of one repeat unit were obtained. Hence, it was not possible to determine the number of copies of repeated elements present in different individuals from the length of the PCR product, which would have allowed investigation of possible correlation between copy number and geographical origin. As individuals seem to differ in their genome size by units of repeated elements, this characteristic copy number of repeated elements could be a heritable feature of individuals from distinct locations and therefore potentially be a useful marker. Reasons for the failure of long-PCR could be a) the preferential amplification of shorter fragments (one copy compared to several copies), due to suboptimal PCR conditions, or b) repeated elements differ within one individual, but not only on different mitochondrial molecules, also within the copies present on one molecule, leading to high sequence diversity between different copies, and altering priming sites. Further trials to adjust the PCR conditions for long-PCR might be necessary to provide more clarity.

Sequence diversity, resulting in additional priming sites, between copies of repeated elements within the mitochondrial genome of some individuals could also have caused the amplification of additional, smaller bands in some samples. In a few cases, additional bands persisted (in all repetitions) even after re-amplification of a

gel-extracted fragment. The template fragment might in fact have consisted of a mixture of same-sized PCR products amplified from different copies with differing sequence, or additional priming sites within a single template fragment could have lead to the generation of smaller PCR products. If this was true, it would point towards a high degree of mutational re-arrangements within repeated elements of different individuals. The high sequence similarity of the ends of the repeated element found between the samples from Brittany and the Isle of Man does not exclude this possibility. Within the repeated element different regions could have different mutation rates. The formation of hairpin structures for transcription initiation (as proposed by Rigaa *et al.* (1995)) would involve flanking domains which form the stem, and which would therefore have to maintain a high degree of complementarity, and a central part forming the loop, which would be expected to show more variation. Thus, certain parts of the sequence of a repeated element could be more conserved than others.

If reliable amplification of a single copy of a repeated element was possible, it would enable population genetic studies to be carried out by means of restriction analysis of the fragment with four-base-cutters. To assess the variability and suitability of the repeated element for population genetic analyses, PCR products consisting of one single fragment (with no additional smaller bands) were cut in the present study with different restriction enzymes. Two out of ten enzymes revealed polymorphisms within the repeated element, and it is likely that screening more enzymes would detect further polymorphisms. Nevertheless, before conducting population studies, one has to rule out the possibility that the polymorphic banding pattern generated could, in theory, be due to sequentially distinct, but same-sized copies of repeated elements present in one PCR product.

Future work could also concentrate on the inheritance and phylogeny of repeated elements. Determination of the number of repeated elements in different populations could take place by amplification of the entire sequence between two conserved priming sites located in neighbouring (conserved) genes. If repeated

elements are truly non-coding and selectively neutral they will be potentially useful for the determination of mutation rates and for population genetic studies by means of sequencing or restriction analysis.

Since mitochondrial DNA molecules of pectinids are unusually large, the question of their selective advantage arises. As not much of the basic mtDNA genome can be deleted without destroying function, mutations tend to increase rather than decrease mtDNA size. A literature survey of mitochondrial genome size variation, carried out by Rand (1993) showed that length variation within species seems to be more common in ectotherms than in endotherms; the latter also tended to have smaller mtDNA. The metabolic differences between endotherms and ectotherms could influence the evolution of mitochondrial genome size. Since the mitochondria are the site of 90 % of oxidative metabolism (Richter *et al.*, 1988), species with higher metabolic rates have higher oxidative damage to their mtDNA, and more frequent replication of the mitochondrial genome could encorporate a kind of purifying selection to maintain undamaged molecules. A higher rate of turnover of mtDNA would result in a more rapid response to selection associated with replicating less DNA. Reduced mutation pressures (lower oxidative damage, lower replication rates) could generate greater mtDNA size variation in ectotherms relative to endotherms (Rand, 1993), and allow longer mitochondrial genomes in ectotherms.

This does not explain the presence of repeated elements in the genomes of only a few organisms in the first place. The similarity in the repeated unit organization in *Pecten maximus* and *Placopecten magellanicus* suggests that structural constraints exist on the repeated unit, and that this domain could have a regulatory role (La Roche *et al.*, 1990; Rigaa *et al.*, 1995) which, considering the location of the repeated units at the control region, is likely to be an involvement in the regulation of transcription and replication of the mitochondrial genome. If truly derived from the control region, additional copies of repeated sequences could cause a more efficient binding of DNA polymerase, as they stabilize secondary structures (Buroker *et al.*, 1990), leading to an advantage in the initiation of transcription that

could outweigh the burden of polymerising additional nucleotides in the otherwise very efficiently packed mitochondrial genome.

The possible function of repeated elements surely deserves further investigation. Their base composition, enabling the formation of secondary structures of certain parts, could provide clues as to their origin. As pointed out by Rigaa *et al.* (1995), an inverted repeat could be an indication of the insertion of a foreign sequence. Comparison of sequence similarity to transposable elements would be of considerable interest.

CHAPTER 7: GENERAL DISCUSSION

7.1. Synopsis of results

7.1.1. Manx locations

Results of both mtDNA-RFLPs and RAPD analyses indicated genetic structuring on a small geographical scale between Manx beds of *Pecten maximus*, most clearly in the 1995 samples and between East Douglas and the other locations. The RFLPs on mitochondrial DNA fragments showed significant values of variance components among the Manx populations in DPM95, and values close to significance in PMA95. Phi_{ST} values were still significant after Bonferroni correction for DPM95 in the case of the pairwise comparisons Douglas - Bradda, Douglas - Chickens, and Douglas - Ramsey (the same pairs were significant *before* Bonferroni correction in the PMA95 fragment). Significant heterogeneity of variances, indicating unequal genetic diversity in different Manx populations, was present in PMA95, and in the combined fragments com95 *before* Bonferroni correction. In com95, the variance component between the Manx populations was significant, and phi_{ST} values were significant *before* Bonferroni correction for Douglas - Chickens and Douglas - Ramsey. In addition, the UPGMA cluster analyses based on corrected nucleotide diversity (nucleotide divergence) showed Douglas separated from all other Manx locations in both year-classes (except for PMA96), a feature which was confirmed by the RAPD UPGMA and PCOORD analyses, especially for the 1995 samples. RAPD results indicated a significant variance component between the Manx locations for the 1995 samples, and a value close to significance for the 1996 samples. Phi_{ST} values were significant (after Bonferroni correction) between Bradda - Ramsey for the 1995 samples (and *before* Bonferroni between Bradda - Douglas for the 1996 samples). The two year-classes tended to plot separately in the analyses for both RAPD (separation on the third axis of PCOORD) and mtDNA-RFLPs (separation of year-classes (except Targets) in UPGMA).

7.1.2. All regions

In general, no significant correlation between phenotypic and geographic distance was apparent from the RAPD data. The population from Mulroy Bay was significantly different from all other populations, as indicated by the phi_{ST} values, and supported by the UPGMA and the PCOORD for both RAPD and mtDNA. This confirmed the expected genetic differentiation resulting from restricted gene flow due to the relative isolation of the enclosed Mulroy Bay population; lowest values for haplotype diversity and mean nucleotide diversity amongst all populations provided further evidence for the effects of isolation. In contrast, the population from Plymouth did not exhibit pronounced differences from the Manx populations, although it is geographically further away. However, in the RAPD analysis some of the pairwise phi_{ST} values between Plymouth and Manx locations were significant (after Bonferroni), and Plymouth clustered separately from the other locations in the PCOORDs and UPGMAs. This trend was not confirmed by the mitochondrial data, where Plymouth tended to cluster with the Manx locations, particularly with Targets, in both year-classes. Plymouth showed relatively high values of mean nucleotide sequence diversity.

7.2. Discussion

In this study, nuclear and mitochondrial DNA was analysed for genetic differences between populations. Both methodological approaches agreed in as much as they detected high levels of variation within populations, but mtDNA RFLP analysis generally showed a greater number of significant phi_{ST} values than RAPD. The enhanced evolutionary rate, and the smaller effective population size of mtDNA (in animals with separate sexes approximately one-quarter that of nuclear DNA, but for hermaphrodites only one-half) makes it more susceptible to stochastic events, potentially causing a higher degree of differentiation between subpopulations (Avise *et al.*, 1987). In the extreme case, a population going through a bottleneck could lose most of its mtDNA variability, but maintain a significant fraction of its nuclear DNA variability, therefore showing no significant genetic differentiation for nuclear markers, but potentially high genetic differentiation between mitochondrial markers.

Family-specific mortality can be another cause of reduced levels of diversity in subsequent generations, by reducing effective population size: in species in which the offspring of individual females are grouped into egg masses or schools, localized mortality of such aggregations may have effects similar to bottlenecks. It is not clear to what extent this applies to *P. maximus* larvae. Particularly in areas with strong current systems, one would expect that offspring from different individuals would get well mixed and distributed. However, it is possible that fertilization success varies considerably between spawnings of different individuals, which would have an effect analogous to family-specific mortality of larvae.

Several essential points emerge from the results of the study of *P. maximus*: there is substantial genetic diversity within all populations, although Douglas and Mulroy have relatively lower levels. The population from Mulroy differs from all other populations. This was largely expected and supported the earlier findings of Wilding *et al.* (in press). Mulroy Bay is an enclosed body of water, described as a 'type C lough' by Milne (1972), with an entrance sill and a further sill separating the upper and lower lochs. The structure of such lochs results in reduced water transfer in and out, causing confinement of larvae and self-recruitment. Another possibility is that the Mulroy Bay population has been through a bottleneck. Linked to the use of tributyltin (TBT) on salmon nets in the mid 1980s, scallop spat settlement decreased dramatically, but recovered subsequently following the ban on the anti-foulant (Minchin *et al.*, 1987). This event might have affected genetic variability. *P. maximus* from Mulroy Bay have also been reported to differ in colour from other Irish populations (Minchin, 1991b).

Regarding the Manx populations only, a number of statistically significant results suggest that Douglas is genetically different; weak trends also indicate a low (mostly statistically non-significant) degree of population differentiation between the other Manx locations, a phenomenon which has also been reported by Wilding (1996), and which should not be dismissed completely. Overall, the data indicate that recruitment to populations is temporally inconsistent and that gene flow exists

between Manx sites over distances in the range of tens of kilometres, but that, in some years more than in others, genetic exchange is limited between beds off the east and the west coast of the Isle of Man. The fact that the two commonest haplotypes were found in all populations suggests higher gene flow, at least in the past.

In the northern hemisphere populations of many species have existed only since the last Ice Age, some 10 000 to 15 000 years ago. Climatic oscillations over geological time changed the sea level and the major circulation systems. This would also have altered the current systems between sites on a small scale, possibly resulting in variable connectivity between populations. According to Ferguson (1994) and Williams and Benzie (1990), many widespread marine species may have not yet attained equilibrium between genetic drift and migration over their entire range, and substantial genetic differences will not arise in this period of time except through selection or where low effective population size, either continuously or as a result of transient bottlenecks, has resulted in differentiation due to drift. If selection is absent and population size is large, limited exchange is sufficient to prevent differentiation. One reproductively successful immigrant in each subpopulation every other generation is enough to maintain cohesiveness of allelic frequencies across all subpopulations (Wright, 1931). Consequently, absence of genetic structure does not necessarily mean high rates of gene flow. Marine animals with pelagic larvae are thus expected to show little genetic differentiation over large geographic scales and even less over small geographic scales.

However, although intuition suggests that only species with low dispersal potential will exhibit genetic differentiation on a small scale, the true situation seems to be more complex. Genetic differences do not necessarily implicate restricted larval dispersal, and larval dispersal does not necessarily lead to realized gene flow through successful immigration. There are examples in the literature for all cases, including species with high apparent dispersal potential exhibiting high genetic differentiation (e.g. Burton and Feldman, 1982, copepods; Saavedra *et al.*, 1993, oysters; Todd and Lambert, 1993, nudibranchs; Kordos and Burton, 1993, blue crab), as well as genetic

homogeneity in low-dispersal species (e.g. France *et al.*, 1992, hydrothermal vent amphipods; Selander *et al.*, 1970, and Saunders *et al.*, 1985, horseshoe crabs). Different realized patterns of gene flow have been found even in species with similar larval dispersal potential (Palumbi, 1995). This suggests that larvae cannot always be assumed to make full use of their potential for dispersal, or that even if they spend a long period of time drifting in the sea, they might fail to survive following settlement at a distant site or finally settle close to their origin due to circular current systems or other oceanographic features. For instance, larvae of a shrimp have been shown to settle close to their parents, despite an apparent potential for wide dispersal (Knowlton and Keller, 1986).

A hierarchical study of population differentiation in the barnacle *Balanus glandula* by Hedgecock *et al.* (1982), although based on allozymes, showed results very similar to the present study. F-statistics revealed that 96 % of the total genetic diversity was accounted for by variation within populations, and only 4 % was due to variation between populations. As these 4 % proved to be statistically significant, they contradict the suggestion that geographically distant populations of species with high dispersal capacity are genetically homogeneous. Similarily, an allozyme study on several year-classes of anchovy populations revealed low F_{ST} values but statistically significant genetic heterogeneity (Hedgecock *et al.*, 1989); like in the present study, inconsistencies existed among sites and between years, with no obvious microgeographic clustering of genotypes, a feature which was best described by the term 'chaotic patchiness' (Johnson and Black, 1982).

According to Hedgecock (1994b), the occurrence of genetic differentiation on a small scale despite a potential for high gene flow can be explained by temporal variation in the genetic composition of recruits; this could be due to localized selection on larval and spat populations, such that only certain genotypes survive to recruit to a certain area. Another cause could be that the large number of offspring produced results in large variation in the reproductive success of individuals, thus limiting the effective population size. Mortality rates and distribution factors of

larvae, which influence the variance in offspring numbers among individuals, depend on oceanographic conditions. Another source of variance in reproductive success is variation in fertilization rate between batches of eggs produced by different females. Turbulent diffusion in the sea rapidly reduces the concentration of externally-spawned gametes to the point where sperm-egg encounters virtually cease. Rates of fertilization thus commonly vary between 0 and 100% within single broadcast-spawning species (Levitan and Petersen, 1995), depending on the proximity of potential mates and on whether dispersing gamete clouds happen to meet during a critical interval, potentially lasting only a matter of seconds, immediately following spawning (Denny and Shibata, 1989).

Heavy fishing of *P. maximus* may substantially reduce population size, and if the grounds are at least partly self-sustaining, this could possibly result in transient bottlenecks, which would not affect nuclear diversity as much as mitochondrial diversity. This could be the case for the Douglas 1995 population, which exhibited lowest nucleotide and haplotype diversity amongst the Manx populations. Obviously, if populations are only partly self-sustaining, and the restraining event has occurred only recently, differences between them might not be very pronounced.

The Irish Sea has been the focus of many hydrodynamic models, almost every one suggesting a different current system and implying a different pattern of larval dispersal. In particular, around the Isle of Man, the current systems are highly variable from season to season and the extreme weather conditions occurring there can change current flow on a local scale, imposing different constraints on annual recruitment (Prestidge and Taylor, 1995). In the model proposed by Ramster and Hill (1969) the main current around the Isle of Man approaches from the south (Figure 7.1), suggesting a possible supply of larvae from the extensive fishing grounds south of the island or even from Anglesey or Cardigan Bay (Macleod *et al.*, 1985; Murphy, 1986; Duggan, 1987). On the other hand, a computer simulation of currents carried out by Backhaus and Hainbucher (1987) suggested that conditions can change, especially during summer, which is the main spawning-time in *P. maximus* and

therefore relevant to larval dispersal. Included in the model were the effects of atmospheric forcing, tide, stratification, temperature, and salinity. Contrary to previous models, this simulation indicates that in June and July a general southward flow occurs (Figure 7.2), which could transport larvae from the north and may act, together with the south west Irish Sea front that is established at this time (Pingree and Griffiths, 1978), as a barrier to larval transport from the south. It further confirms that small local fluctuations are a common feature, and that current systems either side of the Isle of Man could be regarded as partially isolated (Coombs *et al.*, 1994), which is due to the bathymetry of the Irish Sea: a deep basin in the west, connected to the Celtic Sea in the south via St. George's Channel and to the Malin Shelf via the North Channel, and rather shallow water in the east. This corresponds to the regions of stratified and mixed water masses present during summer (Pingree and Griffiths, 1978, Figure 7.3.) and suggests the existence of two separate recruitment regions west and east of the Isle of Man (Lewis, 1992; Allison, 1993). More recently, Brown and Gmitrowicz (1995) confirmed the different water flow on the two sides of the Irish Sea by studying currents across the North Channel. From late April onwards, they measured a southward flow west of the Isle of Man, and a northward flow in the east. Strong tidal currents in the east cause thorough mixing of the water, while stratification occurs on the west side (Edwards and Burkill, 1995); the two areas are separated by fronts (Simpson and Hunter, 1974).

These different oceanographic features on the two sides of the Isle of Man influence phytoplankton abundance, and larval distribution, spat settlement and growth of scallops. In 1992, the settlement year for the 1995 3-year-old scallops, spat settlement on artificial collectors was very poor (Zhang, 1996). According to Zhang (1996), this could have been due to a low mean summer chlorophyll *a* concentration (which is used as an indicator for phytoplankton abundance) in 1991, and which would have directly depressed the storage of energy reserves for gametogenesis in the following year. Possibly the conditions leading to poor recruitment in summer 1992 also had an influence on the degree of self-recruitment of

local populations, leading to more pronounced genetic differences between the Manx populations in the 1995 samples.

Despite having a prolonged larval lifespan of several weeks (Le Pennec, 1982), both pectinid species *P. maximus* and *Aquipecten opercularis* show some degree of population genetic structuring around the British Isles, including minor differentiation between relatively closely located Isle of Man fishing grounds. This suggests that, although mainly depending on the annual variation in conditions, some mechanism of larval retention close to the source population may restrict local genetic exchange. Of the various possibilities there is some relevant information concerning two: larval retention in eddies and gyres, and vertical migration.

Recently, Hill *et al.* (1996), using free-drifting, satellite-tracked buoys, documented a cyclonic, near-surface gyre present only in spring and summer above the deep western basin of the Irish Sea. This gyre may act as a retention system for the larvae of the burrowing decapod crustacean *Nephrops norvegicus* living in the seabed of that area. Larval entrainment over the *P. maximus* beds in the subsidiary northern gyre of this system could explain the relative distinctiveness of the population from Peel in the UPGMA clusters for PMA96, com96 and com9596 (mtDNA data).

In the eastern Irish Sea, local water-body retention times can extend to many months (Heaps and Jones, 1977). While Williamson (1952) described a clockwise gyre based on plankton distributions, Ramster and Hill (1969), carrying out direct current measurements, found an anti-clockwise gyre in the Liverpool Bay. Heaps and Jones (1977) suggested that both situations can occur, but are controlled by different factors, and further complicated by a two layer estuarine flow pattern in Liverpool Bay (Figure 7.4). Data on the concentration and distribution of Caesium effluent from Sellafield were used to illustrate potential residence times of water in various areas of the Irish Sea by means of a simple box model (Figure 7.5.), (Heaps and Jones, 1977). The relatively long residence time for the box east of the Isle of Man

could restrict the dispersal pattern of the *P. maximus* population off Douglas. A small tidal circulation system in Douglas Bay (Aldridge and Davies, 1993) might also retain larvae in this area.

Larvae of the related species *Placopecten magellanicus* are known to undertake small amplitude vertical migrations (Tremblay and Sinclair, 1990), possibly in response to light (Kaartvedt *et al.*, 1987; Silva and O'Dor, 1988), pressure (Cragg, 1980) or temperature (Tremblay and Sinclair, 1990). Recently, Manuel *et al.* (1996) showed that veligers of this species obtained from different hydrographic regimes exhibited significantly different vertical migration patterns and depth distribution, which could assist retention over, or return to, adult beds. These differences could be due to selection for different behaviours in different populations (Manuel *et al.*,1996). *P. maximus* spat smaller than 500 µm seem to have the ability to actively alter their sinking rate. The exact mechanisms used are uncertain; suggestions are that the secretion of byssus threads or extension of the foot could allow spat to vary their rate of descent (Beaumont and Barnes, 1992). If *P. maximus* larvae show a behaviour similar to *P. magellanicus*, they might conceivably remain in their local area by diurnal vertical migration between water layers moving in opposite directions, a flow pattern that seems to occur particularly in the two-layer-system east of the Isle of Man, where gyres flow in opposite directions at different depths (Heaps and Jones, 1977). It is not known how much time, under natural conditions, *P. maximus* larvae actually spend free-drifting before settlement; estimates have only been made under laboratory conditions. Considering the wide range of environmental conditions larvae from different locations might experience, the time until settlement is probably very variable. However, the results presented here suggest that extensive mixing, either due to strong current systems and/or long dispersal times, are the rule rather than the exception for *P. maximus*.

Although some studies on pectinids have detected population differentiation, it has always been at a very low level, which is in agreement with the high dispersal capacity of the planktonic larvae and with the present results. Previous allozyme

studies on *P. maximus* found genetic homogeneity between populations both within the Irish Sea and around the UK (see Chapter 1): Huelvan (1985) and Beaumont *et al.* (1993) could not detect any heterogeneity between *P. maximus* populations from Scotland, Ireland and Brittany. However, sample sizes were relatively small and allozymes generally provide lower resolution than DNA-based techniques. For the closely-related species *A. opercularis*, a few more allozyme studies have been carried out, some of which showed signs of slight population differentiation. The most convincing demonstration of small-scale differentiation is the work by Lewis (1992), who found distinct *A. opercularis* populations west and east of the Isle of Man, therefore indicating a situation broadly similar to this study. However, in Lewis' (1992) study, the populations from Douglas and Ramsey seemed genetically similar. Although not directly comparable to this work on a different species, using different methods, Lewis' apparent 'east-coast-cluster' is contrary to the findings of this study, in which Ramsey seemed distinct from Douglas in both year-classes. The fact that Douglas was distinct from all other Manx locations in the present study is also in disagreement with the results published by Wilding *et al.* (in press), who could not distinguish the Manx *P. maximus* populations Peel, Douglas and Chickens. Both Lewis (1992) and Wilding *et al.* (in press) analysed several year-classes together, so that more pronounced differentiation in one particular year-class could have been obscured.

In addition to the molecular findings presented here, other evidence for genetic differentiation in *P. maximus* populations includes the persistent differences in reproductive cycles observed following transplantation of animals from different geographical areas (Mackie and Ansell, 1993). The occurrence of genetic differentiation raises the possibility that some exploited beds are largely self-recruiting, although substantial population differentiation might be prevented by influxes of larvae from elsewhere. The number of incoming larvae could be large enough to avoid pronounced genetic differences, but too small to sustain the population, leaving the populations potentially susceptible to recruitment overfishing, with important implications for fishery management. The slow and incomplete

recovery of the fished-out Cardigan Bay (Wales) stock may be an example of recruitment overfishing of a self-recruiting population of *P. maximus* (Darby and Durance, 1989). Compared with other fishing grounds, where some year-classes can be completely absent, recruitment to Manx beds has been numerically relatively consistent from year to year, without complete failure of recruitment in any one year-class (Brand and Prudden, 1997). In view of the over-exploitation of commercial beds, this persistence is most likely due to inflow of larvae transported from further away or from stocks of scallops on local grounds inaccessible to dredges, providing a reservoir of breeding individuals. Larvae originating from different areas may mix in the turbulent current systems of the Irish Sea, and, depending on the dominant current at spawning time, larvae from different areas might dominate different year-classes. In addition to 'topping up' the settlement supplied by the local populations, inflow of possibly highly diverse recruits may account for the high levels of genetic heterogeneity observed, and may also underlie annual changes.

The relative genetic homogeneity generally shown by pectinid populations should not lead to the conclusion that populations that are heavily fished will always recover due to inflow from larvae from elsewhere. The constantly declining catch rates around the Isle of Man oppose such a view and indicate that, although the lack of pronounced genetic differences between populations could be due to inflow of larvae from elsewhere, this is not suffcient to replenish the population.

Scallops form large accumulations on suitable ground. Individuals living in the middle of such accumulations are more likely to have their eggs fertilized by neighbouring animals, whereas individuals located at the borders are more susceptible to gametes drifting in from further away, or to fertilization failure. Settlement of larvae from elsewhere is also more likely to occur at the borders, simply because there is more space available. The sampling scheme employed in this study did not take scallops from exactly the same places in both years. Therefore, the relatively large differences between the year-classes could be partly due to scallops being fished from different areas within a bed.

A general problem with molecular methods in the context of fishery management is that they are only useful if significant population differences are detected. Molecular genetics is very sensitive to rates of gene flow. A small number of migrants per generation is sufficient to homogenize the gene pool, at least empirically, given the relatively small sample sizes usually employed. Therefore, two populations may be effectively self-recruiting yet not be genetically distinct. Gene flow rates of 1%, 5 %, 10 % and 50 % would all probably mean that populations cannot be distinguished genetically, and appear panmictic, yet as far as a fishery manager is concerned, gene flow rates of 10 % or less may justify treatment as separate stocks (Carvalho and Hauser, 1994). Moreover, as pointed out by Ward and Grewe (1994): 'failure to reject a null hypothesis does not necessarily mean that the null hypothesis is true. For example, the rejection of the null hypothesis of a single genetic stock implies the existence of two or more stocks, each of which should be managed separately, but non-rejection does not necessarily indicate a single genetic stock. There could be a single panmictic stock, or there could be a multitude of separate stocks exchanging just sufficient individuals to prevent the detection of such differentiation. Fishery managers would be better advised to treat such stocks as discrete units rather than as a single large population. Furthermore, if populations have become recently separated, then they will not have had time to accumulate genetic differences'.

7.3. Future work

The concept of scallop populations occurring in distinct 'beds' around the Isle of Man can lead to the incorrect assumption that those beds are actually geographically separated areas. In practice, scallops occur more or less all around the Isle of Man, and beds designated with different names are not necessarily separated by any natural boundaries. Areas where the seabed is unsuitable for fishing are likely to harbour a mature population of breeding individuals. Those areas can be inside, between or at the borders of fished beds and their larvae can supply fished populations. Future genetic surveys should therefore be based on a more biologically

realistic assessment of scallop distribution and density over the entire seabed, rather than concentrating on traditional, commercially-based subdivisions.

The present study included genetic comparison between different year-classes, in an attempt to elucidate patterns of recruitment. However, under the constraints of a three year PhD programme this involved analysis of only two, successive, year-classes, between which there can be no direct ancestor-descendant relationship since *P. maximus* takes more than one year to mature. It would undoubtedly be better to monitor patterns of genetic differentiation over a longer time-span. Intervals of two to ten generations may be suitable for the assessment of temporally changing genetic patchiness (Waples, 1989; Hedgecock, 1994). Even so, characterisation of definite local recruitment patterns in scallops might still prove elusive. Being a long-lived and iteroparous species, each new year-class of *P. maximus* will presumably be the product of interbreeding between several previous cohorts. Furthermore, annual variation in current patterns in areas such as the Irish Sea may ensure that the sources of larval input to a particular location commonly differ from year to year.

Further studies, including specimens from potential source populations to the north and south, are required to elucidate the broader affinities of Isle of Man scallop stocks. A large-scale survey over the whole geographical range of the species would enable broad genetic patterns to be described. To establish the amount of genetic variation at different spatial scales, *P. maximus* populations from open-waters and enclosed embayments (sea loughs, fjords, rias) could be compared. The genetic divergence on a larger scale is of potential significance for the choice of brood stock in hatcheries supplying spat for commercial ongrowing (or for enhancement of wild stocks), and may also be relevant to the management of commercial collection of wild spat for similar purposes. As pointed out by Lewis and Thorpe (1994), self-recruiting populations may become adapted to local conditions, resulting in reduced fitness when transplanted to other areas. For instance, *P. maximus* from Mulroy Bay might have already become adapted to their environment, and might not perform optimally in alternative conditions.

The analysis of mitochondrial DNA in this study revealed low levels of population differentiation, and in practice presented fewer problems than the RAPD analysis. Although the mitochondrial genome is inherited as a single locus, its genes show different mutation rates. One of the fragments used in this study included the most conserved mitochondrial rRNA genes, and the second fragment was of anonymous origin. Additional fragments could provide better resolution, as they could have mutation rates higher than those of the fragments employed in this study. In addition to increasing the number of DNA markers, larger sample sizes would be an advantage. Now that several markers have already been developed, future projects could concentrate on screening more individuals from a larger selection of locations.

Nevertheless, comparison of mitochondrial and nuclear DNA markers in the same study can be informative. Microsatellites could be employed as nuclear markers, although, considering their laborious and expensive development, direct sequencing of variable sections could be contemplated instead, as this would provide the highest possible resolution. DNA-fragments to be sequenced have to be chosen carefully, with the aim of finding highly polymorphic markers; non-coding sequences like those of the repeated elements in the mitochondrial genome or introns in the nuclear genome could prove to be useful.

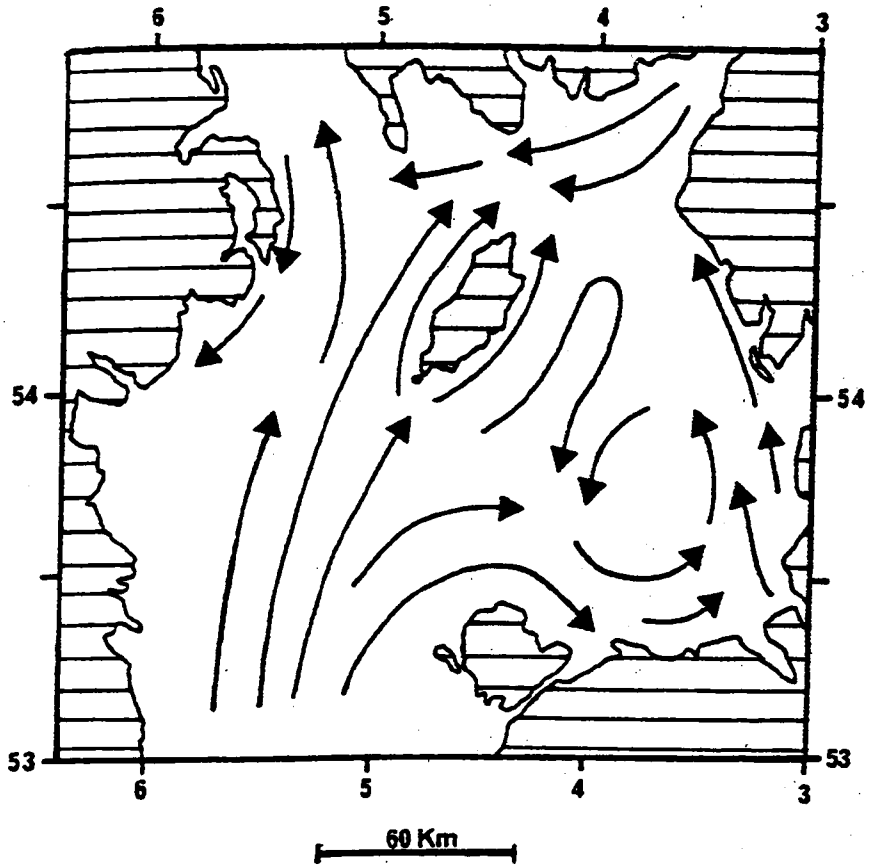


Figure 7.1. Main flow of surface currents in the northern Irish sea, based on a model by Ramster and Hill (1969); from Macleod *et al.*, 1985.

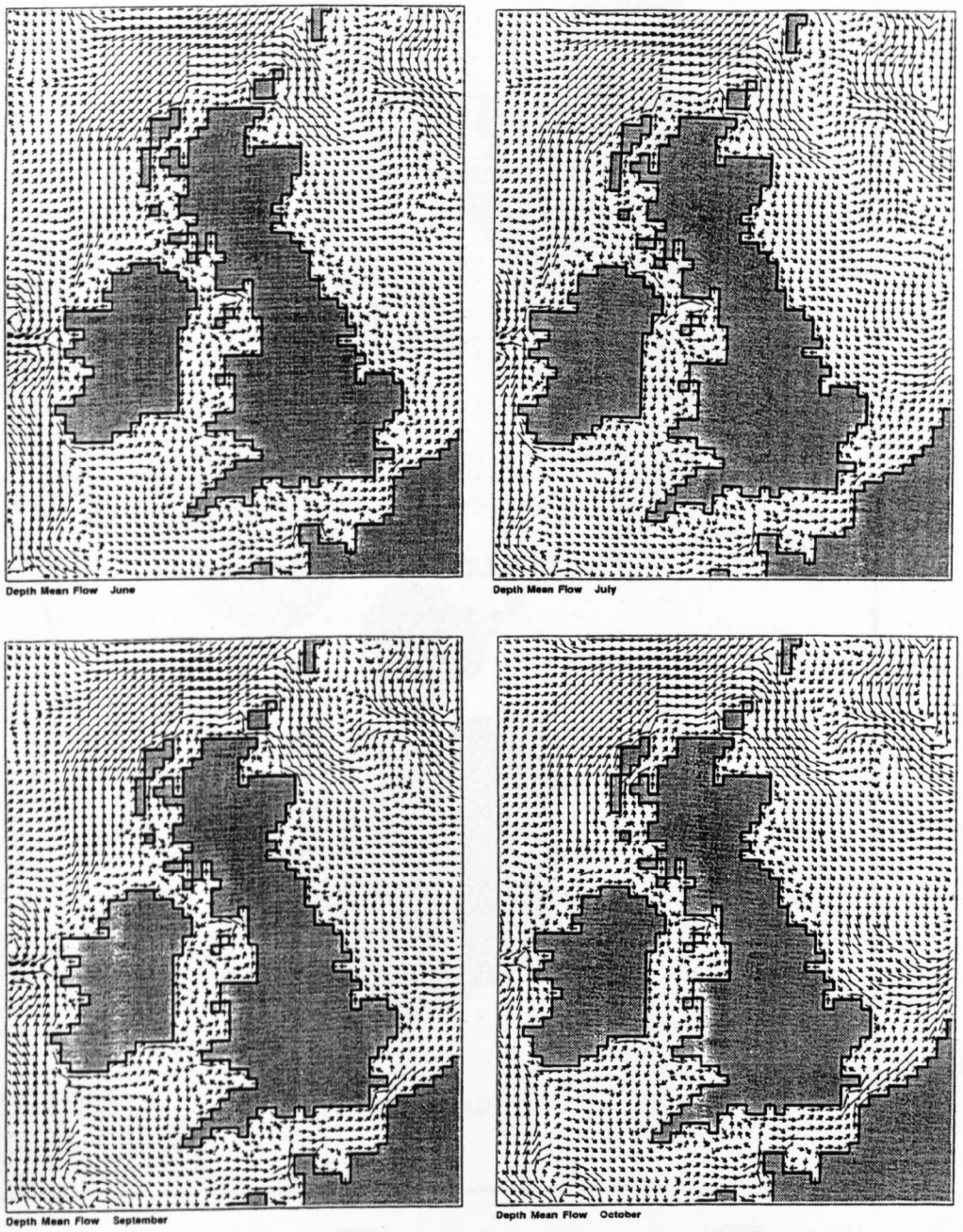


Figure 7.2. Predicted current vectors averaged over the whole water column during the spawning time for *P. maximus*. Backhaus 3D non-linear finite difference general circulation model (from Backhaus and Hainbucher, 1987).

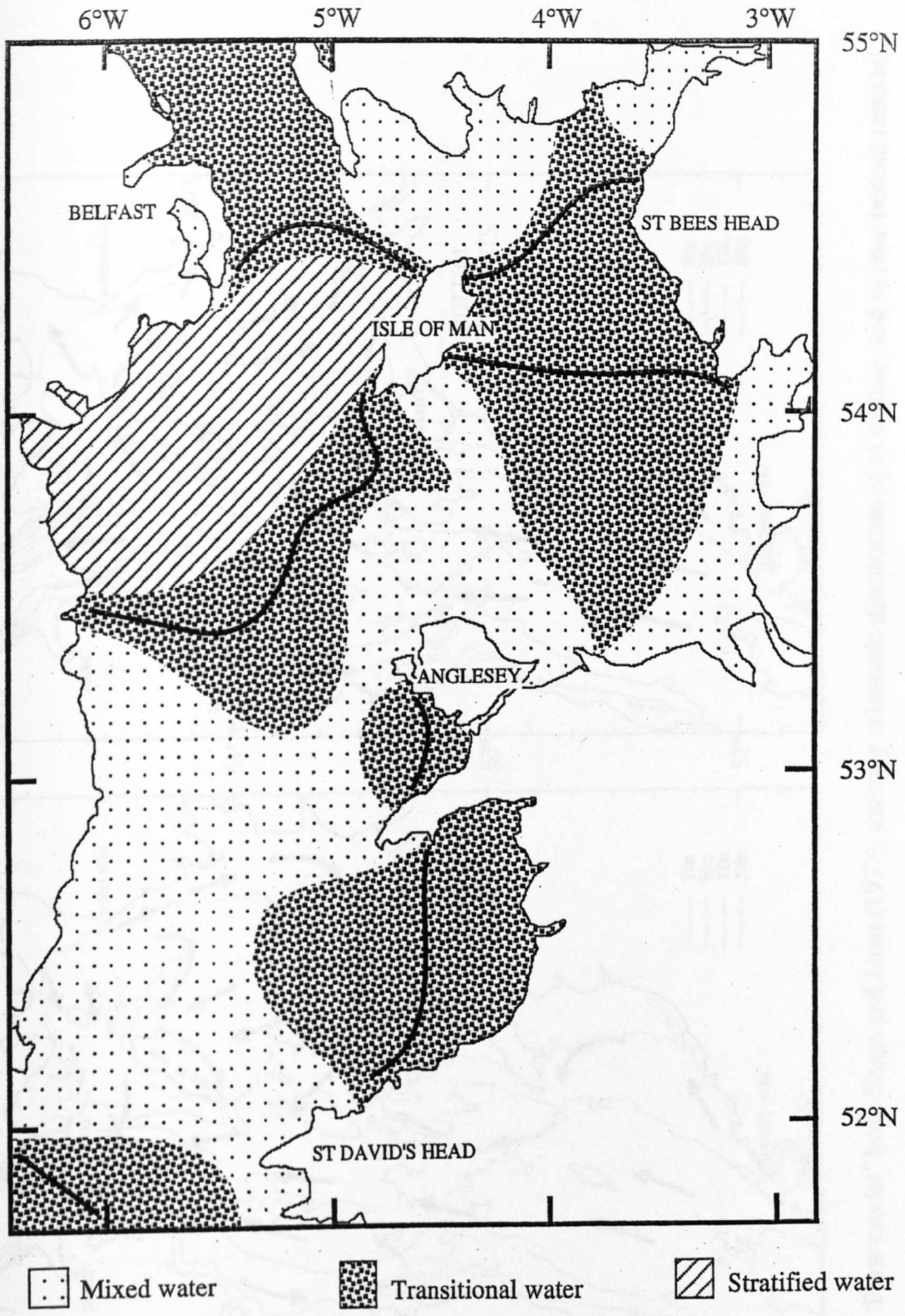


Figure 7.3. Summer hydrographic conditions as predicted from the model of Pingree and Griffiths (1978), showing areas of mixed, transitional and stratified water and frontal regions. The approximate position of the fronts is indicated by the bold lines.

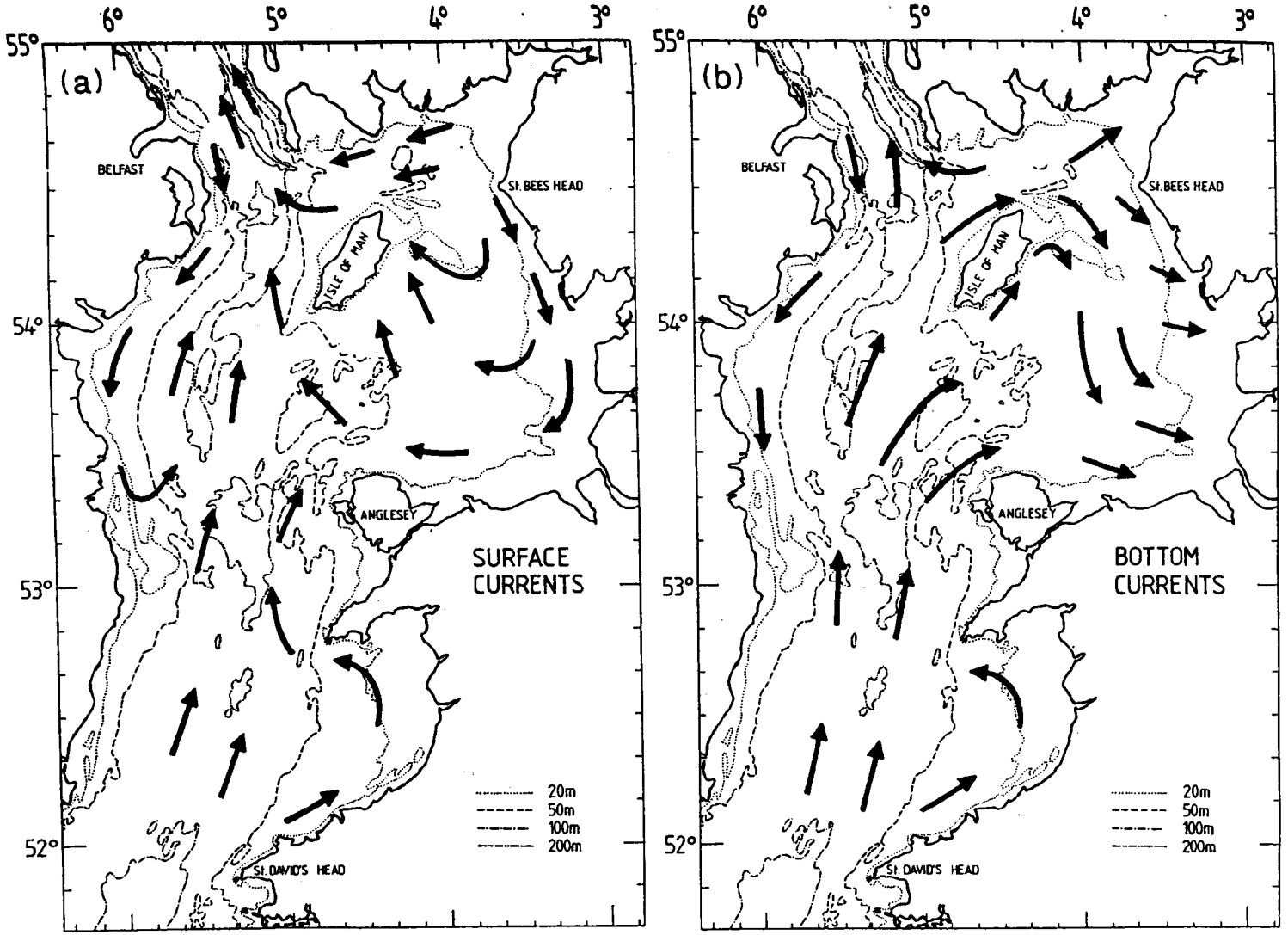


Figure 7.4. 'Flow-model' from Heaps and Jones (1977), showing schematic distribution of a) surface, and b) near-bottom residual currents. Note the two-layer circulation system in Liverpool Bay.

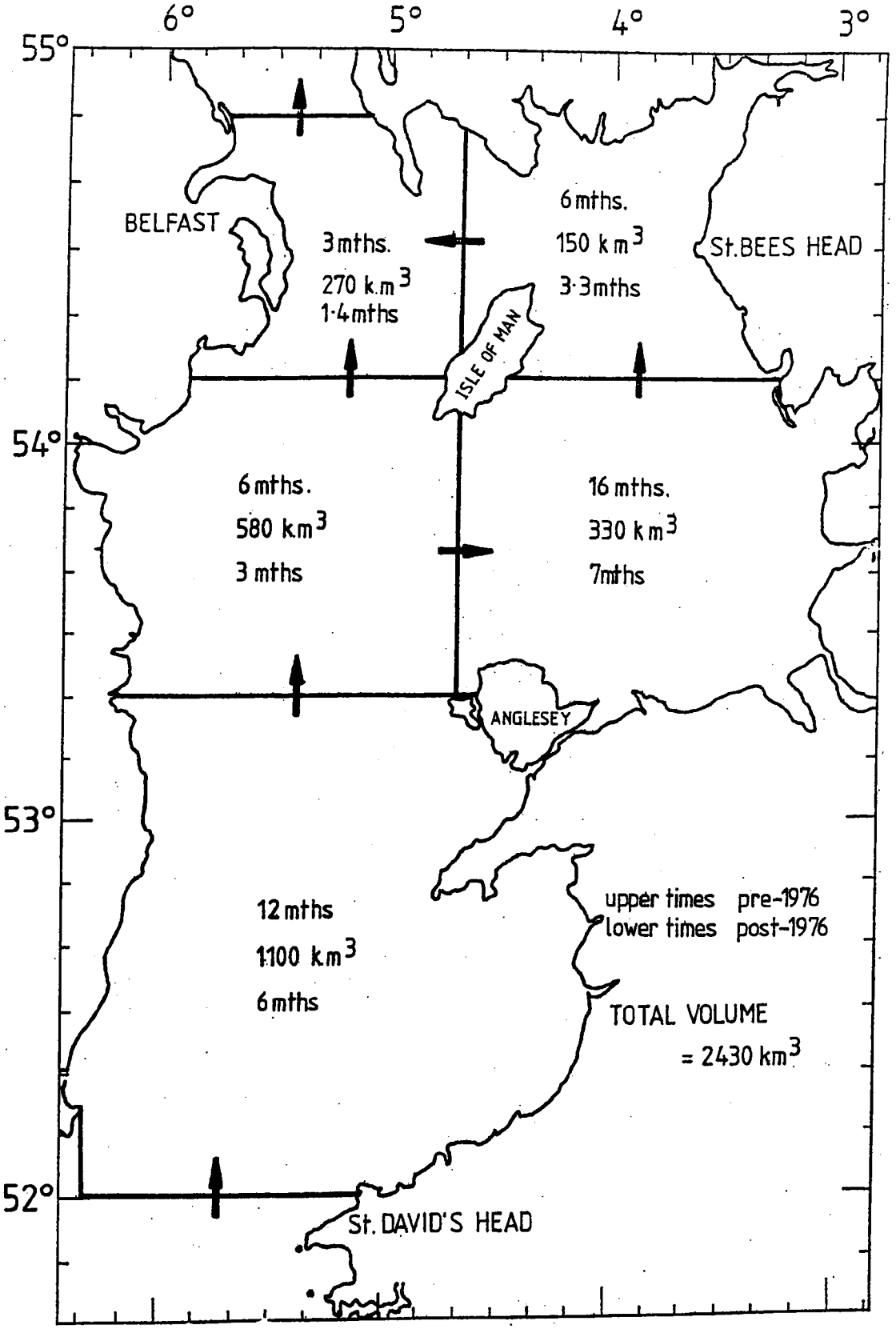


Figure 7.5. 'Box-model' from caesium effluent data, showing water residence times in areas of the Irish sea (from Heaps and Jones, 1977).

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Appendices

RAPD data 1995

Primer-bands: R06-3 R12-3 F01-5 F04-3 F06-5 R03-4 R04-3 F02-4 R01-4 F10-3 Y14-3 Y18-5 Y11-6

b1 0 1 1 1 0 0 0 1 0 1 0 0 1 1 0 1 0 0 0 0 0 1 1 0 1 1 1 0 1 1 0 1 1 1 0 1 0 0 1 0 0 0 1 1 1 0 0 1 0 0 1
b2 0 0 1 0 1 0 0 1 0 0 1 1 0 1 0 1 1 0 1 1 0 1 0 0 1 1 0 1 1 0 0 0 1 1 0 1 0 0 0 1 0 1 0 1 0 0 0 0 0 1 1
b3 0 0 1 1 1 1 1 1 1 0 0 0 1 0 1 0 0 1 0 0 1 1 1 1 0 1 1 0 0 0 1 0 0 1 0 0 0 0 1 1 1 0 0 0 1 1 0 0 1 1 0 1
b4 0 0 1 0 0 0 0 1 0 0 1 1 0 1 1 1 0 0 1 0 0 1 1 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 0 0 0 1 1 0 1 0 1 1 0 0 1 0 1 1
b5 1 0 1 0 0 1 1 1 1 0 0 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 0 0 1 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 1 0 1 1 0 0 1 1 0 1 1
b6 0 0 1 0 0 0 0 1 0 0 1 1 0 1 0 1 0 1 0 0 0 1 0 0 1 1 1 1 1 1 0 1 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 1 1 0 0 1 0 0 1
b7 0 0 1 0 1 1 0 1 0 0 1 1 0 1 0 1 0 1 0 1 1 1 1 1 1 0 1 1 0 0 1 0 1 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 1 1 0 1 1
b8 0 0 1 1 1 1 0 0 0 1 1 1 0 1 0 1 0 0 0 0 0 1 1 0 1 1 0 1 1 0 0 1 1 0 1 1 0 0 0 0 0 0 1 0 1 0 0 1 0 1 1
b9 1 1 1 0 1 0 0 1 0 0 1 0 0 1 0 1 1 1 0 1 0 1 1 0 1 1 0 1 0 1 0 0 1 0 0 0 0 0 0 1 0 1 0 0 1 1 0 1 0 1 1
b10 0 1 1 1 0 0 0 1 0 0 1 1 0 1 0 1 0 0 0 0 0 1 1 1 1 1 0 1 0 1 0 0 0 0 0 1 0 0 1 0 0 0 0 1 0 0 0 1 0 1 1
b11 0 1 0 1 0 1 0 1 1 1 0 0 0 0 0 1 0 1 0 1 0 0 0 0 1 0 0 1 0 1 0 1 0 1 0 0 0 0 0 0 0 0 1 0 0 0 1 1 1 1 1 1 0 0 1
b12 0 0 0 1 0 1 0 1 0 1 0 0 1 1 1 1 0 1 0 0 0 0 1 1 1 0 0 0 0 1 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0 1 0 1 0 1 1
b13 0 1 1 1 1 1 0 0 1 0 0 1 1 0 1 0 0 0 0 0 1 0 1 1 1 0 1 0 0 1 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 1
b14 0 0 1 1 0 1 0 1 0 1 0 0 1 1 0 1 0 1 0 1 0 1 0 0 0 1 1 0 1 1 0 1 1 0 0 1 1 1 0 0 0 0 1 0 0 1 1 0 1 1 0 1 0 1 1
b15 1 0 1 1 1 1 1 0 1 0 1 1 1 0 1 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 0 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 0 1 0 1 0 1 0 0 1 0 1 1
b16 0 0 1 1 0 1 0 1 0 0 1 0 0 1 0 1 1 0 0 1 1 0 0 0 0 0 0 1 1 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 1 1
b17 1 1 0 0 1 1 0 1 0 1 0 1 1 1 0 1 0 0 0 0 1 0 0 1 0 0 1 0 0 1 1 0 1 1 0 0 0 1 0 0 0 0 0 0 0 0 1 0 1 1 1 0 1 1
b18 0 1 1 0 1 0 0 1 0 0 0 0 0 1 0 1 0 1 0 0 0 1 1 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 1 1 1 1
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RAPD data 1996

Primer bands: R06-3 R12-3 F01-5 F04-3 F06-5 R03-4 R04-3 F02-4 R01-4 F10-3 Y14-3 Y18-5 Y11-6

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t6 0 0 1 0 0 0 0 1 0 0 1 0 0 1 0 1 0 1 0 0 1 1 1 0 1 1 1 1 0 0 0 0 0 1 0 0 0 1 1 0 0 0 0 0 1 0 1 1 0 1 1
t7 1 1 1 0 0 0 0 1 0 1 1 1 0 1 1 0 1 0 1 0 1 1 0 1 0 1 0 0 1 1 0 0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0 0 1 0 1 1
t8 0 1 1 0 0 1 0 0 0 0 0 1 1 0 1 0 1 0 0 0 0 1 1 0 1 1 1 0 0 0 1 0 0 0 0 1 0 1 0 1 0 0 0 0 1 1 0 0 1 0 1 1
t9 0 1 1 1 0 1 0 1 0 1 0 1 0 1 0 1 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 0 1 1 0 1 1 1 0 0 0 0 1 1 0 1 0 1 0
t10 0 0 0 1 0 1 1 0 0 0 0 0 1 1 0 1 0 0 0 1 1 0 1 1 1 0 1 1 0 1 0 1 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 1 1 0 1 0 1 1
t11 0 0 1 0 1 1 1 0 1 0 1 1 0 0 1 0 1 1 1 1 0 0 0 1 0 0 1 1 0 1 1 1 1 0 0 1 0 0 1 0 0 1 1 0 0 1 1 0 0 1 1 0 1 1
t12 1 0 1 1 1 0 0 0 0 0 0 1 1 0 1 0 1 0 1 0 1 0 0 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 1 1 1 0 0 0 0 0 1 0 0 1 0 1 1
t13 0 1 1 1 0 1 1 1 1 0 0 1 0 0 1 0 1 0 1 0 1 0 0 0 0 1 0 1 1 0 0 0 0 0 1 1 0 0 0 0 0 1 1 0 0 0 0 0 1 1 0 0 1 0 1 0 0 1 0 1 1
t14 0 0 0 1 1 1 0 1 0 0 1 0 0 1 0 1 0 1 0 1 0 0 0 1 1 0 0 1 1 1 1 0 1 0 1 1 1 1 0 0 1 0 0 1 0 1 0 0 0 1 1 0 1
t15 1 1 1 0 0 1 0 1 0 0 1 1 0 1 0 0 0 1 0 0 0 0 1 1 1 1 0 0 0 0 1 0 1 0 0 0 0 1 0 1 1 0 0 0 0 0 0 1 0 1 1
t16 0 0 0 1 1 0 0 1 0 0 1 0 0 1 0 1 0 1 0 0 0 1 0 0 1 1 0 1 1 1 0 1 1 0 0 0 0 1 1 1 0 0 1 0 0 0 1 1 1 0 1 1
t17 0 0 1 1 1 1 1 0 1 0 1 1 1 0 1 0 0 1 1 0 0 0 0 1 0 1 1 1 1 0 1 0 1 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 1 0 1 1
t18 0 0 1 1 1 1 1 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 0 0 1 1 0 0 0 0 0 0 0 1 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0 1 1
m1 1 0 0 1 0 1 0 1 0 1 0 1 0 1 0 0 0 1 0 0 0 0 0 0 0 1 1 0 1 1 1 0 0 1 1 1 1 0 1 1 1 0 1 1 1 0 1 0 1 0 0 0 1 0 1 1
m3 1 1 0 1 0 1 0 1 0 0 1 1 0 1 1 1 0 1 1 1 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 1 0 0 0 1 0 1 1
m4 0 0 0 1 1 1 0 1 0 0 1 0 0 1 0 1 0 1 1 1 0 1 0 0 0 1 1 1 1 0 0 0 1 1 0 1 0 1 0 1 0 0 0 1 1 0 0 1 1 0 1 1
m5 1 0 1 1 1 1 0 1 0 0 1 0 0 1 0 1 0 1 0 1 0 0 0 1 0 0 0 1 1 0 1 1 0 0 1 1 1 0 0 1 0 0 0 1 0 1 0 0 1 1 0 1 1
m6 1 0 1 1 1 1 0 1 0 0 1 0 0 1 1 1 0 1 0 1 0 1 1 0 1 0 1 1 0 0 0 0 1 0 0 1 0 1 1 0 0 0 0 1 0 0 1 0 0 1 1
m7 1 0 1 1 1 1 1 1 1 0 1 0 1 0 0 1 1 0 1 0 0 0 1 1 0 0 1 1 1 1 0 0 0 1 0 0 1 1 1 1 1 1 0 1 0 1 1 0 0 1 1 1 1
m9 0 0 1 1 0 1 0 1 0 0 1 1 0 1 0 1 0 1 1 1 0 1 0 0 0 1 1 0 1 1 1 0 1 0 0 0 0 0 1 0 0 1 0 0 0 0 0 1 1 0 1 1
m10 1 0 1 1 1 1 0 1 0 1 0 1 0 0 0 1 0 1 0 1 0 1 1 0 0 1 1 1 0 1 0 0 0 1 0 1 1 0 1 0 0 0 1 1 0 0 0 1 0 1 1
m11 0 1 0 1 1 1 1 0 1 0 0 0 1 0 0 1 1 0 1 0 1 0 1 1 1 0 0 0 1 1 0 0 0 1 0 0 0 0 0 1 0 0 1 0 0 0 0 0 1 0 1 1
m12 1 0 1 1 1 1 0 1 0 0 1 1 0 0 1 1 0 1 0 0 0 1 0 0 1 1 0 1 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 1 1 0 1 1 1 1 1
m13 0 0 1 1 1 1 0 1 1 0 1 0 0 1 0 1 0 1 0 1 0 1 1 0 1 1 0 1 1 0 0 0 1 0 1 0 0 0 1 1 0 0 0 1 0 0 0 1 0 1 1
m14 0 0 1 1 1 1 1 1 1 0 1 1 1 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 1 1 1 0 0 1 0 0 0 0 0 1 1 0 1 0 0 0 0 1 1 0 1 1
m16 0 0 1 1 1 1 0 1 0 0 1 1 0 1 0 1 0 0 0 1 0 1 0 1 1 0 0 1 1 1 0 1 0 1 1 0 0 0 0 0 1 1 1 0 0 0 1 0 0 1 1 0 1 1
m17 1 1 1 1 1 0 0 1 0 1 0 1 0 0 0 1 0 0 0 0 0 1 1 0 1 1 1 1 0 1 0 1 1 0 0 0 0 0 1 1 0 0 1 0 1 1 0 0 0 1 1
p1 0 1 0 1 1 1 0 1 0 1 1 1 0 1 0 1 0 1 0 1 1 0 1 0 1 1 0 0 1 0 0 0 0 0 1 0 0 1 1 1 0 0 0 0 0 0 1 1 0 1 1
p2 0 0 1 1 1 1 0 1 0 1 1 1 0 1 0 1 0 1 0 1 0 1 1 1 1 1 1 0 1 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 1 0 1 1 1 1 1 1
p3 0 0 1 1 1 0 0 0 0 0 1 1 0 1 0 1 0 1 0 1 0 0 0 0 1 0 0 0 1 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 1 0 1 1 0 1

p4 1 0 1 1 1 0 1 1 1 0 0 0 0 1 0 1 0 1 0 1 0 1 0 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1
p5 0 0 1 1 1 1 0 1 0 1 1 1 0 0 0 0 0 1 1 1 0 1 0 0 1 1 0 0 1 0 0 0 1 1 1 0 0 0 0 0 0 0 0 1 1 0 0 1 0 1 1
p7 0 0 1 1 1 1 0 1 0 0 1 1 1 1 0 1 0 1 0 1 0 1 0 0 1 1 0 1 0 0 0 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 0 1 1
p8 0 0 1 1 1 1 0 1 0 0 1 0 0 1 0 1 0 0 1 1 0 1 0 1 1 1 0 0 1 1 0 0 1 0 0 1 0 0 0 0 0 1 0 0 0 0 0 1 0 1 1
p9 0 0 1 1 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 0 1
p10 0 0 0 1 1 1 0 1 1 1 1 1 1 1 1 0 0 1 1 0 1 0 0 1 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 1
p11 0 0 1 1 1 1 1 0 0 0 1 1 0 1 1 0 1 1 0 1 1 0 1 0 0 0 1 1 0 1 0 0 0 0 1 1 0 1 0 0 0 1 1 0 0 1 0 1 1
p12 1 0 1 1 1 1 0 1 1 1 1 1 1 1 1 0 0 0 1 0 1 0 1 1 1 1 1 0 1 0 0 0 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0
p13 0 0 1 1 1 1 0 1 0 1 1 0 1 1 0 0 0 1 0 1 0 1 0 0 1 0 0 0 1 0 1 0 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 1 1
p14 0 1 1 1 1 0 0 1 0 0 0 1 0 1 0 1 0 1 0 1 0 1 1 1 1 0 0 0 1 0 0 0 1 0 0 1 1 1 1 1 0 0 0 0 0 0 0 1 0 0 1
p15 1 0 0 1 1 0 0 1 0 1 1 1 1 1 0 1 0 1 0 1 1 0 1 1 1 1 0 0 1 0 0 1 1 0 1 0 1 1 1 1 0 0 0 0 1 0 1 1 0 1 0
p16 0 0 0 1 1 1 0 1 0 0 1 1 0 1 0 1 0 1 0 1 0 1 1 0 1 1 0 1 0 0 0 0 1 0 0 0 0 0 1 0 0 0 0 0 1 0 0 1 0 1 1
p17 1 1 1 1 1 0 0 1 0 0 1 0 0 1 0 1 0 1 0 1 1 1 0 0 0 1 0 1 0 0 0 0 1 0 0 0 0 0 1 1 0 1 0 1 1 0 1 0 0 1 1
p18 0 0 1 1 1 1 0 1 0 1 1 1 0 1 0 0 0 1 0 1 1 0 0 0 1 1 0 1 0 0 0 1 1 0 0 0 1 1 1 1 0 0 0 0 0 0 0 1 0 1 1

Solutions for isolation of mitochondria and extraction of mtDNA

Isotonic buffer, pH 7.5
0.5 M sucrose
0.15 M KCl
2 mM EDTA
25 mM Tris-HCl

1 M sucrose, pH 7.5
1 M sucrose
5 mM EDTA
10 mM Tris-HCl

1.5 M sucrose, pH 7.5
1 M sucrose
5 mM EDTA
10 mM Tris-HCl

STE, pH 8
0.1 M NaCl
0.05 M Tris-HCl
0.01 M EDTA

100 ml 10 % CTAB
4.1 g NaCl
10.0 g CTAB
dissolved in 100 ml H_2O

Restriction enzyme profile listing for DPM

MspI

A	100111
B	100011
C	101111
D	000111
E	110111

RsaI

A	0110110111
B	1110110111
C	0110010111
D	0110111111
E	0111110111

DraI+PvuII

A	0111
B	1111
C	0101
D	0011

HaeIII

A	101111
B	101101
C	111111

TaqI

A	110111
B	110101
C	100111
D	110011
E	111111

Enzyme profile listing for PMA

TaqI

A	0011
B	0001
C	0111
D	1001

MspI

A	01101
B	01100
C	11100
D	01111
F	00100

RsaI

A	10011
B	10001
C	11011
F	10010
G	00011
I	10111

DraI+PvuII

A	0010
B	1010
C	1110
D	0011

HaeIII

A	1001011
B	1011011
C	1000011
D	1011111
E	1101011

Presence / absence of restriction sites for DPM95
Order of restriction enzymes: Msp1/RsaI/Dra+Pvu/HaeII/Taq1

B2	100111 0110110111 0111 101111 110111
B3	100111 0110110111 1111 101101 110111
B4	100111 0110111111 1111 101101 100111
B5	100111 0110110111 1111 101101 110101
B6	100111 0110110111 1111 101101 110111
B7	100111 0110110111 0111 101111 110111
B8	100111 0110110111 1111 101101 110111
B9	101111 0110110111 0111 101111 110111
B10	100111 0110110111 0111 101111 110111
B11	100111 0110110111 0111 101111 110111
B12	100111 0110110111 0011 101111 110111
B13	100111 0110110111 1111 101101 110111
B14	100111 0110110111 1111 101101 110111
B15	100111 1110110111 1111 101101 110111
B16	100111 0110110111 0111 101111 110111
B17	100111 0110110111 1111 101101 110111
B18	100111 0110110111 0111 101111 110111
B19	100111 0110110111 1111 101101 110101
B20	100111 0110110111 1111 101101 110111
B21	100111 0110110111 1111 101101 110111
B22	100111 0110110111 0111 101111 110111
B23	100111 0110110111 0111 101111 110111
B24	100111 0110110111 1111 101101 110111
C1	100111 1110110111 1111 101101 110111
C2	100111 0110110111 1111 101101 110111
C3	100111 0110110111 1111 101101 110111
C4	100111 0110110111 0111 101111 110111
C5	100111 0110110111 1111 101101 110111
C7	100111 0110110111 0111 101111 110111
C8	100111 0110110111 0111 101111 110111
C9	100111 0110110111 0111 101111 110111
C10	100111 0110110111 1111 101101 110101
C11	100111 0110110111 0111 101101 110111
C12	100111 0110110111 0111 101111 110111
C13	100111 0110110111 1111 101101 110111
C14	100111 0110110111 1111 101101 110101
C15	100111 0110110111 1111 101101 110011
C16	100111 0110110111 0111 111111 110111
C17	100111 0110110111 1111 101101 110111
C18	100111 0110110111 0111 101111 110111
C19	100111 0110110111 0111 101111 110111
C20	100111 0110110111 0101 101111 110111
C21	100111 0110010111 0111 101111 110111
C22	100111 0110110111 0111 101111 110111
C23	100111 0110110111 1111 101101 110111
C24	100111 0110110111 1111 101101 110111
D1	100111 0110110111 1111 101101 110111
D2	000111 0110110111 0111 101111 110111
D3	100111 0111110111 0111 101111 110111
D4	100111 0110110111 0111 101111 110111

D5	100111 0110110111 0111 101111 110111
D6	100111 0110110111 0011 101111 110111
D7	100111 0110110111 0111 101111 110111
D8	100111 0110110111 0111 101111 110101
D9	100111 0110110111 0111 101111 110111
D10	100111 0110110111 0111 101111 110111
D11	100111 0110110111 0111 101111 110111
D12	100111 0110110111 0111 101111 110111
D13	100111 0110110111 0111 101111 110111
D14	100111 0110110111 0111 101111 110111
D15	100111 0110110111 0111 101111 110111
D16	100111 0110110111 1111 101101 110111
D17	100111 0110110111 0111 101111 110011
D18	100111 0110110111 0011 101111 110111
D19	100111 0110110111 0101 101111 110111
D20	100111 0110110111 0111 101111 110111
D21	100111 0110110111 0111 101111 110111
D22	100111 0110110111 0111 101111 110111
D23	100111 0110110111 0111 101111 110111
D24	100111 0110110111 1111 101101 110111
R1	100011 0110110111 0111 101101 110111
R2	100111 0110110111 1111 101101 110111
R3	100111 1110110111 0111 101111 110111
R5	100111 0110110111 0111 101111 110111
R6	100111 0110110111 1111 101101 110111
R7	100111 0110110111 0111 101111 110111
R8	100111 0110110111 1111 101101 110111
R9	100111 0110110111 1111 101101 110111
R10	100111 0110110111 0111 101111 110111
R11	100111 0110110111 1111 101101 110111
R12	100111 0110110111 1111 101101 110111
R13	100111 0110110111 1111 101101 110111
R14	100111 0110110111 1111 101101 110111
R15	110111 0110110111 0111 101111 110111
R16	101111 0110110111 1111 101101 110111
R18	100111 0110110111 0111 101111 110101
R19	100111 0110110111 1111 101101 110101
R20	100111 0110110111 1111 101101 110101
R21	100111 0110110111 0111 101111 110111
R22	100111 0110110111 0111 101111 110111
R23	100111 0110110111 0101 101111 110111
R24	100111 0110110111 1111 101101 110111
T1	100111 0110110111 0111 101111 110111
T2	100111 0110110111 0111 101101 110111
T3	100111 0110110111 1111 101101 110111
T4	100111 0110110111 0111 101111 110111
T5	100111 0110110111 0111 101111 110111
T6	100111 0110110111 0111 101111 110111
T7	100111 0110110111 1111 101101 110111
T8	100111 0110110111 1111 101101 110111
T9	100011 0110110111 1111 101101 110111
T12	100111 0110110111 0111 101111 110111
T13	100111 0110110111 0011 101111 110111
T14	100111 0110110111 0111 101111 110111

T15	100111 0110110111 0111 101111 110111
T17	100111 0110110111 0111 101111 110111
T18	100111 0110110111 1111 101101 110111
T19	100111 0110110111 1111 101101 110111
T20	100111 0110110111 1111 101101 110101
T21	100111 0110110111 0111 101111 110111
T22	100111 0110110111 0111 101111 110111
T23	100111 0110110111 1111 101101 110111
T24	100111 0110110111 0111 101111 110111

Presence / absence of restriction sites for DPM96
Order of restriction enzymes: MspI/RsaI/DraI+PvuII/HaeII/TaqI

B1	100111 0110110111 0111 101111 110111
B2	100111 0110110111 1111 101101 110111
B3	100111 0110110111 0111 101111 110111
B4	100011 0110110111 1111 101101 100111
B5	100111 0110110111 1111 101101 110111
B6	100111 0110110111 0111 101111 110111
B7	100111 1110110111 0111 101111 110111
B8	100111 0110110111 0111 101111 110111
B9	100111 0110110111 0111 101111 110111
B10	100111 0110110111 0111 101111 110111
B11	100111 0110110111 0111 101111 110111
B12	000111 0110110111 0111 101111 110111
B13	100111 0110110111 1111 101101 110111
B14	100111 0110110111 0111 101111 110111
B16	100111 0110110111 1111 101101 110111
B17	100111 0110110111 1111 101101 110011
B18	100111 0110110111 0111 101111 110111
B19	100111 0110110111 0111 101111 110111
B20	100111 0110110111 0111 101111 110111
B21	100111 0110110111 1111 101101 110111
B22	100111 0110110111 0111 101111 110111
B23	100111 0110110111 0111 101111 110111
B24	100111 0110110111 0111 101111 110111
C1	100111 0110110111 0111 101111 110111
C2	100111 0110110111 0111 101111 110111
C3	100111 0110110111 0111 101111 110111
C4	100111 0110110111 1111 101101 110111
C5	100111 0110110111 0111 101111 110111
C6	100111 0110110111 0111 101111 110111
C7	100111 0110110111 1111 101101 110111
C8	100111 0110110111 0111 101111 110111
C9	100111 0110110111 0111 101111 110111
C10	100111 0110110111 0111 101111 110111
C11	100111 0110110111 0111 101111 110111
C12	100111 0110110111 0111 111111 110111
C13	100111 0110110111 0111 101111 110111
C14	100111 0110110111 0011 101111 110111
C15	100111 0110110111 1111 101101 110111
C16	100111 0110110111 0111 101111 110111
C17	100111 0110110111 1111 101101 110111
C18	100111 0110110111 0111 101111 110111
C18	100111 0110110111 1111 101101 110111
C20	100111 0110110111 0111 101111 110111
C21	100111 0110110111 1111 101101 110101
C22	100111 0110110111 1111 101101 110111
C23	100111 0110110111 1111 101101 110111
C24	100111 0110110111 0011 101111 110111
D1	100111 0110110111 0111 101111 110111
D2	100111 0110110111 0101 101111 110111
D3	100111 0110110111 0111 101111 110111

D4	100111 0110110111 0111 101111 110111
D5	100111 0110110111 0111 101111 110111
D6	100111 0110110111 1111 101101 110111
D7	100111 0110110111 1111 101101 110111
D8	100111 0110111111 0111 101111 110111
D9	100111 0110110111 0111 101111 110111
D10	100111 0110010111 0111 101111 110111
D11	100111 0110110111 0111 101111 110111
D12	100111 0110110111 0111 101111 110111
D13	100111 0110110111 0111 101111 110111
D14	100111 0110110111 0111 101111 110111
D15	100111 0110110111 1111 101101 110111
D16	100111 0110110111 0111 101111 110111
D17	000111 0110110111 0111 101111 110111
D18	101111 0110110111 0111 101111 110111
D19	100111 0110110111 0111 101101 110111
D20	100111 0110110111 0111 101111 110111
D21	100111 0110110111 0111 101111 110111
D22	100111 0110110111 0111 101111 110111
D23	100111 0110110111 0111 101111 110111
D24	100111 0110110111 0111 101111 110111
R1	100111 0110110111 1111 101101 110111
R2	100111 0110110111 0111 101111 110111
R3	100111 0110110111 0111 101111 110111
R4	100111 0110110111 1111 101101 110111
R5	100111 0110110111 0111 101111 110111
R6	100111 0110010111 0111 101111 110111
R7	100111 0110110111 1111 101101 110111
R8	100111 0110110111 1111 101101 110111
R9	100111 0110110111 1111 101101 110111
R10	100111 0110110111 0111 101111 110111
R11	100111 0110110111 0111 101111 110111
R12	100111 0110110111 0111 101111 110111
R13	100111 0110110111 0111 101111 110111
R14	100111 0110110111 0111 101111 111111
R15	100111 0110110111 0111 101111 110111
R16	100111 0110110111 0111 101111 110111
R17	100111 0110110111 0111 101111 110111
R18	100111 0110110111 0111 101111 110111
R19	100111 0110110111 0111 101111 110111
R20	100111 0110110111 0111 101111 110111
R21	100111 0110110111 0111 101111 110111
R22	100111 0110110111 1111 101101 110111
R23	100111 0110110111 1111 101101 110111
R24	100111 1110110111 1111 101101 110111
T1	100111 0110110111 0111 101111 110111
T2	100111 0110110111 1111 101101 110111
T3	100111 0110110111 0111 101111 110111
T4	100111 0110110111 0111 101111 110111
T5	100111 0110110111 1111 101101 110111
T6	100111 0110110111 0111 101111 110111
T7	100111 0110110111 1111 101101 110111
T8	100111 0110110111 0111 101111 110111
T9	100111 0110110111 1111 101101 110111

T10	100111 0110110111 0111 111111 110111
T11	100111 0110110111 1111 101101 110111
T12	100111 0110110111 1111 101101 110111
T13	100111 0110110111 1111 101101 110111
T14	100111 0110110111 1111 101101 110111
T15	100111 0110110111 0111 101111 110111
T16	100111 0110110111 0111 101111 110111
T17	100111 0110110111 0111 101111 110111
T18	100111 0110110111 0111 101111 110111
T19	100111 0110110111 0111 101111 110111
T20	100111 0110110111 0111 101111 110111
T21	100111 0110110111 1111 101101 110101
T22	100111 0110110111 0111 101111 110111
T23	100111 0110110111 1111 101101 110111
T24	100111 0110110111 0111 101111 110111
E1	100111 0110110111 1111 101101 110111
E2	100111 0110110111 0111 101111 110111
E3	100111 0110110111 0111 101111 110111
E4	100111 0110110111 0111 101111 110111
E5	100111 0110110111 0111 101111 110111
E6	100111 0110110111 1111 101101 110111
E7	100111 0110110111 0111 101111 110111
E8	100111 0110110111 0111 101111 110111
E9	100111 0110110111 0111 101111 110111
E10	100111 0110110111 0111 101111 110111
E11	100111 0110110111 0011 101111 110111
E12	100111 0110110111 1111 101101 110111
E13	100111 0110110111 0111 101111 110111
E14	100111 0110110111 1111 101101 110111
E15	100111 0110110111 1111 101101 110111
E16	100111 0110110111 1111 101101 110111
E17	100111 0110110111 0111 101111 110111
E18	100111 0110110111 1111 101101 110101
E19	100111 0110110111 0111 101111 110111
E20	100111 0110110111 1111 101111 110111
E21	100111 0110110111 0111 101111 110111
E22	100111 0110110111 0111 101111 110111
E23	100111 0110110111 0111 101111 110101
E24	100111 0110111111 0111 101111 110111
M1	100111 0110110111 0111 101111 110111
M2	100111 0110110111 0111 101111 110111
M3	100111 0110111111 1111 101101 110101
M4	100111 0110110111 0111 101111 110111
M5	100111 0110110111 0111 101111 110111
M6	100111 0110110111 0111 101111 110111
M7	100111 0110110111 1111 101101 110111
M8	100111 0110110111 0111 101111 110111
M9	100111 0110110111 0111 101111 110111
M10	100111 0110110111 0111 101111 110111
M11	100111 0110110111 0111 101111 110111
M12	100111 0110110111 1111 101101 110111
M13	100111 0110110111 0111 101111 110111
M14	100111 0110110111 0111 101111 110111
M15	100111 0110110111 0111 101111 110111

M16 100111 0110110111 1111 111111 110101
M17 100111 0110110111 0111 101111 110111
M18 100111 0110110111 0111 101111 110111
M19 100111 0110110111 0111 101111 110111
M20 100011 0110110111 0111 101111 110111
M21 100111 0110110111 0111 101111 110111
M22 100111 0110110111 1111 101101 110111
M23 100111 0110110111 1111 101111 110111
M24 100111 0110110111 0111 101111 110111
P1 100111 0110110111 0111 101111 110111
P2 100111 0110110111 1111 101101 110111
P3 100111 0110110111 0111 101101 110111
P4 100111 0110110111 0111 101111 110111
P5 100111 0110110111 0111 101111 110111
P6 100111 0110110111 1111 101101 110111
P7 100111 0110110111 0111 101111 110111
P8 100111 0110110111 0111 101111 110111
P9 100111 0110110111 1111 101101 110111
P10 100111 0110010111 0111 101111 110111
P11 100111 0110110111 0111 101111 110111
P12 100111 0110110111 0111 101111 110111
P13 100111 0110110111 0111 101101 110111
P14 100111 0110110111 1111 101101 110111
P15 100111 0110110111 0111 101111 110101
P16 100111 0110110111 1111 101101 110111
P17 100111 0110110111 0011 101111 110111
P18 100111 0110110111 1111 101101 110111
P19 100111 0110110111 0111 101111 110111
P20 100111 0110110111 1111 101101 110111
P21 110111 0110110111 0111 101111 110111
P22 100111 0110110111 0111 101111 110111
P23 100111 0110110111 1111 101101 110111
P24 100111 0110110111 0111 101111 110111

Presence / absence of restriction sites for PMA95
Order of restriction enzymes: TaqI/ MspI/RsaI/DraI+PvuII/HaeIII

B1	0011 01101 10011 0010 1001011
B2	0011 01101 10011 0010 1001011
B3	0001 01100 10001 1010 1011011
B4	0011 01100 10011 0010 1011111
B5	0011 01111 10011 0010 1011011
B6	0001 01100 10011 0010 1011011
B7	0011 01101 10011 0010 1001011
B8	0001 01100 10011 0010 1011011
B9	0011 01101 10011 0010 1001011
B10	0011 01101 10011 0010 1001011
B11	0011 01101 10011 0010 1001011
B12	0011 01101 10011 0010 1001011
B13	0011 11100 10011 0010 1011011
B14	0001 01100 10011 1010 1011011
B15	0011 01100 10011 0010 1011011
B16	0011 01101 10011 0010 1001011
B17	0001 01100 10011 0010 1011011
B18	0011 01101 10011 0010 1001011
B19	0001 01100 10011 0010 1011011
B20	0011 00100 10011 0010 1011011
B21	0001 01100 10011 0010 1011011
B22	0001 01101 10011 0010 1001011
B23	0011 01101 10011 0010 1001011
B24	0011 01100 10011 0010 1011111
C1	0001 01100 10011 0010 1011011
C2	0001 01100 10001 1010 1011011
C3	0001 01100 10001 1010 1011011
C4	0011 01101 10001 0010 1001011
C5	0001 01100 10011 0010 1011011
C6	0011 01101 10001 0010 1001011
C7	0011 01101 10011 0010 1001011
C8	0011 01101 10011 0010 1001011
C9	0011 01101 10011 0010 1001011
C10	0001 01100 10001 0010 1011011
C11	0001 01100 10001 1010 1011011
C12	0011 01101 10011 0010 1001011
C13	0001 01100 10011 0010 1011011
C14	0011 01111 10011 0010 1011011
C15	0001 01100 10011 0010 1011011
C16	0011 01101 10011 0010 1001011
C17	0001 01100 10011 0010 1011011
C18	0011 01101 10011 0010 1001011
C18	0011 01101 10011 0010 1001011
C20	0011 01101 10011 0010 1001011
C21	0011 01101 10011 0010 1001011
C22	0011 01101 10011 0010 1001011
C23	0001 01100 10001 1110 1011011
C24	0001 01100 10011 1010 1011011
D1	0001 01100 10111 1010 1011011
D2	0011 01101 10011 0010 1001011

D3	0011 01101 10011 0010 1001011
D4	0011 01101 10011 0010 1001011
D5	0011 01101 10011 0010 1001011
D6	0011 01101 10011 0010 1011011
D7	0011 01101 10011 0010 1001011
D8	0011 01101 10011 0010 1001011
D9	0001 01101 10011 0010 1001011
D10	0011 01101 10011 0010 1001011
D11	0011 01101 10011 0010 1001011
D12	0011 01101 10011 0010 1001011
D13	0011 01101 10011 0010 1001011
D14	0011 01101 10111 0010 1001011
D15	0011 01101 10011 0010 1001011
D16	0001 01100 10001 1010 1011011
D17	0011 01101 10011 0010 1001011
D18	0011 01101 10011 0010 1011011
D19	0011 01101 10011 0010 1001011
D20	0011 01101 00011 0010 1001011
D21	0011 01101 10001 0010 1001011
D22	0001 01101 10011 0010 1011011
D23	0011 01100 10011 0010 1001011
D24	0011 01101 10011 0010 1001011
R1	0011 01101 10011 0010 1001011
R2	0001 01100 10001 1010 1011011
R3	0001 01101 11011 0010 1001011
R4	0011 01101 10011 0010 1001011
R5	0011 01101 10111 1110 1001011
R6	0011 11100 10011 0010 1011011
R7	0011 01101 00011 0010 1001011
R8	0001 01100 10011 0010 1011011
R9	0001 01100 10001 0010 1011011
R10	0011 01101 10011 0010 1001011
R11	0001 01100 10011 0010 1011011
R12	0001 01100 10011 0010 1011011
R13	0001 01100 10011 0010 1011011
R14	0001 01100 10011 0010 1011011
R15	0011 01101 10011 0010 1001011
R16	0011 01100 10011 0010 1011011
R17	0001 01100 10011 0010 1011011
R18	0011 01101 10011 0010 1001011
R19	0011 01101 10011 0010 1001011
R20	0011 01111 10011 0010 1011011
R21	0011 01101 10011 0010 1001011
R22	0011 01101 10011 0010 1001011
R23	0011 01101 10011 0010 1001011
R24	0001 01100 10011 0010 1011011
T1	0011 01101 10011 0010 1001011
T2	0011 01101 10011 0010 1001011
T3	0001 01100 10011 0010 1011011
T4	0011 01101 10011 0011 1001011
T5	0011 01100 10011 0010 1001011
T6	0011 01101 10011 0010 1011011
T7	0001 01100 10011 0010 1000011
T8	0001 01100 10011 0010 1011011

T9	0001 01100 10011 0010 1011011
T12	0011 01101 10011 0010 1001011
T13	0011 01101 10011 0010 1001011
T14	0011 01101 10011 0010 1001011
T15	0011 01101 10011 0010 1001011
T16	0001 01100 10011 0010 1011011
T17	0011 01101 10011 0010 1001011
T18	0001 01100 10011 0010 1011011
T19	0011 11100 10011 0010 1011011
T20	0011 01111 10011 0010 1011011
T21	0011 01101 10011 0010 1001011
T22	0011 01101 10011 0010 1001011
T23	0001 01100 10001 0010 1011011
T24	0011 01101 10011 0010 1001011

Presence / absence of restriction sites for PMA96
Order of restriction enzymes: TaqI/MspI/RsaI/DraI+PvuII/HaeIII

B1	0001 01101 10011 0010 1001011
B2	0011 01100 10011 0010 1011011
B3	0011 01100 10011 0010 1001011
B4	0011 01100 10011 0010 1011011
B5	0001 01100 10011 0010 1011011
B6	0011 01101 10111 0010 1001011
B7	0011 01101 10011 0010 1001011
B8	0011 01101 10011 0010 1001011
B9	0011 01101 00011 0010 1001011
B10	0011 01101 10011 0010 1001011
B11	0011 01101 10011 0010 1001011
B12	0011 01101 10011 0010 1001011
B13	0001 01100 10011 0010 1000011
B14	0011 01101 10011 0010 1011011
B16	0001 01100 10001 0010 1011011
B17	0001 01100 10011 0010 1011011
B18	0011 01101 10011 0010 1001011
B19	0011 01101 10011 0010 1001011
B20	0011 01101 10011 0010 1001011
B21	0001 01100 10011 0010 1011011
B22	0011 01101 10011 0010 1001011
B23	0011 01101 10011 0010 1001011
B24	0011 01101 10011 0010 1001011
C1	0011 01101 10011 0010 1001011
C2	0011 01101 10011 0010 1001011
C3	0011 11100 10011 0010 1001011
C4	0001 01100 10011 0010 1011011
C5	0011 01100 10011 0010 1001011
C6	0011 01101 10011 0010 1001011
C7	0011 01100 10011 0010 1011011
C8	0011 01101 10011 1010 1001011
C9	0111 01101 10111 0010 1001011
C10	0011 01101 10011 0010 1001011
C11	0011 01100 10011 0010 1001011
C12	0011 01101 10111 0010 1001011
C13	0011 01101 10011 0010 1001011
C14	0011 01101 10011 0010 1001011
C15	0001 01100 10011 0010 1011011
C16	0011 01101 10011 0010 1000011
C17	0001 01100 10001 1010 1011011
C18	0011 01101 10011 0010 1001011
C19	0001 01100 10001 1010 1011011
C20	0011 01101 10011 0010 1001011
C21	0011 01111 10011 0010 1011011
C22	0001 01100 10011 0010 1011011
C23	0001 01100 10011 0010 1011011
C24	0011 01101 10011 0010 1001011
D1	0001 01100 10011 0010 1011011
D2	0011 01101 10011 0010 1001011
D3	0011 01101 10011 0010 1001011

D4	0011 01101 10011 0010 1101011
D5	0011 01101 10011 0010 1001011
D6	0001 01100 10011 0010 1011011
D7	0001 01101 10011 0010 1011011
D8	0011 01101 10011 0010 1001011
D9	0011 01101 10111 0010 1001011
D10	0011 01101 10011 0010 1001011
D11	0011 01101 10011 0010 1001011
D12	0011 01101 10011 0010 1001011
D13	0011 01101 10011 0010 1011011
D14	0011 01101 10011 0010 1001011
D15	0011 01100 10011 0010 1011011
D16	0011 11100 10011 0010 1001011
D17	0011 01101 10011 0010 1001011
D18	0011 01101 10011 0010 1001011
D19	0001 01100 10011 0010 1011011
D20	0011 01101 10011 0010 1001011
D21	0001 01100 10001 0010 1011011
D22	0011 01101 10011 0010 1001011
D23	0011 01101 10011 0010 1001011
D24	0011 01101 10011 0010 1001011
R1	0011 01100 10011 0010 1011011
R2	0001 01101 10011 0010 1001011
R3	0111 01101 10011 0010 1001011
R4	0011 01100 10011 0010 1011011
R5	0001 01101 10011 0010 1001011
R6	0011 01101 10011 0010 1001011
R7	0011 01111 10001 0010 1011011
R8	0001 01100 10011 0010 1011011
R9	0001 01100 10011 0010 1011011
R10	0001 01101 10011 0010 1001011
R11	0011 01101 10011 0010 1001011
R12	0011 01101 10011 0010 1000011
R13	0011 01101 10011 0010 1001011
R14	0011 01101 10011 0010 1001011
R15	0011 01101 10011 0010 1001011
R16	0011 01101 10011 0010 1001011
R17	0011 01101 10011 0010 1001011
R18	0011 01101 10011 0010 1001011
R19	0011 01101 10011 0010 1001011
R20	0011 01101 10011 0010 1001011
R21	0011 01101 10011 0010 1000011
R22	0001 01100 10011 0010 1011011
R23	0001 01100 10011 0010 1011011
R24	0011 01100 10011 0010 1011011
T1	0011 01101 10011 0010 1001011
T2	0011 01100 10011 0010 1011011
T3	0011 01101 10011 0010 1001011
T4	0011 01101 10011 0010 1001011
T5	0001 01100 10111 1010 1011011
T6	0011 11100 10011 0010 1001011
T7	0001 01100 10001 1010 1011011
T8	0011 01101 10011 0010 1001011
T9	0001 01100 10011 0010 1011011

T10	0011 01101 10011 0010 1001011
T11	0001 01100 10011 0010 1011011
T12	0001 01100 10011 0010 1011011
T13	0011 01100 10011 0010 1011011
T14	0011 01100 10111 0010 1011011
T15	0011 01101 10011 0010 1001011
T16	0011 01101 10011 0010 1011011
T17	0011 01101 10011 0010 1001011
T18	0011 01101 10011 0010 1001011
T19	0011 01101 10011 0010 1001011
T20	0011 01101 10011 0010 1001011
T21	0011 01111 10011 0010 1011011
T22	0011 01101 10011 0010 1001011
T23	0001 01100 10001 1010 1011011
T24	0011 01101 10011 0010 1001011
E1	0011 11100 10011 0010 1001011
E2	0011 01101 10011 0010 1001011
E3	0001 01101 10011 0010 1001011
E4	0011 01101 10011 0010 1001011
E5	0001 01101 10011 0010 1001011
E6	0011 01100 10011 0010 1011011
E7	0001 01101 10011 0010 1011011
E8	0011 01101 10011 0010 1001011
E9	0011 00100 10011 0010 1001011
E10	0001 01101 10011 0010 1001011
E11	0011 01101 10011 0010 1001011
E12	0001 01100 10011 0010 1011011
E13	0001 01101 10011 0010 1001011
E14	0001 01100 10011 0010 1011011
E15	0001 01100 10011 0010 1011011
E16	0001 01100 10011 0010 1011011
E17	0011 01101 10011 0010 1001011
E18	0011 01100 10011 0010 1011011
E19	0011 01101 10011 0010 1001011
E20	0001 01101 10011 0010 1001011
E21	0011 01101 10011 0010 1001011
E22	0011 01101 10011 0010 1001011
E23	0001 01101 10011 0010 1001011
E24	0011 01101 10011 0010 1011011
M1	0011 01101 10011 0010 1001011
M2	0011 01101 10011 0010 1001011
M3	0011 01111 10011 0010 1011011
M4	0011 01101 10111 0010 1001011
M5	0011 01101 10011 0010 1001011
M6	0011 01101 10011 0010 1001011
M7	0011 01100 10011 0010 1001011
M8	0011 01101 10011 0010 1001011
M9	0011 01101 10011 0010 1001011
M10	0011 01101 10011 0010 1001011
M11	0011 01101 10011 0010 1001011
M12	0001 01100 10011 0010 1011011
M13	0011 01101 10011 0010 1001011
M14	0011 01101 10011 0010 1001011
M15	0011 01101 10011 0010 1001011

M16 0011 01111 10011 0010 1011011
M17 0011 01101 10011 0010 1001011
M18 0011 01101 00011 0010 1001011
M19 0001 01101 10011 0010 1001011
M20 0011 01101 10011 0010 1001011
M21 0011 11100 10011 0010 1001011
M22 0011 01100 10011 0010 1001011
M23 1001 01100 10011 0010 1011011
M24 0011 01101 10011 0010 1001011
P1 0011 01101 10011 0010 1001011
P2 0001 01100 10011 0010 1011011
P3 0011 01100 10011 0010 1011011
P4 0011 01101 10011 0010 1001011
P5 0011 01101 10011 0010 1001011
P6 0001 01100 10011 0010 1011011
P7 0011 01101 10011 0010 1001011
P8 0011 01100 10011 0010 1001011
P9 0001 01101 10011 0010 1011011
P10 0011 01101 10011 0010 1001011
P11 0011 01100 10001 0010 1001011
P12 0011 01101 10011 0010 1001011
P13 0011 01111 10011 0010 1011011
P14 0011 01101 10011 0010 1011011
P15 0011 01100 10011 0010 1001011
P16 0011 01101 10011 0010 1011011
P17 0011 01100 10011 0010 1001011
P18 0001 01100 10011 0010 1001011
P19 0001 01100 10011 0010 1001011
P20 0001 01100 10011 0010 1011011
P21 0011 01101 10011 0010 1001011
P22 0011 01101 10011 0010 1001011
P23 0011 01100 10001 0010 1011011
P24 0011 01111 10011 0010 1011011

Haplotypes observed for DPM95

Order of restriction enzymes: Msp1/RsaI/Dra+Pvu/HaeII/Taq1

1 100111 0110110111 0111 101111 110111
2 100111 0110110111 1111 101101 110111
3 100111 0110111111 1111 101101 100111
4 100111 0110110111 1111 101101 110101
5 101111 0110110111 0111 101111 110111
6 100111 0110110111 0011 101111 110111
7 100111 1110110111 1111 101101 110111
8 100111 0110110111 0111 101101 110111
9 100111 0110110111 1111 101101 110011
10 100111 0110110111 0111 111111 110111
11 100111 0110110111 0101 101111 110111
12 100111 0110010111 0111 101111 110111
13 000111 0110110111 0111 101111 110111
14 100111 0111110111 0111 101111 110111
15 100111 0110110111 0111 101111 110101
16 100111 0110110111 0111 101111 110011
17 100011 0110110111 0111 101101 110111
18 100111 1110110111 0111 101111 110111
19 110111 0110110111 0111 101111 110111
20 101111 0110110111 1111 101101 110111
21 100011 0110110111 1111 101101 110111
22 100111 0110111111 1111 101101 110101
23 100111 0110110111 1111 111111 110101
24 100011 0110110111 0111 101111 110111
25 100111 0110110111 1111 101111 110111

Haplotypes observed for DPM96

Order of restriction enzymes: Msp1/RsaI/Dra+Pvu/HaeII/Taq1

1 100111 0110110111 0111 101111 110111
2 100111 0110110111 1111 101101 110111
3 100011 0110110111 1111 101101 100111
4 100111 1110110111 0111 101111 110111
5 000111 0110110111 0111 101111 110111
6 100111 0110110111 1111 101101 110011
7 100111 0110110111 0111 111111 110111
8 100111 0110110111 0011 101111 110111
9 100111 0110110111 1111 101101 110101
10 100111 0110110111 0101 101111 110111
11 100111 0110111111 0111 101111 110111
12 100111 0110010111 0111 101111 110111
13 101111 0110110111 0111 101111 110111
14 100111 0110110111 0111 101101 110111
15 100111 0110110111 0111 101111 111111
16 100111 1110110111 1111 101101 110111
17 100111 0110110111 1111 101111 110111
18 100111 0110110111 0111 101111 110101
19 100111 0110111111 1111 101101 110101
20 100111 0110110111 1111 111111 110101
21 100011 0110110111 0111 101111 110111
22 110111 0110110111 0111 101111 110111

Haplotypes observed for PMA95
Order of restriction enzymes: Taq1/ Msp1/RsaI/Dra+Pvu/HaeIII

1 0011 01101 10011 0010 1001011
2 0001 01100 10001 1010 1011011
3 0011 01100 10011 0010 1011111
4 0011 01111 10011 0010 1011011
5 0001 01100 10011 0010 1011011
6 0011 11100 10011 0010 1011011
7 0001 01100 10011 1010 1011011
8 0011 01100 10011 0010 1011011
9 0011 00100 10011 0010 1011011
10 0001 01101 10011 0010 1001011
11 0011 01101 10001 0010 1001011
12 0001 01100 10001 0010 1011011
13 0001 01100 10001 1110 1011011
14 0001 01100 10111 1010 1011011
15 0011 01101 10011 0010 1011011
16 0011 01101 10111 0010 1001011
17 0011 01101 00011 0010 1001011
18 0001 01101 10011 0010 1011011
19 0011 01100 10011 0010 1001011
20 0001 01101 11011 0010 1001011
21 0011 01101 10111 1110 1001011
22 0011 01101 10011 0011 1001011
23 0001 01100 10011 0010 1000011
24 0011 11100 10011 0010 1001011
25 1001 01100 10011 0010 1011011
26 0011 01100 10001 0010 1001011
27 0001 01100 10011 0010 1001011
28 0011 01100 10001 0010 1011011

Haplotypes observed for PMA96

Order of restriction enzymes: TaqI/MspI/RsaI/DraI+PvuII/HaeIII

1 0001 01101 10011 0010 1001011
2 0011 01100 10011 0010 1011011
3 0011 01100 10011 0010 1001011
4 0001 01100 10011 0010 1011011
5 0011 01101 10111 0010 1001011
6 0011 01101 10011 0010 1001011
7 0011 01101 00011 0010 1001011
8 0001 01100 10011 0010 1000011
9 0011 01101 10011 0010 1011011
10 0001 01100 10001 0010 1011011
11 0011 11100 10011 0010 1001011
12 0011 01101 10011 1010 1001011
13 0111 01101 10111 0010 1001011
14 0011 01101 10011 0010 1000011
15 0001 01100 10001 1010 1011011
16 0011 01111 10011 0010 1011011
17 0011 01101 10011 0010 1101011
18 0001 01101 10011 0010 1011011
19 0111 01101 10011 0010 1001011
20 0011 01111 10001 0010 1011011
21 0001 01100 10111 1010 1011011
22 0011 01100 10111 0010 1011011
23 0011 00100 10011 0010 1001011
24 1001 01100 10011 0010 1011011
25 0011 01100 10001 0010 1001011
26 0001 01100 10011 0010 1001011
27 0011 01100 10001 0010 1011011

Haplotypes observed for com95

Order of restriction enzymes:

DPM: MspI, RsaI, DraI/PvuII, HaeIII, TaqI

PMA: TaqI, MspI, RsaI, DraI/PvuII, HaeIII

1 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
2 100111 0110110111 1111 101101 110111 0001 01100 10001 1010 1011011
3 100111 0110111111 1111 101101 100111 0011 01100 10011 0010 1011111
4 100111 0110110111 1111 101101 110101 0011 01111 10011 0010 1011011
5 100111 0110110111 1111 101101 110111 0001 01100 10011 0010 1011011
6 101111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
7 100111 0110110111 0011 101111 110111 0011 01101 10011 0010 1001011
8 100111 0110110111 1111 101101 110111 0011 11100 10011 0010 1011011
9 100111 0110110111 1111 101101 110111 0001 01100 10011 1010 1011011
10 100111 1110110111 1111 101101 110111 0011 01100 10011 0010 1011011
11 100111 0110110111 1111 101101 110101 0001 01100 10011 0010 1011011
12 100111 0110110111 1111 101101 110111 0011 00100 10011 0010 1011011
13 100111 0110110111 0111 101111 110111 0001 01101 10011 0010 1001011
14 100111 0110110111 1111 101101 110111 0011 01100 10011 0010 1011111
15 100111 1110110111 1111 101101 110111 0001 01100 10011 0010 1011011
16 100111 0110110111 0111 101111 110111 0011 01101 10001 0010 1001011
17 100111 0110110111 1111 101101 110101 0001 01100 10001 0010 1011011
18 100111 0110110111 0111 101101 110111 0001 01100 10001 1010 1011011
19 100111 0110110111 1111 101101 110011 0001 01100 10011 0010 1011011
20 100111 0110110111 0111 111111 110111 0011 01101 10011 0010 1001011
21 100111 0110110111 0101 101111 110111 0011 01101 10011 0010 1001011
22 100111 0110010111 0111 101111 110111 0011 01101 10011 0010 1001011
23 100111 0110110111 1111 101101 110111 0001 01100 10001 1110 1011011
24 100111 0110110111 1111 101101 110111 0001 01100 10111 1010 1011011
25 000111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
26 100111 0111110111 0111 101111 110111 0011 01101 10011 0010 1001011
27 100111 0110110111 0011 101111 110111 0011 01101 10011 0010 1011011
28 100111 0110110111 0111 101111 110101 0011 01101 10011 0010 1001011
29 100111 0110110111 0111 101111 110111 0011 01101 10111 0010 1001011
30 100111 0110110111 0111 101111 110011 0011 01101 10011 0010 1001011
31 100111 0110110111 0111 101111 110111 0011 01101 00011 0010 1001011
32 100111 0110110111 0111 101111 110111 0001 01101 10011 0010 1011011
33 100111 0110110111 0111 101111 110111 0011 01100 10011 0010 1001011
34 100111 0110110111 1111 101101 110111 0011 01101 10011 0010 1001011
35 100011 0110110111 0111 101101 110111 0011 01101 10011 0010 1001011
36 100111 1110110111 0111 101111 110111 0001 01101 11011 0010 1001011
37 100111 0110110111 0111 101111 110111 0011 01101 10111 1110 1001011
38 100111 0110110111 1111 101101 110111 0001 01100 10001 0010 1011011
39 110111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
40 101111 0110110111 1111 101101 110111 0011 01100 10011 0010 1011011
41 100111 0110110111 1111 101101 110101 0011 01101 10011 0010 1001011
42 100111 0110110111 0111 101101 110111 0011 01101 10011 0010 1001011
43 100111 0110110111 0111 101111 110111 0011 01101 10011 0011 1001011
44 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1011011
45 100111 0110110111 1111 101101 110111 0001 01100 10011 0010 1000011
46 100011 0110110111 1111 101101 110111 0001 01100 10011 0010 1011011
47 100111 0110111111 1111 101101 110101 0011 01111 10011 0010 1011011
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53 100111 01101101111 0111 101101 110111 0011 01100 10011 0010 1011011
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60 100111 01101101111 1111 101101 110111 0001 01100 10011 0010 1001011
61 100111 01101101111 0111 101111 110111 0001 01100 10011 0010 1001011
62 100111 01101101111 1111 101101 110111 0011 01100 10001 0010 1011011
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Haplotypes observed for com96

Order of restriction enzymes:

DPM: MspI, RsaI, DraI/PvuII, HaeIII, TaqI

PMA: TaqI, MspI, RsaI, DraI/PvuII, HaeIII

1 100111 0110110111 0111 101111 110111 0001 01101 10011 0010 1001011
2 100111 0110110111 1111 101101 110111 0011 01100 10011 0010 1011011
3 100111 0110110111 0111 101111 110111 0011 01100 10011 0010 1001011
4 100011 0110110111 1111 101101 100111 0011 01100 10011 0010 1011011
5 100111 0110110111 1111 101101 110111 0001 01100 10011 0010 1011011
6 100111 0110110111 0111 101111 110111 0011 01101 10111 0010 1001011
7 100111 1110110111 0111 101111 110111 0011 01101 10011 0010 1001011
8 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
9 100111 0110110111 0111 101111 110111 0011 01101 00011 0010 1001011
10 000111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
11 100111 0110110111 1111 101101 110111 0001 01100 10011 0010 1000011
12 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1011011
13 100111 0110110111 1111 101101 110111 0001 01100 10001 0010 1011011
14 100111 0110110111 1111 101101 110011 0001 01100 10011 0010 1011011
15 100111 0110110111 0111 101111 110111 0011 11100 10011 0010 1001011
16 100111 0110110111 0111 101111 110111 0011 01101 10011 1010 1001011
17 100111 0110110111 0111 101111 110111 0111 01101 10111 0010 1001011
18 100111 0110110111 0111 111111 110111 0011 01101 10111 0010 1001011
19 100111 0110110111 0011 101111 110111 0011 01101 10011 0010 1001011
20 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1000011
21 100111 0110110111 1111 101101 110111 0001 01100 10001 1010 1011011
22 100111 0110110111 1111 101101 110101 0011 01111 10011 0010 1011011
23 100111 0110110111 0111 101111 110111 0001 01100 10011 0010 1011011
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27 100111 0110111111 0111 101111 110111 0011 01101 10011 0010 1001011
28 100111 0110010111 0111 101111 110111 0011 01101 10011 0010 1001011
29 101111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
30 100111 0110110111 0111 101101 110111 0001 01100 10011 0010 1011011
31 100111 0110110111 0111 101111 110111 0001 01100 10001 0010 1011011
32 100111 0110110111 0111 101111 110111 0111 01101 10011 0010 1001011
33 100111 0110110111 1111 101101 110111 0011 01111 10001 0010 1011011
34 100111 0110110111 0111 101111 111111 0011 01101 10011 0010 1001011
35 100111 1110110111 1111 101101 110111 0011 01100 10011 0010 1011011
36 100111 0110110111 1111 101101 110111 0001 01100 10111 1010 1011011
37 100111 0110110111 0111 111111 110111 0011 01101 10011 0010 1001011
38 100111 0110110111 1111 101101 110111 0011 01100 10111 0010 1011011
39 100111 0110110111 1111 101101 110111 0011 11100 10011 0010 1001011
40 100111 0110110111 0111 101111 110111 0001 01101 10011 0010 1011011
41 100111 0110110111 0111 101111 110111 0011 00100 10011 0010 1001011

42 100111 0110110111 1111 101101 110101 0011 01100 10011 0010 1011011
43 100111 0110110111 1111 101111 110111 0001 01101 10011 0010 1001011
44 100111 0110110111 0111 101111 110101 0001 01101 10011 0010 1001011
45 100111 0110111111 0111 101111 110111 0011 01101 10011 0010 1011011
46 100111 0110111111 1111 101101 110101 0011 01111 10011 0010 1011011
47 100111 0110110111 1111 101101 110111 0011 01100 10011 0010 1001011
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60 100111 0110110111 1111 101101 110111 0011 01100 10001 0010 1011011
61 100111 0110110111 0111 101111 110111 0011 01111 10011 0010 1011011

Haplotypes observed for the combined fragments and year-classes

Order of restriction enzymes:

DPM: MspI, RsaI, DraI/PvuII, HaeIII, TaqI

PMA: TaqI, MspI, RsaI, DraI/PvuII, HaeIII

1 100111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
2 100111 0110110111 1111 101101 110111 0001 01100 10001 1010 1011011
3 100111 0110111111 1111 101101 100111 0011 01100 10011 0010 1011111
4 100111 0110110111 1111 101101 110101 0011 01111 10011 0010 1011011
5 100111 0110110111 1111 101101 110111 0001 01100 10011 0010 1011011
6 101111 0110110111 0111 101111 110111 0011 01101 10011 0010 1001011
7 100111 0110110111 0011 101111 110111 0011 01101 10011 0010 1001011
8 100111 0110110111 1111 101101 110111 0011 11100 10011 0010 1011011
9 100111 0110110111 1111 101101 110111 0001 01100 10011 1010 1011011
10 100111 1110110111 1111 101101 110111 0011 01100 10011 0010 1011011
11 100111 0110110111 1111 101101 110101 0001 01100 10011 0010 1011011
12 100111 0110110111 1111 101101 110111 0011 00100 10011 0010 1011011
13 100111 0110110111 0111 101111 110111 0001 01101 10011 0010 1001011
14 100111 0110110111 1111 101101 110111 0011 01100 10011 0010 1011111
15 100111 1110110111 1111 101101 110111 0001 01100 10011 0010 1011011
16 100111 0110110111 0111 101111 110111 0011 01101 10001 0010 1001011
17 100111 0110110111 1111 101101 110101 0001 01100 10001 0010 1011011
18 100111 0110110111 0111 101101 110111 0001 01100 10001 1010 1011011
19 100111 0110110111 1111 101101 110011 0001 01100 10011 0010 1011011
20 100111 0110110111 0111 111111 110111 0011 01101 10011 0010 1001011
21 100111 0110110111 0101 101111 110111 0011 01101 10011 0010 1001011
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85 100111 0110110111 0111 101111 110111 0011 01111 10011 0010 1011011

Cloning - Media

LB Agar (per liter)
10 g NaCl (Sigma)
10 g casein enzymatic hydrolysate (Sigma)
5 g yeast extract (Sigma)
20 g agar (Sigma)
adjusted to pH 7.0 with 5 N NaOH
deoinized water added to a final volume of 1 liter
autoclaved

prior to pouring the plates, 133 µl filter-sterilized IPTG (0.5M) (Promega), 133 µl x-gal (250 mg/ml) (Promega) and 500 µl filter-sterilized ampicillin (50 mg/ml)(Sigma) per 500 ml LB-agar were added (plus 6.25 mg tetracycline for SURE cells (Sigma))

LB-liquid medium
10 g NaCl (Sigma)
10 g casein enzymatic hydrolysate (Sigma)
5 g yeast extract (Sigma)
adjusted to pH 7.0 with 5 N NaOH
deoinized water added to a final volume of 1 liter
autoclaved
10 µl ampicillin (50 mg/ml, Sigma) were added per 10 ml liquid medium before incubating
(plus 125 µg tetracycline for SURE cells (Sigma))

SOB medium (1 liter)
20 g casein enzymatic hydrolysate
5 g yeast extract
0.5 g NaCl
autoclaved
prior to use 10 ml of 1M $MgCl_2$ and 10 ml of 1M $MgSO_4$ were added

SOC medium (100 ml)
1 ml of 2 M glucose was added to 100 ml SOB-medium and the solution was filter-sterilized (0.22 µm filters, Nalgene)

```
  1  CCNTCANNNC  CNGGTNAGCC  NGTGCGTGTT  TTTCAAGAGG  AAAAATGGGG
 51  GTGCCACAGG  AATTTACTCA  CTAAGTACAA  AATATATTTT  TTGTAAAAAA
101  AAGTTTTTTT  TCCCAAAAAA  GTCGGGTTTT  TNNGGATAAT  TNCTCTTATN
151  NGGTGAAAAA  TGGCACGTCC  AAAAAAGGAA  AAAAANNCGC  GAAAAAATTG
201  TGGAAAAAAC  GTGGGAAANC  CNGGGNGTTT  NGGNCCCCNC  CCNCNCNAAA
251  NNCCTCCCNN  CGNCGCAAN
```

```
  1 GANACGTGGC TACCCTGCCC AAACAAGTGG GGCACTACTA TAACCCCCTC
 51 TTNGGATCCT AAATCAAACA CATCCATCTG CCAACTTGGC AATTTCTACT
101 AAACCACTAA TCGTTCATGT TCACCAGTCG GTCTGGGGGA AGCAGGGGGA
151 TGGAATATCC CCTGCCCCAC NATAGGCCCN NCANCCCANC CNNGNGGGNT
201 GNGNANANNN TNNTCCNCNN GGGGGNNNCG GGNCCCCCNC NNNNAAAANN
251 NCCNCCNNNG NNNNN
```

1	CNNCTANGNC	NGTGCGTGTT	TTTCNAGAGG	AAAAATGGGG	GNGCCACAGG
51	AATTTACTCA	CTAAGTACAA	AATATATTTT	TTGTAAAAAA	AAGTTTTTTT
101	TCCCAAAAAA	GTCGGGGTNT	NCNGGGTAAT	TTCTTTTATT	TGGGGNAAAA
151	TNGGACNGCC	AAAAAAGGNA	AAAAAAACCC	CAAAAAAATT	TTGGAAAAAC
201	CTGGGAAAAA	GGGGGNTNCG	GGCCCCCCCN	CNNTCAANCN	NN

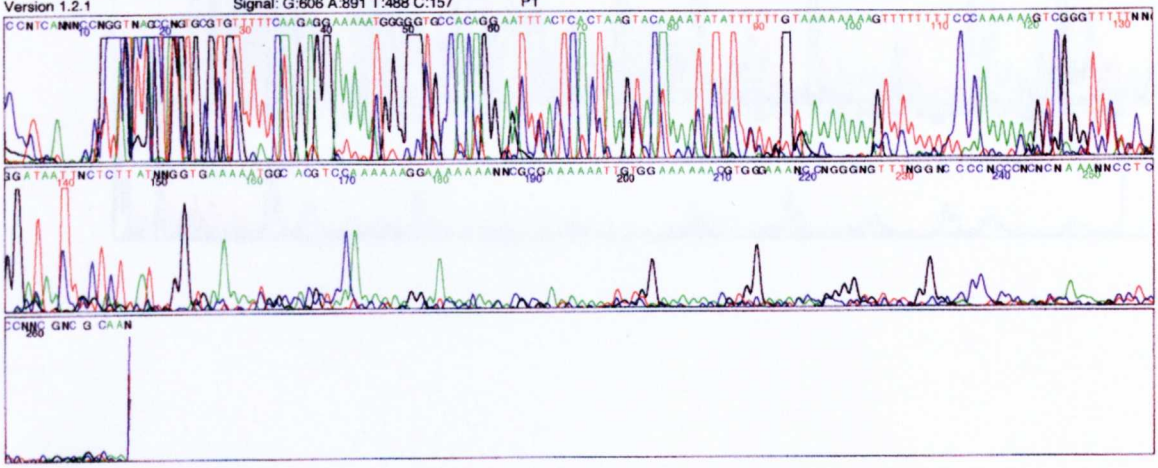
```
  1  GCCNNNGGGG GNNCNTTNNA NATNTGCTAC CCTGCCCAAA CNAGTGGGGC
 51  ACTACTATAA CCCCCTCTTT GGATCCTAAA TCAAACACAT CCATCTGCCA
101  ACTTGGCAAT TTCTACTAAA CCACTAATCG TTCATGTTCA CCAGTCGTTT
151  TGGGGGAAGN AGNGGGNTGG ATATCCCCNC CNCCNANATG ANCCCCNCAN
201  CCCANCCNNG NNGGNTGNNN NNNNNNNNNN NNNNNGGGGG GNNNNGGGNN
251  CCCNNNNNNN NANNNNNNCN CNNNNNNNNN NN
```

Applied Biosystems
Model 373A
Version 1.2.1

160595 01
DyeTerminator{AnyPrimer}
Lane 1
Signal: G:606 A:891 T:488 C:157

Points 806 to 3800 Base 1: 806
SEQ MATRIX #1726
DNA2
P1

Mon, May 15, 1995 17:31
X: 0 to 3162 Y: 0 to 1200
Spacing: -12.00



Applied Biosystems
Model 373A
Version 1.2.1

160595 02
DyeTerminator{AnyPrimer}
Lane 2
Signal: G:264 A:414 T:340 C:169

Points 811 to 3800 Base 1: 811
SEQ MATRIX #1726
DNA2
P2

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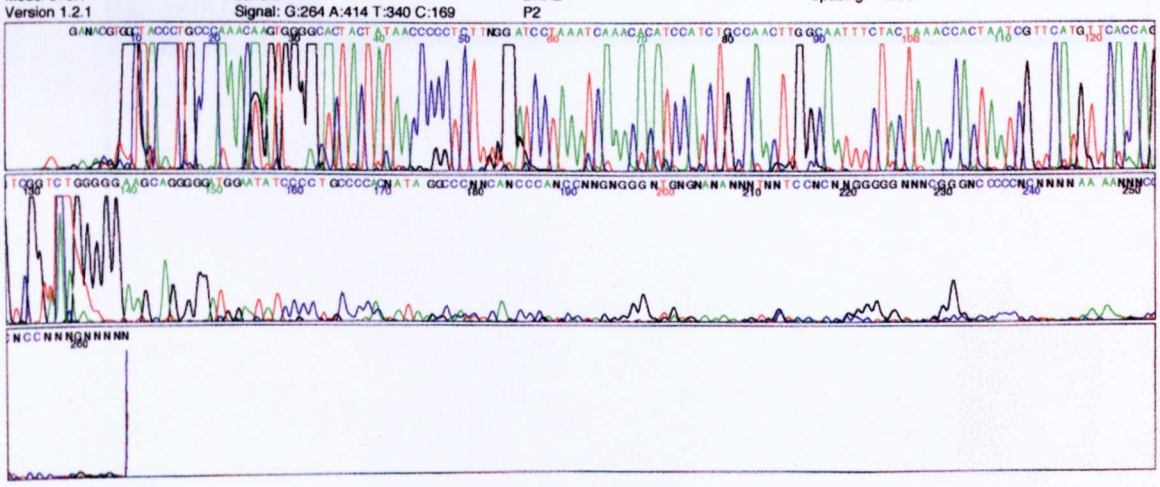
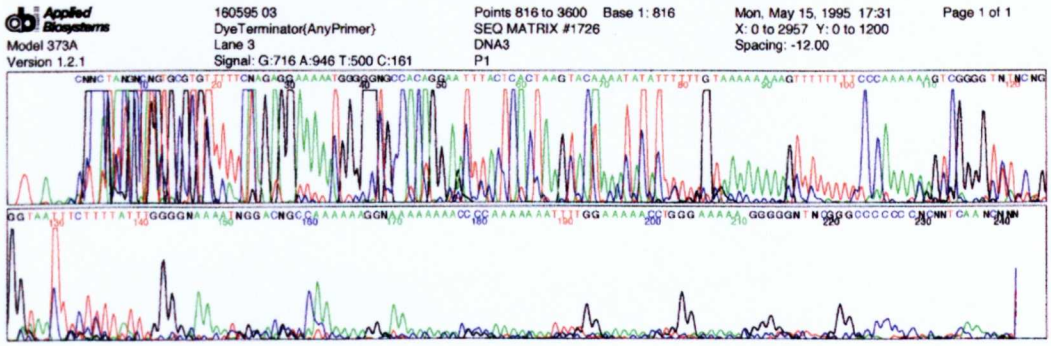
X: 0 to 3157 Y: 0 to 1200
Spacing: -12.00

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Version 1.2.1
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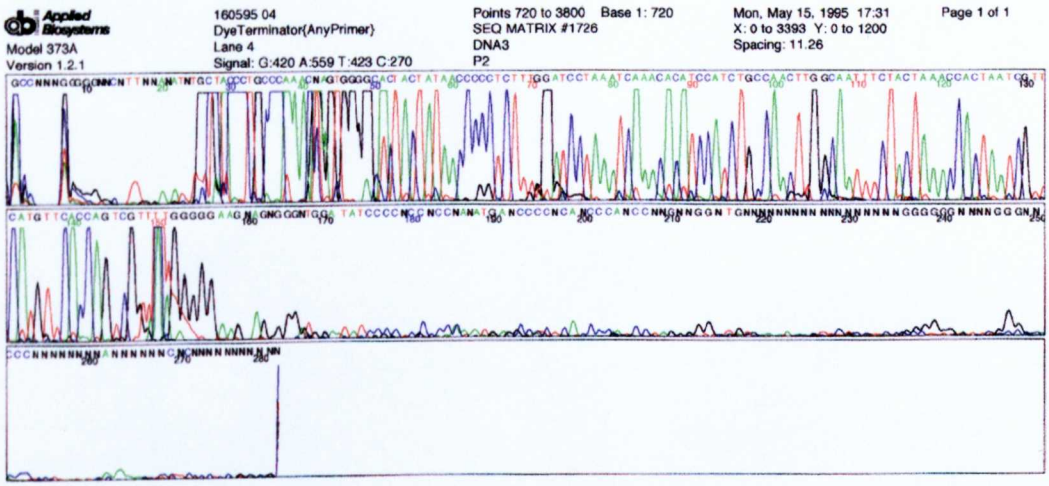
DyeTerminator{AnyPrimer}
Lane 3
Signal: G:716 A:946 T:500 C:161
Points 816 to 3600 Base 1: 816
SEQ MATRIX #1726
DNA3
P1
Mon, May 15, 1995 17:31
X: 0 to 2957 Y: 0 to 1200
Spacing: -12.00
Page 1 of 1


Applied Biosystems
Model 373A
Version 1.2.1
160595 04
DyeTerminator{AnyPrimer}
Lane 4
Signal: G:420 A:559 T:423 C:270
Points 720 to 3800 Base 1: 720
SEQ MATRIX #1726
DNA3
P2
Mon, May 15, 1995 17:31
X: 0 to 3393 Y: 0 to 1200
Spacing: 11.26
Page 1 of 1