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Population genetics of the scallop Pecten maximus : morphological, allozyme electrophoresis and mitochondrial DNA approaches.

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POPULATION GENETICS OF THE SCALLOP *PECTEN MAXIMUS*: MORPHOLOGICAL, ALLOZYME ELECTROPHORESIS AND MITOCHONDRIAL DNA APPROACHES

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by

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Although considerable evidence exists to suggest that certain populations of scallops, *Pecten maximus* (L.) are reproductively isolated, attempts to date to differentiate populations using data from allozyme electrophoresis have been unsuccessful. In order to further investigate the population structure of this species, genetic differentiation was quantified using mitochondrial DNA (mtDNA) markers and the results compared to morphological comparisons and allozyme variation. A multivariate morphological study revealed little variation in shell shape although animals from Brest and La Trinité (Brittany, France) appeared to have longer hinges. A population from Saint Brieuc Bay, Brittany which exhibits differences in reproductive cycle from neighbouring populations could not be separated on the basis of shell shape although unquantified differences in shell colour were noted. Allele frequencies at 7 loci assessed by allozyme electrophoresis were essentially homogeneous throughout the sample range in accord with previous studies and provided little evidence for population subdivision. Length variation of the mtDNA was extensive, therefore variability was assessed through a PCR approach in order that this did not cause analytical problems. On the basis of sequence divergence data there was convincing evidence that *P. maximus* from Mulroy Bay, Eire, a semi-enclosed sea lough, were genetically differentiated from all other samples. This could not be unequivocally attributed to a reduction in gene flow since the sample consisted of an ongrown single spatfall. Although no distinct pattern of mtDNA haplotype frequencies was apparent, the frequency of the commonest haplotype varied between sites with a pattern similar to that of allozyme allele frequencies in *Aequipecten opercularis*, a scallop species with a similar distribution and life history for which there is evidence of considerable population subdivision. In comparisons of *P. maximus* and the closely related *P. jacobaeus* no consistent differences were detected in morphology, allozymes or mtDNA data. The taxonomic standing of *P. jacobaeus* is brought into question.

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Contents

1.0 Introduction

1.1 The stock concept in fishery management

Successful fishery management policy is based upon maximisation of harvested biomass without impairment of the future prospects of the fishery (Pitcher and Hart, 1990). Fishery models, developed to aid in the achievement of this aim are typically based on the concept of a sustainable yield such that only a harvestable surplus (replenished by annual recruitment) is removed. When a species is effectively panmictic, that is a single reproductive unit with no subdivision into smaller breeding units, then such models can be based on the species as a whole. Localised harvesting may then not seriously affect the total fishery since individuals or recruits can freely move throughout the range to replenish the population. However if reproductive barriers exist, thus restraining breeding and migration among sub populations then models must account for this. If population structure is ignored or is not revealed then there is the possibility that fishing pressure will be exerted at a level suitable for the total population but beyond the capacity of a population, that is, to some extent, reproductively isolated. This danger is particularly relevant to self sustaining populations, for which recruitment comes solely from within and these populations would be particularly prone to recruitment overfishing (Sinclair *et al.*, 1985) whereby extensive fishing activity reduces the breeding stock to such a level that the mature spawning population is unable to effectively provide adequate future recruitment to replenish the population. Identification of self-recruiting populations is vital if fishery collapse is to be avoided. However although a population may not be completely self sustaining, where migration is to some extent restricted, even small quantities of migrants may be insufficient to replenish locally over fished populations and ignorance of this may have serious consequences. Detection of breeding structure is therefore essential for effective management of fishery resources.

In fisheries management, the term stock has been used to describe sub-populations of a species, although the precise description of what constitutes a stock is not clear (Carvalho and Hauser, 1995). Various definitions, including the animals occupying a physical region, those exploited in a specific area, or groups of individuals with similar morphologies have been used but do not necessarily

accurately depict the true nature of a stock, that is, a group of individuals with a sound genetic basis (Ovenden, 1990). Differences in reproductive, physiological or morphological parameters can all aid in stock identification and variation among regions may indicate curtailment of gene flow, but determination of actual genetic structure is vital in understanding the extent and pattern of population subdivision.

1.2 Assessment of stock structure

Assessment of the capacity for gene flow and hence the breeding structure of a species can proceed through either a direct or indirect approach (Slatkin, 1987, 1990). The former method relies on estimation of gene flow either by tagging organisms and tracing movements (mark-recapture), or extrapolating from estimates of dispersal distances and breeding success. In the case of broadcast spawning benthic invertebrates with planktotrophic larvae, such an approach is fraught with difficulties since not only is accurate hydrographic data vital, but estimated dispersal capacity may not accurately reflect gene flow. The behaviour of larvae both during dispersal and at settlement is often an unknown quantity. For instance the nature of larval migration through the water column during the day and hence the extent of passive transport in currents is unknown which will affect the actual extent of migration. Larvae may also have distinct settlement preferences which may mean that dispersal potential does not equate to realised dispersal.

The indirect approach utilises allele frequencies and DNA sequence data to estimate the level and pattern of gene flow required to have produced the observed pattern. Indirect methods have an advantage over direct methods in the spatial scale that can be studied. Also, because the observed pattern of genetic variation will be a result of gene flow (or lack of it) over numerous generations there will be no bias from the timing of the study as can occur if a direct approach is taken. However with this method it is impossible to distinguish the effects of ongoing gene flow from recent, historical connection of populations. Ideally both approaches would be undertaken in concert but it is often only practical to adopt indirect methods.

1.3 Population structure of marine species

Assessment of population subdivision using genetic techniques has been an important tool for studying the population structure of aquatic species.

The marine environment appears to be continuous and essentially homogeneous and since many marine species undergo a larval phase during which passive drifting occurs, the capacity for gene flow appears extensive. Furthermore, populations of marine species are usually large which would slow divergence of isolated populations (Palumbi, 1994) and therefore genetic homogeneity throughout a species' range is expected. Indeed population genetic studies have shown that many aquatic species appear to act as large panmictic units (Palumbi, 1992). However for other species there is an apparent structure of more or less distinct and discrete subpopulations (Allendorf, 1983; Burton, 1983; Hedgecock, 1986). Such population subdivision is possible because the sea is not in fact homogeneous, gross current patterns do not paint a full picture and barriers to gene flow do occur (Palumbi, 1994). These can be obvious, such as land masses separating populations in different ocean basins, or more difficult to detect in the case of hydrographic features such as fronts, thermoclines and gyres. Other more subtle factors must also be considered. For example, gene flow may be curtailed among populations that experience differential water temperatures throughout the year. If spawning occurs at particular and different water temperatures over a species' range, interbreeding may not occur and, if larval settlement is also temperature related, a complete reproductive barrier may exist (Ovenden, 1990).

1.4 Factors affecting the distribution of genetic variation

Interpretation of population genetic data requires consideration of all factors that can influence the nature and extent of allele frequencies.

Dobzhansky (1937) defined evolution as "a change in the genetic composition of populations through time". A precondition of such change is genetic variation among individuals. Understanding the mechanisms of evolution such as gene flow thus requires information on the nature, extent and pattern of genetic variation within and among populations. New variation enters a population either on an evolutionary timescale through mutation, or as a result of gene flow through interbreeding. Various factors can then affect how this variation becomes partitioned. Where populations are to some extent geographically isolated, genetic divergence between them will occur at a rate dependent on the levels of migration, mutation, selection and population size (Allendorf, 1983). The absolute

level of differentiation will depend on the effects of these factors over the time since separation. Understanding the genetic structure of populations will aid in evaluating the contribution of these various processes, in particular gene flow, which will then allow inferences to be made concerning the nature of recruitment among populations.

When migration involves the exchange of reproductively successful individuals, it is analogous to gene flow. Individuals that migrate but do not subsequently mate can have little influence. Lack of genetic structure is possibly due to extensive gene flow, although the homogenising effect of this on allele frequencies is opposed by the processes of selection and genetic drift which can themselves lead to genetic divergence among populations. Natural selection occurs where genotypes have differential success in contributing offspring to the next generation. A number of forms of selection are pertinent both to the maintenance of variation within a population and the distribution among sub-populations. Variation can be maintained by frequency dependent selection (Ayala and Campbell, 1974) or balancing selection, for example, heterozygous advantage which will act to maintain stable allele frequencies and thus prevent divergence (Allendorf, 1983). This can lead to an erroneous conclusion of high gene flow when this does not actually occur (e.g. Karl and Avise, 1992). Directional selection occurs when particular alleles are favoured in different sub-populations and therefore acts to increase divergence (Allendorf, 1983) and can produce apparent divergence of populations in spite of continued gene flow, for example, selection at the *Lap* locus in *Mytilus edulis* (Koehn *et al.*, 1980; Hilbish and Koehn, 1985).

It is important that the nature and extent of selection be recognised since the use of loci subject to selection can lead to erroneous conclusions concerning the pattern of gene flow (e.g. Koehn *et al.*, 1980; Karl and Avise, 1992). Ideally a locus that is unaffected by selection (i.e. selectively neutral) should be used to avoid misinterpretation of the pattern of genetic variation.

Stochastic (chance) processes may also have substantial effects on genetic variation within and (as a consequence) among populations. Genetic drift is defined as chance changes in allele frequencies as a result of random sampling among gametes from generation to generation. It has greater influence at smaller

population sizes when sampling effects are elevated (Allendorf, 1983) but is negligible if the population is large. Each generation allele frequencies are able to randomly drift and since there is no tendency in the next generation to restore the original frequencies then over short periods on the evolutionary timescale there is the potential for substantial shifts in allele frequencies. Indeed at low population sizes, alleles can rapidly approach fixation under the influence of genetic drift alone The effects of genetic drift become pronounced in smaller populations, particularly where small numbers of individuals found a new population (e.g. Knight *et al.*, 1987). Thus in species where negligible gene flow occurs among populations, strikingly different allele frequencies may develop when population sizes are low.

The level of gene flow necessary to maintain similar allele frequencies between geographically isolated populations has important implications for interpretation of population subdivision in a fishery context. If only limited numbers of migrants are sufficient to equilibrate gene frequencies then population genetic data may be of little value, since low leakage of migrants between stocks, although adequate for affecting genetic parameters would likely be insufficient for repopulating or enhancing low or overfished populations. It has been estimated that the magnitude of divergence in the absence of natural selection is dependent on the absolute number of migrants and not the proportion of exchange (Allendorf, 1983) such that a given number of migrants will result in equal amounts of genetic divergence irrespective of the population size. Thus, the same extent of genetic divergence is expected between two populations containing 1000 animals, exchanging 25 individuals (migration rate, m=2.5%) as between populations with N=50 exchanging 25 migrants (m=50%). This is because the forces of migration (gene flow acting to homogenise allele frequencies) and genetic drift (promoting divergence) oppose each other, thus larger populations are less affected by genetic drift and proportionately fewer migrants are needed to counteract drift, whilst at lower population size, populations will diverge rapidly through drift necessitating a higher frequency of migrants to stabilise allele frequencies. In fact, Kimura and Ohta (1971b) have estimated that only one migrant per generation, irrespective of population size, is required to maintain the presence of all alleles in all sub-populations. This would however not maintain identical allele frequencies, only the

continued presence of all alleles. In order to maintain similar frequencies among sub-populations requires a much higher number of migrants. Through the use of computer simulations Allendorf and Phelps (1981) estimated that for populations containing 200 individuals then even with 10 migrants per generation divergence of allelic frequencies could occur and indeed allelic divergence occurred in 50% of simulations in which 20 subpopulations exchanged 50 individuals per generation. Therefore the extent of allele frequency differences should yield important information concerning population structure.

Interpretation of data on the genetic structure of populations requires that all these processes (gene flow, genetic drift, selection) be considered. However processes that can result in similar outcomes such as differential selection in different populations and reduced gene flow with genetic drift are difficult to separate (Cabe and Alstad, 1994) particularly where a possible mechanism through which selection can act is recognised and this can make interpretation of genetic variation complex. Indeed the influence of selection on the observed levels of genetic variation within species is unknown and the debate over whether most variation is maintained by natural selection (the selectionist argument e.g. Powers, 1991) or stochastic processes (the neutralist theory e.g. Kimura and Ohta, 1971a; Kimura, 1990) although once heated and polarised has still not been resolved (Dillon, 1991a; Avise, 1994; Cabe and Alstad, 1994). Analysis of results of accumulated studies have failed to answer this question. Nevo *et al.* (1984) came to the conclusion that selection was responsible for the variation seen in around 1000 diverse species whilst Skibinski *et al.* (1993) implicated neutral processes for over 95% of the variation within their database of allozyme variation. The assumption of selective neutrality is, however, the simplest and most straight-forward way to interpret observed variability and often accepted as the "null-hypothesis" before entertaining selection based arguments (Avise, 1994). However it is impossible to prove. Selection by contrast can be demonstrated but this is, for a number of reasons, extremely difficult. Firstly a number of types of selection can operate and without detailed studies there is no way of choosing amongst them. Selective forces of the magnitude likely to operate on most loci are also difficult to measure and the occasional observation of strong selection at a locus is not

adequate proof of the selectionist hypothesis which would require demonstration at many or indeed most loci. Suggestion of the action of selection at a locus is also inadequate since it is virtually impossible to separate selection at a locus from the effect of selection of linked genes that may happen to contain a single selected locus (the "hitch-hiking" effect). Also, importantly, different loci usually provide similar levels of divergence as expected under the neutral model. Natural selection will operate differently for each locus and each allele at a locus whilst stochastic processes should be uniform over all loci and alleles (Lewontin and Krakauer, 1973). Many statistical treatments of allele frequencies also assume selective neutrality as a null hypothesis since this is the most pragmatic approach in the absence of alternative information (Lewis and Thorpe, 1994). However assumptions of the neutral theory cannot be tested experimentally since there is no way of proving selective neutrality.

1.5 Characters of use in stock identification

Many aspects of fishery resources have been used to suggest that stock structure exists (Ihssen *et al.*, 1981). These include physiology, behaviour, population parameters (e.g. growth rate), parasites, tagging, cytogenetics, immunology and serology (de Ligny, 1969; Hodgins, 1972; Utter *et al.*, 1974) allozyme electrophoresis and DNA based methods. Three of the most widely used methods are detailed below.

1.5.1 Morphology

Morphological variation is generally widespread among individuals but the genetic basis of such variability is often unclear. Early attempts at studying genetic variation were based on the limited numbers of phenotypic polymorphisms including mutant forms of a select few experimental animals, for which direct genetic control due to segregation in a Mendelian fashion could be attributed on the basis of laboratory or controlled crosses. Such traits require that the effect of an allelic mutation be so great as to allow assignment of phenotypes to genotypic classes. There are limited numbers of such traits in certain animals (e.g. variants of *Drosophila melanogaster*, Lewontin, 1974 p33) but their paucity makes their usefulness limited and the necessity for breeding studies limits such methods and makes the search for variation laborious and time consuming. However, the

extension of these studies using for example traits linked to chromosomal abnormalities in *Drosophila* (Wright and Dobzhansky, 1946) banding patterns on the shells of *Cepaea* (Cain and Sheppard, 1950,1954) or melanism in Lepidoptera (Kettlewell, 1955) when undertaken in concert with experimental approaches, revealed for the first time the selective significance of variation.

Many morphological characters for which a Mendelian mode of inheritance could not be shown do, however, appear through breeding studies and artificial selection to have high heritabilities. Heritability is the proportion of phenotypic variation within a population due to genetic differences among individuals (Allendorf, 1983) indicative of substantial genetic control (Avise, 1994). This variation however is difficult if not impossible to characterise due to the influence of factors such as polygenic control, dominance, linkage, epistasis and, perhaps most importantly, genotype-environment interactions. However, evidence of genetic control suggests not only that quantification of such characters may provide some of the necessary data on genetic variation but also that substantial genetic variation is present within populations. Morphological characters often exhibit the same population specific pattern as genetic characters, for example, shell shape in *Mytilus* separates populations in a manner akin to allozymes and mtDNA (Karakousis and Skibinski, 1992).

1.5.2 Allozymes

The first method to allow assessment of genetic variation at large numbers of unlinked loci from which valid genetic interpretations could be taken was allozyme electrophoresis. Early studies on purified proteins (Smithies, 1955) had shown that variant forms of proteins could be separated by passage through a medium under the influence of an electrical field (electrophoresis). During protein electrophoresis separation occurs on the basis of amino acid composition, size, configuration, structure and the pH and viscosity of the medium. Proteins being made up of amino acid chains carry an electrical charge of magnitude and polarity dependent on the constituent amino acids. If mutation alters a codon within an organism's DNA which after transcription and translation produces an amino acid substitution in the coded polypeptide itself resulting in a charge shift (or size or conformational change) for the protein then the resultant variant molecule may be differentiated

from others in the population through electrophoresis. When coupled with specific histochemical staining methods (Hunter and Markert, 1957) purification of proteins prior to separation became redundant as only proteins capable of reacting with the substrate were detected. Staining methods have now been developed for a multitude of enzymes (Shaw and Prasad, 1970; Schaal and Anderson, 1974; Harris and Hopkinson, 1976; Aebersold *et al.*, 1987; Benson and Smith, 1988; Dillon, 1991b).

Within diploid organisms there are two copies of most protein coding genes, one on each chromosome of an homologous pair. In some cases multiple molecular forms of proteins with the same function are encoded at different loci whilst a locus can encode a number of variants. The term isoenzyme (or isozyme) has been coined to describe all polymers of subunits encoded by different loci or allelic alternatives at the same locus, whilst alloenzymes (or allozymes) are the multiple forms of enzymes catalysing the same reaction and encoded at the same locus. Thus allozymes are allelic forms of isozymes. Since there is often codominant expression of alleles at allozyme loci such that for an individual heterozygous at a particular locus, each of the alleles is expressed, genotype designation is possible and allele frequencies can be calculated from population data, allowing direct estimates of variability from the primary gene products of loci.

1.5.2.1 Hardy-Weinberg equilibrium

Because of the nature of the data provided by allozyme electrophoresis in which heterozygotes and homozygotes are differentiable, genotype frequencies can be tested against those expected based on the allele frequencies in the sample (Lessios, 1992). The expected values for genotype frequencies can be calculated by the expansion of $(p + q + r + ...)^2$ where p, q and r are the allele frequencies in the sample. Comparison of observed and expected genotype frequencies can provide important information about the breeding structure of a population as first recognised, independently by Hardy and Weinberg in 1908 (Richardson *et al.*, 1987). The Hardy-Weinberg rule thus states that in an idealised population, that is, of effectively infinite size, mating at random and undergoing no mutation, immigration, emigration or selection, allele frequencies should conform to Hardy-Weinberg expectations from generation to generation. Comparison

of observed and expected genotype frequencies is typically undertaken with a χ^2 test. A significant difference provides an indication that the studied population does not comply with one of the underlying assumptions. Deviations from Hardy-Weinberg equilibrium, particularly due to deficiencies of heterozygotes, are often exhibited by populations of bivalves. A number of factors have been proposed to explain this (Zouros and Foltz, 1984b) including selection, inadequate scoring of genotypes (including that due to null alleles, Foltz, 1986; Hoare and Beaumont, 1995) and the Wahlund effect, a purely mathematical phenomenon resulting from sampling of two or more discrete populations with differing allele frequencies. Zouros and Foltz (1984b) also suggested that differences in spawning times could produce heterozygote deficiencies in aquatic organisms. Since mutation is unlikely to occur on the time scales relevant to most population studies it is dismissed as a likely cause of deviations when detected. The influence of null alleles on heterozygote deficiency in bivalves may be considerable. Although null alleles (alleles encoding defective enzymes) should be rapidly selected out particularly when homozygous, at certain loci relatively high frequencies are observed (see Hoare and Beaumont, 1995).

Conformation of allele frequencies to Hardy-Weinberg expectations can also be performed using F statistics (see below).

1.5.2.2 Quantification of population subdivision and gene flow

One of the major applications of allozyme data has been in the study of population subdivision and gene flow and a number of statistics can be used for the quantification of this. Wright (1951, 1965; summarised by Neigel, 1994) developed the basic theories that relate migration to the observed distribution of genetic variation in which fertilisation is viewed as a process in which pairs of gametes are sampled to form zygotes. Wright represented individual gametes as variables with a value determined by ancestry such that only gametes traceable to a common ancestor (i.e. identical by descent) have the same value. A correlation coefficient can then be defined for a pair of gametes combining to form the zygote. If combination occurs at random the coefficient will be zero whilst positive values indicate that gametes of common ancestry combine more frequently than expected. Wright's (1951) F statistics represent these correlation coefficients in terms of the correlations between gametes uniting to form a zygote. F_{IT} and F_{IS} are defined as

the correlation between the 2 uniting gametes, relative to the total population and the subpopulation respectively. As such they provide convenient measures of the concordance with Hardy-Weinberg equilibrium of the subpopulation and population respectively. F_{ST} however is a convenient indicator of population subdivision since it is defined as the correlation between random gametes within a population, relative to gametes of the total population.

Wright made no reference to actual genetic variation for these equations, only the ancestry of gametes, but the two are obviously related since gametes with a common ancestry will be more likely to carry the same alleles. Consequently F statistics can be estimated using allele frequency and genotype frequency data. F_{IT} and F_{IS} typically labelled as fixation indices (F_I), describing departures from Hardy-Weinberg genotypic frequencies within local populations (F_{IS}) and the total population (F_{IT}) can be quantified using observed (h_{obs}) and (h_{exp}) frequencies of heterozygotes at a locus (Nei, 1973, 1977).

$$F_I = 1 - \left(\frac{h_{obs}}{h_{\exp}} \right)$$

F_{ST} can be defined in terms of the variance of allele frequencies between populations. If allele frequencies vary among populations this indicates that gametes are combining more frequently with gametes from within the population than expected if the total population represents the gamete pool.

$$F_{ST} = \frac{\sigma^2}{p(1-p)}$$

Where σ^2 is the variance in allele frequencies among populations and p is the mean allozyme frequency. Thus F_{ST} is the ratio of the observed variation in allele frequency among subpopulations to the maximum variance. Values range from 0 (a genetically homogeneous unstructured population) to 1 (a population divided into isolated sub-populations with no alleles in common).

Because of the direct effect that migration (gene flow) has upon the distribution of genetic variation within a species, values of F_{ST} can be used to measure the rate of migration (the proportion of individuals that enter a population as migrants). Of course only individuals that actually reproduce once they have entered the population can have an effect on gene flow and genetic variation and

thus the term effective number of migrants is used (N_em). Since migration brings gametes that originated outside the sampled population into the sample it lowers F_{ST}. If both genetic drift and migration are occurring among a large group of populations F_{ST} will approach an equilibrium value:

$$F_{ST} \approx \frac{1}{4N_em+1}$$

where N_e is the effective size of each population and m the proportion of migrants.

Wright's formulae for the estimation of F-statistics have been criticised by Nei (1977) because "it is not clear whether the same definition applies in the presence of selection and migration particularly when there are multiple alleles" (F_{ST} is strictly applicable to only a diallelic locus). Wright also assumed that there was an infinitely large number of subpopulations when in fact the number is often small. A number of alternative formulae have been proposed, perhaps the most useful being Nei's (1973) gene diversity measures. Nei (1973) showed that the total gene diversity could be partitioned into the diversity within and among subpopulations when gene diversity is defined as the heterozygosity expected under Hardy-Weinberg equilibrium. Gene diversity analysis makes no assumptions about the pedigrees of individuals, past selection or migration or the number of subpopulations. Nei's G_{ST}, which is a multiallelic alternative to F_{ST} (Nei, 1973), was then defined as the ratio of the gene diversity within a local population to that of the total population and is designed to be applied to the average gene diversity for a large number of loci among a finite number of subpopulations (Nei, 1977).

Neigel (1994) describes F_{ST} as a "somewhat abstract definition"..."useful as a standard basis for comparison of different methods" and therefore suggests F_{ST} should be maintained as a purely theoretical demographic parameter, that is, a "correlation between gametes" and G_{ST} as the statistical calculation of this from genetic data. Since genetic data are used to estimate G_{ST} which is itself a calculation of F_{ST} then accurate estimates of F_{ST} from different data (e.g. allozymes and DNA sequence) should agree, thus making it possible to take independent measurements of F_{ST} and relate them to the underlying demographic processes.

1.5.2.3 Allozyme electrophoresis in population genetics

Harris' (1966) and Lewontin and Hubby's (1966) description of extensive variation at allozyme loci provided the first evidence for widespread genetic variability within natural populations. Since then there has been a burgeoning of studies using the technique for examining aspects of the population biology of species. Early applications of the method were aimed at gathering a substantial quantity of data on the levels of variability within species in an effort to describe the quantity and significance of variability within species (Lewontin, 1974) and populations (Nevo *et al.*, 1984). The main thrusts of research in bivalve biology have concentrated on searching for population substructure (see for example, Buroker *et al.*, 1975; Ward, 1978; Beaumont, 1982a; Logvinenko *et al.*, 1982; Skibinski *et al.*, 1983; Grant and Cherry, 1985; Guinez and Galleguillos, 1985; Macleod *et al.*, 1985; Gang *et al.*, 1988; Fevolden, 1989; Von Brand and Kijima, 1990; Beaumont, 1991a; Borsa *et al.*, 1991; Galleguillos and Troncoso, 1991; Beaumont *et al.*, 1993; Saavedra *et al.*, 1993; King *et al.*, 1994; Leclair and Phelps, 1994; Igland and Nævdal, 1995), examining taxonomic relationships (see Thorpe and Solé-Cava, 1994 and references therein), correlating allozymic variability with physiological traits such as growth rate (Ward, 1978; Garton *et al.*, 1984; Zouros and Foltz, 1984a; Koehn *et al.*, 1988; Alvarez *et al.*, 1989; Volckaert and Zouros, 1989; Gaffney *et al.*, 1990; Beaumont, 1991c; Pogson and Zouros, 1994), examining hybrid zones and species complexes (Väinölä and Varvio, 1978; Koehn, 1991) and attempting to unravel the causes of heterozygote deficiencies that are often exhibited by bivalve populations (Skibinski *et al.*, 1983; Zouros and Foltz, 1984a,b; Mallet *et al.*, 1985; Foltz, 1986; Gaffney *et al.*, 1990; Beaumont, 1991c; Borsa *et al.*, 1991; Fairbrother and Beaumont, 1993; Gaffney, 1994).

1.5.2.4 Criticisms of allozyme methods

Although there are considerable benefits of allozyme methods they are certainly not without their problems. Firstly, allozyme electrophoresis suffers from low resolution such that an electromorph may actually consist of a number of variant alleles which may be indistinguishable due either to deficiencies in the technique used, inadequate scoring of genotypes (which can be subjective), or as a consequence of the redundancy of the genetic code. In attempts to atone for this problem of hidden variation, studies have been conducted on the efficacy of

various elaborations to the basic electrophoretic technique for aiding resolution of variants (reviewed by Johnson, 1977; Ayala, 1982; Coyne, 1982; Ayala, 1983). These include heat inactivation of polypeptides (*in vivo* or *in vitro*) prior to electrophoresis (Singh *et al.*, 1976), urea denaturation (Milkman, 1976; Ayala, 1982), sequential electrophoresis during which a combination of various buffers, buffer pHs, gel types or gel concentrations are used (Singh *et al.*, 1976; Aquadro and Avise, 1982; Beaumont and Beveridge, 1983), use of monoclonal antibodies (Slaughter *et al.*, 1981) and peptide mapping (Fletcher *et al.*, 1978; Ayala, 1982). Inadequate genotype scoring can only be addressed by adequate choice of loci for which alleles have been confirmed through breeding studies. Alleles at allozyme loci may also remain unobserved since the DNA sequence encodes a non-functional enzyme or sub-unit. Such "null-alleles" will consequently not be scored, leading variation to be missed and heterozygotes to be mis-scored with consequent effects on comparisons of genotype frequencies with Hardy-Weinberg expectations (1.3.2.1).

The problem of missed variation is more difficult to solve. Variation at "silent" positions in the DNA sequence cannot be detected with allozyme methods. Allozymes, being the phenotypic expressions of variability in the DNA reveal variability only through inferences from the primary gene products of loci. Much of the variability at the DNA level is invisible to allozyme electrophoresis for a number of reasons. Firstly 16 of the 20 amino acids are neutral in charge thus mutations resulting in interchanges may prove indiscernible. Undetectable variation may also result from the degeneracy of the genetic code. A mutation in the DNA sequence of a gene need not alter the transcribed amino acid sequence since each amino acid is coded for by more than one triplet. Thus, for example a mutation in the codon AGA specifying arginine, to AGG would not be detectable since AGG also specifies arginine. Such redundancy is particularly commonplace at the third position in a codon where there is often 4 fold degeneracy (i.e. the position can be occupied by any of the 4 nucleotides without changing the resultant amino acid). Neel *et al.* (1986) have estimated that approximately 1/3 of all amino acid substitutions in a polypeptide produce alterations in molecular charge detectable by

electrophoresis whilst ½ should be detectable by allozyme electrophoresis through charge shifts or detectable alterations in polypeptide conformation.

Secondly the loci under study may not be a representative sample of the genome. If this is so then problems will be caused for accurately assessing the level of variation across the genome as a whole (Lewontin, 1974) but it will not affect, the use of allozymes in searches for population substructure since it does not matter which loci are used, only that they reveal a true picture of any subdivision. Any locus which serves this purpose will suffice and it may only take the inclusion of a single locus to change the interpretation of a study's results (Beaumont, 1991a). The possible problem occurs because electrophoresis is undertaken in a buffer solution and allozyme detection methods rely on histochemical stains, thus the majority of studied loci encode soluble enzymatic proteins. Such loci make up only a small proportion of the eukaryotic genome (Powell, 1975) and thus are unlikely to be a random sample. It is far more difficult to examine genes for structural or regulatory proteins. A final problem with allozyme methods is that it is the protein products of genes, not the genes themselves that are being visualised as electromorphs on the gel and therefore it is the phenotype not the genotype which is studied. The allelic forms of the enzymes (allozymes) may be functionally different and may therefore be subject to selection. It can prove difficult to disentangle the effects of selection from those of other processes (Cabe and Alstad, 1994). Selection acting on loci is difficult to prove but strong selection can push a population out of Hardy-Weinberg equilibrium (see 1.3.2.1) at a locus therefore if disequilibrium is detected selection can be suspected. In some instances it has proven possible to show that selection was responsible for changes in gene frequency, for example, at the *Lap* locus of *Mytilus edulis* (Koehn *et al.*, 1980; Hilbish and Koehn, 1985) and *Gpi* of *Colias* butterflies (Watt, 1977; Watt *et al.*, 1983, 1985).

Despite these disadvantages, allozyme electrophoresis continues to be applied to multitudinous species, often in preference to other methods, primarily due to the speed and cost-effectiveness of data acquisition.

1.5.3 DNA based methodology

One major criticism of allozyme techniques is that with a few exceptions, assessable loci encode soluble enzymes. Methodologies which target the DNA directly should not be so constrained.

The genome of metazoan animals comprises a discrete set of chromosomes packed into the nucleus and a comparatively tiny DNA molecule in each of the mitochondria within the cytoplasm. Nuclear DNA (nDNA) is extremely complex, tightly bound with proteins and contains large quantities of non coding regions between genes (intergenic spaces) and also within genes (introns). Large segments of the genetically useful DNA are duplicated to form either repeated sequences or gene families which can differ in copy number, length, sequence composition and dispersion around the chromosomes. In contrast, the mitochondrial (mt) DNA of animals has a characterised and conserved organisation. Mitochondrial DNA is often selected ahead of nDNA because it is easier to extract and study by restriction fragment length polymorphisms (RFLP) methods, the traditional workhorse technique for studying variation, it is highly polymorphic and it is haploid. However mtDNA is a single locus whereas within nuclear DNA there is considerable choice of locus. The ability to study many independent loci is an important advantage of nDNA methods and may compensate for the slower rate of evolution of many nDNA loci in comparison with mtDNA. Choice of technique will have considerable bearing on choice of marker.

Methods for analysis of nDNA are reviewed by Kreitman (1991), Lessa and Applebaum (1993) and Ward and Grewe (1995).

1.5.3.1 Restriction fragment length polymorphisms

The second major breakthrough for molecular population genetics came with the discovery of type II restriction endonucleases in the study of RFLPs. These are enzymes isolated from certain strains of bacteria which cut DNA at distinct palindromic (typically) 4, 5 or 6 base pair sequences (Halford, 1983), for example, *Bam*HI isolated from *Bacillus amyloliquefaciens* H cuts the DNA wherever the sequence G↓GATCC occurs (cleavage occurring at the position of the arrow). Over 400 REs have been isolated which with their combinations of different cutting sequences should allow screening of a large portion of the genome for nucleotide variation. When used on different organisms, if different patterns of cleavage within

a DNA sequence occur (after resolution by electrophoresis different cleavage patterns are termed restriction fragment length polymorphisms or RFLPs) then a mutation (substitution, insertion, deletion or translocation) within the recognition

Figure 1.1: The effect of different kinds of sequence changes on restriction fragment length polymorphisms (RFLPs). (a) DNA fragments a-h are generated by restriction enzyme digestion, (b) separated using electrophoresis and (c) sizes determined using a calibration curve based on a marker with fragments of known size run alongside the samples (S=size standard). Vertical arrows indicate cleavage sites . Asterisks indicate boundaries of rearrangements. From Hillis and Moritz (1990).

sequence can be inferred (see Figure 1.1). Two patterns differ if they have at least 1 fragment of different mobility although usually patterns differ in the position of 3 bands due to the loss or gain of a cleavage site. For example if organism A has a RFLP profile consisting of a band of 800bp which does not occur in organism B for which bands of 450bp and 350bp are seen then the two patterns differ in the nature of three bands. This can be explained by the gain of a restriction site within organism B or the loss of the restriction site in A. Restriction patterns may differ by less than 3 bands due to migration of bands off a gel, coincident mobility of bands on a gel or deletion/addition within fragments. Having established fragment sizes from REs, restriction maps can be constructed indicating the positions of various enzyme cut sites on a DNA sequence. Double digests (the use of two different restriction enzymes together) are needed for this in which the number and sizes of fragments from double digests are compared with those from digestions using the enzymes on their own to calculate the position of restriction sites relative to each other on the stretch of DNA studied.

With the development of appropriate statistical techniques for the treatment of RFLP data, measures of relatedness at the DNA level can be estimated (Upholt, 1977; Lansman *et al.*, 1981; Nei, 1987; Roff and Bentzen, 1989; Lynch and Crease, 1990; Lynch, 1991). By applying a range of restriction enzymes to animals from different populations an estimate of divergence at the DNA level can be ascertained.

1.5.3.2 Direct staining of RFLP patterns

DNA separated electrophoretically can be stained with fluorescent stains such as ethidium bromide. When restriction digested nuclear DNA is separated and stained then because of the shear size of the nuclear genome (millions of bp) it is extremely likely that restriction enzymes with 4-6bp recognition sequences will cleave the nuclear genome hundreds of times and the underlying RFLPs will consequently appear as an uninterpretable streak due to the continuum of various sizes of bands from multitudinous loci. Direct staining can therefore only be applied to situations where a defined stretch of DNA is studied. Mitochondrial DNA is ideal for this. Because mtDNA exists in many copies per cell, sufficient quantity can usually be isolated such that restriction fragments can be detected directly in the gel. Once the DNA has been cleaved then the resultant fragments are separated using gel

electrophoresis through either polyacrylamide or agarose gels, the concentrations of which can be varied depending on the DNA fragment sizes being separated. Polyacrylamide gels in general offer higher resolution. Following electrophoresis the fragments are visualised using fluorescent dyes such as ethidium bromide that intercalate with the DNA and fluoresce under UV light. Fragment sizes are determined by comparison with size markers such as the *Hin*dIII cut DNA of phage λ. Unfortunately ethidium bromide staining requires large quantities of mtDNA per digest in order for fragments to be visible and the quantity required increases with the number of bands present. Where the amount of DNA is limited an alternative staining method may have to be sought. Hoechst dyes (Wilson Jr. and Tringali, 1990) bind only to DNA and not to RNA and so visualisation is easier, whilst silver staining (Tegelstrom, 1992) requires less DNA due to higher specificity.

1.5.3.3 Blot hybridisation

While allozyme methods allow analysis of the expressed DNA variability at certain protein coding loci, the ability to examine DNA directly potentially permits study of all areas of the genome including non-coding sequences. Direct staining is inappropriate and a method of studying genetic variation at particular loci is required. This is achieved through hybridisation with specific probes, typically using a variant of the classic Southern blot (Southern, 1975). After cleavage with restriction enzymes and agarose electrophoresis the gel separated DNA is transferred onto a membrane (nitrocellulose or more usually charged or uncharged nylon) by capillary action in denaturing buffer (Southern, 1975; Chomczynski, 1992) or vacuum blotting. Once the DNA is fixed to the membrane, a specific labelled (radioactive or non-radioactive) probe is hybridised to reveal the variation at a single locus. After a stringent wash to ensure that the probe hybridises only to complementary sequence on the filter, the underlying RFLP variation can be revealed by exposure to X-ray film. Probes can take a number of forms and repeated hybridisation of the same blot with a number of different probes allows many independent DNA polymorphisms to be studied from a single filter.

In mtDNA studies where insufficient mtDNA is available for direct staining, for instance where the studied organism is extremely small, then blotting may be required. An alternative to hybridisation is to radioactively label the fragments, transfer

to a membrane then reveal RFLPs by autoradiography (Blake and Graves, 1995). Alternatively a radiolabelled, cloned mtDNA can be used to hybridise with the fragments on the gel (e.g. Skibinski and Edwards, 1986; Edwards and Skibinski, 1987; Bachmann and Sperlich, 1991). If cloning is required then the whole molecule can be inserted in a phage or plasmid and used in contrast to nDNA loci in which single loci require isolation prior to cloning.

1.5.3.4 PCR

The latest and possibly most significant development for population studies is the polymerase chain reaction (Mullis *et al.*, 1986; Saiki *et al.*, 1988; White *et al.*, 1989; Mullis, 1990; Bej *et al.*, 1991; Simon *et al.*, 1991; Newton and Graham, 1994), a method for the amplification of defined sequences from complex templates. This allows amplification of a segment of DNA potentially from anywhere within the genome which can then be sequenced (e.g. Beckenbach, 1991; Simon, 1991; Hoelzel and Green, 1992), treated with restriction enzymes (e.g. Bucklin and Kann, 1991; Saperstein and Nickerson, 1991; Boulding *et al.*, 1993; Chow and Inoue, 1993; Cronin *et al.*, 1993; Garnery *et al.*, 1993; Bartlett and Davidson, 1995; Bouchon *et al.*, 1994) or used as a probe.

PCR involves repetitive cycles of denaturation, hybridisation and polymerase extension (Figure 1.2). The template DNA molecule can take a number of forms; a discrete molecule, for example, the mtDNA, or a sequence embedded in a much larger complex such as part of the nDNA, single stranded DNA or even RNA. This flexibility is a result of the specificity of the primers used. When a particular gene is identified for study, primers can be designed to flank this gene so that only it is amplified, the primers "ignoring" everything else. Although it may seem that prior sequence information must be known to design the primers, this is not necessarily the case. Since many sequences are conserved between species then either primers used in other studies can be utilised or they can be designed from known sequences such as for mtDNA genes (Kocher *et al.*, 1989). If non-conserved areas of the genome are to be amplified then primers based on close, conserved areas are used, for example, for the mtDNA D-loop, primers

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Figure 1.2: The polymerase chain reaction. a primers (P) anneal to denatured template DNA (T1 and T2). b The reaction is cycled between denaturing, annealing and extension temperatures. c Repeated cycling results in an exponential amplification of the target sequence. From Hoelzel and Dover (1991).

based on the conserved t-RNA genes can be used to flank the region for example Beckenbach (1991) used primers based on human tRNA genes to amplify the control region of stickleback mtDNA. Primers are designed to fit around the gene and cause initiation of strand synthesis. As a consequence of base specificity these bind only where the sequence closely matches that of the DNA to be amplified. With two primers around the gene on opposing strands double stranded copies of the desired sequence can be made and once a new strand is synthesised it then itself becomes a template and thus there is an exponential generation of DNA. A particular thermal cycle is used so that the double stranded DNA is first separated into single strands at 95°C then cooled to 50°C to allow primers to anneal. Due to their small size and high concentration they quickly find their target sequence. Extension then occurs at the optimum for the DNA polymerase (72°C). *Taq* DNA polymerase (from the bacterium *Thermus aquaticus*) is used (Gelfand and White, 1990). As this is isolated from a thermophilic bacterium, it can withstand the high temperatures required for strand separation and so does not require to be replaced after every cycle. After 20-40 cycles enough DNA will have been generated for most applications and because of the exponential increase in DNA only small initial quantities are needed. Extraction of DNA from tissues is also simplified since a crude DNA preparation is sufficient. Small amounts of tissue are sufficient, for example, larvae (Olson *et al.*, 1991; Geller *et al.*, 1994; Hu and Foltz, 1994; Coffroth and Mulawka III, 1995) and non-destructive sampling can be performed where small samples are taken off an individual before release, for example, fish scales (Whitmore *et al.*, 1992) blood samples (Wayne *et al.*, 1990; Noone *et al.*, 1994) or human hairs (Vigilant *et al.*, 1989; Wilson *et al.*, 1995). The small quantities of tissue required coupled with the primer specificity also allows DNA extraction from dead, partially decayed or preserved samples in which much of the DNA is degraded (Higuchi *et al.*, 1984; Higuchi and Wilson, 1984; Williams, 1995). Higuchi *et al.* (1984) showed that mitochondrial DNA from preserved museum specimens could be extracted and sequenced but was only present in 1% of the amount of that in fresh tissue (although this is not a problem for the PCR). The sequences obtained showed little evidence of post mortem sequence change. The main disadvantage of PCR stems from its power to amplify. Even minute quantities of contaminating DNA may be

amplified and every effort must be taken to prevent this from occurring (Orrego, 1990; Cone and Fairfax, 1993).

1.5.3.5 Sequencing

RFLP studies only allow inference of the nature of sequence changes that have taken place. The type of sequence change responsible cannot be known. Through sequencing of DNA, the ultimate genetic information is obtained, that is, the nucleotide sequence of the DNA. The most common sequencing protocol in use is Sanger *et al.'s* (1977) di-deoxy termination method. In this protocol the DNA being sequenced is rendered single stranded and labelled radioactively using ^{32}P, ^{33}P or ^{35}S (for the relative merits of these see Hengen, 1994). The DNA strands are then extended from a primer using DNA polymerase. Elongation will continue as long as 3′ hydroxyl groups (dATP, dCTP, dGTP and dTTP) are available and the addition of these will leave a 3′ hydroxyl group available for continued strand synthesis. However di-deoxy nucleotide tri-phosphates (ddNTPs) lack a 3′ hydroxyl group and thus if incorporated into the growing DNA strands will act as chain terminators. Therefore if a reaction is set up containing all 4 dNTPs and 1 ddNTP during which the ssDNA of interest is primed by a unique primer, 5′→3′ strand elongation will ensue to yield a mixture of molecules with common 5′ ends but different 3′ ends depending on the site at which the ddNTP was incorporated. The ratio of dNTP to ddNTP is important since excess ddNTP will stop elongation immediately whilst with too little there will be few terminations and consequently too few labelled fragments for resolution on the sequencing gel. If 4 separate reactions are performed containing the same DNA and primer but differing in the ddNTP then the size of each fragment in the mixtures of molecules will be determined by the sequence of the template. If the products of the four sequence reactions are run at the same time in adjacent lanes of a polyacrylamide gel with a capacity to resolve down to a single base pair then at each position in the DNA chain the ddNTP responsible for chain termination can be determined and thus the sequence of the DNA read (see Figure 1.3). Single stranded DNA (ssDNA) is created by either denaturing plasmid DNA, isolating a fragment of interest from a gel and cloning into bacteriophage M13 DNA or using the PCR. If PCR products are to be sequenced then ssDNA can be created by following PCR amplification with a second

round of PCR using unequal primer concentrations (Gyllensten and Erlich, 1988). Alternatively PCR can first be performed using a phosphorylated primer in the amplification. After amplification, λ exonuclease, a 5′→3′ exonuclease is used to digest the strand for which amplification was initiated with the phosphorylated primer. The enzyme requires a terminal 5′ phosphate to initiate digestion and following incubation, leaves ssDNA for sequencing (Kreitman, 1990).

Figure 1.3: Summary of the basis of di-deoxy sequencing. From Hillis *et al.* (1990).

1.6 Markers suitable for population level studies

Since the complete genotype of most diploid organisms is encoded by many thousands of base pairs (bp) it would be unrealistic to attempt to study any significant portion of this from even a single individual and regions of DNA must therefore be selected for study. Due to variations in selective constraints and in the case of comparisons between mtDNA and nDNA, in ploidy level, inheritance and effective population size (see below), various regions of DNA appear to evolve at differing rates (Kreitman, 1991; Avise, 1994). Since there are different rates of evolution within the genome, regions can be chosen for study to address particular questions. For instance highly polymorphic nuclear VNTR (variable numbers of tandem repeats, see below) loci are useful for examining kinship and paternity but are far too variable for inter-specific comparisons and probably for population level questions. Rapidly evolving genes may also accumulate mutations too rapidly for population studies since if a region of DNA rapidly accumulates mutations then at some point nucleotides that have already mutated may mutate again to a different form, or through back mutation, to the original nucleotide. If this has occurred then differentiation of two sequences may be underestimated and consequently misleading. When this occurs the DNA is said to be saturated with mutations (Meyer, 1994). Because of the redundancy of the genetic code, third codon positions in rapidly evolving, functionally unconstrained genes may be prone to saturation particularly for sequence comparisons from widely diverged organisms.

At the other extreme some genes such as the nuclear 18S rRNA appear to be exceptionally functionally constrained and evolve extremely slowly. They are thus useful for examining relationships among higher taxa but of little or no use for population comparisons as the variability is simply too low. Table 1.1 details the potential utility of a variety of markers.

1.6.1 scnDNA loci

Single copy nuclear DNA (scnDNA) loci, that is, those sequences that occur only once per haploid genome (as opposed to repetitive DNA which exist as multiple copies of a repeat unit) are studied using either restriction analysis, blotting and probe hybridisation or through a PCR approach. Useful probes consist of either cloned genes of known function (e.g. various genes used in studying variation

within *Drosophila*, Loukas *et al.*, 1986; the haptoglobin gene in *Spalax* mole rats, Nevo *et al*, 1989; Alcohol dehydrogenase in *Drosophila*, Simmons *et al.* 1989), randomly selected clones from a genomic library (Bhattacharya and Druehl, 1989; Brock and Christiansen, 1989; Glenn Hall, 1990; Wirgin and Maceda, 1991) or randomly selected clones derived from reverse transcription of inserts from a mRNA library (known as cDNA or complementary DNA; Pogson, 1994). Pogson (1994) found that random selection of probes from a cDNA library revealed both useful scnDNA markers and VNTR loci (1.4.2). The advantage of using probes from a genomic DNA library as opposed to a cDNA library is that probes for non-transcribed and flanking sequences, probably of low or no selective value may be uncovered.

Analysis of scnDNA variation through a PCR approach removes the necessity for hybridisation and blotting. Karl and Avise (1992) developed PCR primers for scnDNA of use for green turtles (*Chelonia mydas*) and American oysters (*Crassostrea virginica*). Fragments of restriction digested DNA in the 500-5000bp size range were cloned and the intensity of hybridisation signal with labelled genomic DNA used to ascertain if single copy or repetitive DNA had been cloned. Inserts of scnDNA were sequenced and primers designed which were then used to amplify anonymous scnDNA loci for treatment with restriction enzymes. Although the method required substantial work for development of the technique, following this, many individuals can be analysed for variation and because of the defined nature of the amplified fragments length variation can be easily distinguished from restriction site variability.

The non-coding sequences present between genes also serve as potentially useful markers. Côrte-Real *et al.* (1994) developed primers for the intron of the calmodulin gene situated on the exons of the gene. Because the sequence of the gene sections spanning the intron is essentially evolutionary conserved the primers were shown to work on a variety of organisms. In *Mytilus edulis* the primers amplified two alleles of differing lengths the frequencies of which was shown to vary among populations.

1.6.2 VNTR loci

VNTR or variable numbers of tandem repeat loci are widespread throughout the

Table 1.1: Qualitative comparisons of several common molecular methodologies used to examine different classes of DNA. 'Tissue requirements' refers to constraints upon tissue collection for that method: stringent reflects a number of constraints (e.g. large amounts of tissue, multiple tissue types, high molecular weight DNA etc.), relaxed indicates few constraints. NS indicates a technique is not generally suitable for that application. From Park and Moran (1995).

genome. Alleles for such loci can differ in length due to variation in the numbers of a repeat unit. The two classes of VNTR loci, minisatellite and microsatellite loci are commonly used in studies at the individual and population level because of their high variability (Burke *et al.*,1991; Wright, 1993). Variation is often so extensive that a combination of loci can reveal an individual-specific "DNA fingerprint".

1.6.2.1 Minisatellites

Minisatellite loci consist of tandemly repeated motifs, typically 15-100bp in length. Because different loci often exhibit similarity in the sequence of the repeat, multilocus variation can be examined by probing filter-bound restriction enzyme treated DNA with a probe containing a common core sequence. A restriction enzyme that has no cleavage site within the repeat is used to cleave genomic DNA which is then submitted to electrophoresis, Southern blotted and hybridised to a probe. Jeffreys *et al.'s* (1985) human multilocus minisatellite probes (33.5, 33.6, 33.15) have been useful in examining population structure, for instance in birds (Burke and Bruford, 1987). Alternatively probes can consist of multiple copies of a typical core sequence such as $(CAC)_5$ used at low stringency. The overriding difficulty with the use of multi-locus probes is interpretation of the complex profile (Prodöhl *et al.*, 1992) and particularly identification of homologous bands which causes difficulties for statistical analysis (Lynch, 1991). Human minisatellite probes can also be used to challenge the genomic DNA library of a species, identifying clones containing similar repeats which can then be isolated and used as single locus probes. Taggart and Ferguson (1990) successfully identified four hypervariable single locus probes for use on Atlantic salmon (*Salmo salar*) using such a method. The method for cloning and identifying suitable single locus probes has since been simplified (Prodöhl *et al*, 1994) and may be applicable to many other organisms. Synthetic sequences have also proved useful for detecting single-locus minisatellite variation. May *et al.* (1993) used a $(CCAT)_n$ sequence to detect variation in bottlenecked red kite *Milvus milvus* populations.

Other non-specific probes have been identified as useful for examining variation within populations (Zhang and Tang, 1993). For example the M13 phage contains a sequence detecting minisatellite variation in humans and animals

(Vassart *et al.*, 1987) and has proved to reveal population specific variation in striped bass *Morone saxatilis* (Wirgin *et al.*,1991).

1.6.2.2 Microsatellites

Microsatellites are short stretches (tens to hundreds of bp) composed of tandemly repeated 1-4bp motifs occurring ubiquitously in many organisms (Wright and Bentzen, 1995). Microsatellite loci are typically studied through a PCR approach (Rassmann *et al.*,1991). Size fractionated (<800bp) fragments of nDNA produced by restriction digestion are first cloned, then the resultant colonies challenged with radioactively labelled probes containing typical core microsatellite sequence, for example, $(GT)_n$ or $(GA)_n$. Clones showing a positive signal are isolated, sequenced and from the sequence flanking the repeat unit, PCR primers are designed such that microsatellites can be amplified from individuals and the size of the locus and hence number of repeats determined by electrophoresis of PCR products on a sequencing gel.

Because of their ubiquity and high variability, microsatellites can prove to be particularly useful markers for population analyses (Scribner *et al*, 1994). They are particularly suited for examining variation in species which have little variability for other markers e.g *Bulinus truncatus* (Jarne *et al.*, 1994), inbred species, recently separated populations which exhibit little divergence for other markers or for pedigree analysis (Wright and Bentzen, 1995). Microsatellites have exhibited evidence of geographic differentiation for cod *Gadus morhua* (Wright and Bentzen, 1995) and Atlantic scallop, *Placopecten magellanicus* (E. Kenchington, Marine Gene Probes Lab, Dalhousie University, Halifax, Nova Scotia pers. comm.) species for which allozymes and mtDNA had not exhibited differentiation and for trout *Salmo trutta* (Estoup *et al*, 1993).

1.6.3 rDNA

Nuclear ribosomal DNA (rDNA) consists of tandemly repeated units composed of the genes for the 18S, 5.8S and 28S rRNA separated by transcribed and non transcribed spacer regions (Figure 1.4). Because the rDNA genes are functionally constrained and slowly evolving they are of little use for population comparisons. However the internal transcribed spacer (ITS) regions separating the individual genes should be selectively neutral and therefore potentially a useful marker for

population studies. A potential problem is that polymorphisms may exist between repeat units within a single individual. However rDNA units are believed to evolve by a process of concerted evolution (Schlötterer and Tautz, 1994) resulting in homogeneity of individual repeat units. Mechanisms such as unequal crossing over and gene conversion have been proposed for the process of concerted evolution (Vogler and DeSalle, 1994).

Figure 1.4: Organisation of nuclear ribosomal DNA units (a) rDNA is tandemly repeated. (b) a single repeat consists of the genes for the 18S, 5.8S and 28S rRNA separated by external transcribed spacers (ETS), intergenic spacer (IGS) and (c) internal transcribed spacers (ITS). From Schlötterer and Tautz (1994).

Studies of rDNA variation can proceed using a rDNA unit or individual rDNA genes as probes or by sequencing individual regions. Studies of individual genes is usually constrained to taxonomic comparisons due to the slow evolution of these genes e.g. Tillier *et al.* (1992) used sequences of the 28S rRNA to construct a phylogeny of the Gastropoda.

Sequence variation in the rDNA ITS region has previously been successfully used in both systematics and population level studies (Schlötterer and

Tautz, 1994; Schlötterer *et al.*, 1994, Vogler and DeSalle, 1994). Alternatively RFLP studies of the whole unit can be undertaken. Using a cloned *Drosophila* rDNA as a probe of double enzyme digested DNA, Cutler *et al.* (1991) found evidence for differences between Newfoundland and Scottish salmon and in interspecific comparisons of freshwater snails, differences between species were detected using rDNA RFLPs (Rollinson and Kane, 1991).

1.6.4 Random amplified polymorphic DNA

Random amplified polymorphic DNA or RAPD (Williams *et al.*,1991; Hadrys *et al.*, 1992) is an extension of the PCR technique utilising short primers of arbitrary sequence to amplify anonymous regions of the genome. The primers, usually 10bp long (decamers) are used under low stringency conditions. Due to the small size of the primers there is a high probability that the genome will contain multiple priming sites, close to one another and in inverted orientation on opposing strands. The intervening sequence will then be amplified and when this occurs at numerous sites then the amplified products of various sizes will be generated that can be separated by gel electrophoresis. Fragment numbers and sizes are calculated and compared among organisms using fragment sharing indices to quantify similarity (Kambhampati *et al.*,1992).

RAPD methods have been criticised on a number of grounds. Firstly because the technique is often irreproducible and very sensitive to reaction conditions (Ellsworth *et al.*, 1993; Muralidharan and Wakeland, 1993) and parallel experiments in different laboratories using supposedly identical conditions can give different results (Penner *et al.*, 1993). Despite this, with careful optimisation and standardisation of reaction parameters this problem can be reduced (Bielawski *et al.*, 1995; Patwary *et al.*, 1994). Secondly, RAPDs segregate as dominant markers therefore homozygotes and heterozygotes are indistinguishable, thus RAPD data cannot be used for testing random mating (against Hardy-Weinberg expectations). Thirdly homology of comigrating fragments is difficult to prove without additional tests and this can affect band sharing indices. The overriding advantage of RAPDs is that no prior sequence knowledge is required since primers are arbitrary, thus all primers are potentially of equal use in all species. RAPDs have proved to be useful population markers in *Aedes* mosquitoes (Ballinger-Crabtree *et al.*, 1992;

Kambampathi *et al.*, 1992), grasshoppers (Chapco *et al*, 1992) and fruitflies, *Ceratitis capitata* (Haymer and McInnis, 1993). No population structure was evident when this techniques was used for differentiating scallop, *Placopecten magellanicus* populations (Patwary *et al*, 1994). It is also useful for differentiating at higher taxonomic levels such as subspecies of *Littorina* (Crossland *et al.*, 1992) and coral larvae (Coffroth and Mulawka, 1995).

1.6.5 Mitochondrial DNA

1.6.5.1 Structure

The mitochondrial DNA is a circular, double stranded molecule of deoxyribonucleic acid present at high copy number of around 10 molecules per organelle in humans, thus in a typical human cell there are approximately 8000 mtDNA molecules, whilst in the mitochondria rich eggs of *Xenopus* there can be up to 10^8 molecules per cell (Avise and Lansman, 1983). The two strands of the molecule differ in the higher proportion of the bases guanine and thymine in the heavy (H) strand as opposed to the opposing light (L) strand (Meyer, 1993). MtDNA carries the genetic information for 22 transfer (t) RNAs, 2 ribosomal (r) RNAs (the 12S and 16S sub-units) and 13 proteins involved in the respiratory transport chain; 7 sub-units of the NADH dehydrogenase complex (ND 1-6 and 4L), cytochrome b, 3 sub-units of cytochrome c oxidase (Co I, II and III) and two sub-units of ATP synthetase (ATPase 6 and 8). Most of these genes are encoded by the H strand with only ND6 and 8 tRNAs on the L strand, although in *Mytilus edulis* all the genes are on the H strand alone (Hoffmann *et al.*, 1992). Virtually all organisms studied so far have these genes and these genes alone in their mtDNA with only a few exceptions (e.g. Batuecas *et al.*, 1988; Hoffmann *et al.*, 1992). This genetic complement is far fewer genes than would be needed for autonomous function of the mitochondrion and so there is still heavy reliance on substances carried in from the cytoplasm to the organelle which have been translated from nuclear (n) DNA loci (Glick and Schatz, 1991). Figure 1.5 depicts the structure and organisation of a variety of species' mitochondrial genomes.

The gene order of most organisms is typically conserved (allowing phylogenetic inference from gene order comparisons (Sankoff *et al.*, 1992; Boore and Brown, 1994). There do seem to have been a number of instances of genome reorganisation during invertebrate evolution typically due to transposition of the tRNA genes although

Figure 1.5: Mitochondrial DNA gene arrangements of 3 animal species: *Caenorhabditis elegans* (13,794bp), *Drosophila yakuba* (16,019kb) and *Mus musculus* (16,295kb) from Wolstenholme (1992b). Transfer RNA genes are identified by single letter amino-acid codes. Protein genes: COI-COIII (cytochrome c oxidase subunits); ND1-6 and 4L (NADH dehydrogenase subunits); ATP6 and 8 (ATPase subunits). s-rRNA and l-rRNA (small and large ribosomal subunits). Genes that are transcribed in an anti-clockwise direction are identified by a bent tick and a thick line within the gene box, all other genes are transcribed in a clockwise direction as indicated by the central arrow. The origin of replication of the *M.musculus* and *D.yakuba* mtDNA are indicated by the arrow (o) in the control (C) and A-T rich (A+T) regions respectively.

some changes have been reported in more conserved regions (e.g. see Hyman *et al.*, 1988).

With the exception of length variable regions (see 1.6.5.2) there is little intergenic space in mtDNA (save in *Mytilus*) or non-coding sequences within mtDNA. For example the mtDNA of humans is 16,569 bp long of which 15,368 bp are taken up by genes, leaving only 1201 bp of non-coding sequence part of which is taken up by the replication origins. Only 87 bp are apparently genetically unimportant (Brown, 1990b). The organisation of this compact molecule is such that genes frequently abut directly

without intergenic space and some genes even overlap, for example, ATPase 6 overlaps ATPase 8 (Grivell, 1983).

MtDNA is also unusual in that the genetic code differs from that in nDNA (Kurland, 1992), for example, AGA specifies arginine in nDNA but methionine in the mtDNA. Codon differences can occur between organisms such as AAA in starfish which specifies asparagine and not lysine as in most mtDNA (Batuecas *et al.*, 1988).

MtDNA structure and evolution is reviewed by de Giorgi and Saccone (1989), Wolstenholme (1992a,b) and Wolstenholme *et al.* (1990).

1.6.5.2 Length variation

Typically, metazoan mtDNA molecule is around 16.5 kilobase pairs (kb) long (in plants of the squash and melon family the mtDNA can be up to 2.4 million bp whilst most flowering plants have a length around 2000 kb, Grivell, 1983). The short, invariable size, uniform gene content, constant gene order and absence of introns reported in early studies led Attardi (1985) to describe the molecule as "an extreme example of genetic economy". However more recent research in which a diverse range of organisms has been studied has shown that the length is far more variable. There continue to be more accounts of atypically sized mtDNA in animals from virtually all taxa. Mitochondrial DNA length varies among species from different families, for example, mtDNA length ranges from 12 kb in the spider mite (Fournier *et al.*, 1994) to 41 kb in the sea scallop *Placopecten magellanicus*, the largest animal mitochondrial genome as yet recorded (Gjetvaj *et al.*, 1992), among species within families, for example, size variation in the family Pectinidae (Gjetvaj *et al.*, 1992) in which the presence and magnitude of variation ranges from a size invariant 16.2kb molecule within *Argopecten irradians* to the mtDNA of *P. magellanicus* ranging from 31-41 kb, within species (e.g. *P. magellanicus*) and within individuals (heteroplasmy). Length variations range from short additions and deletions to long repeated sequences although typically extensive length variation has been restricted to invertebrates and lower vertebrates and is rarely seen in higher mammals (Rand, 1993). This has led to the hypothesis that homeotherms due to their greater need for an efficient respiratory system (part of which is translated from mtDNA genes) have tighter selective controls operating on length mutants than do poikilotherms (Meyer, 1993). Smaller size variation of a few base pairs are usually due to changes within homopolymer tracts, for example, addition of 1-

2 cytosines in a run of cytosines possibly due to replication slippage (Moritz *et al.*, 1987). Larger variations are due to additions, deletions or tandem repeated sequences bounded by characteristic flanking sequences, often able to take up specific configurations such as hairpins and cruciforms. These have been implicated in the generation of length variation (e.g. Rand and Harrison, 1989; La Roche *et al.*, 1990; Rigaa *et al*, 1995). *Cnemidophorus* lizards have 64 bp tandem repeats in the control region which are flanked by tRNA genes (Moritz and Brown, 1986) as are the 40 bp repeats of cod (Árnason and Rand, 1992). Nucleotides in a tRNA sequence are able to take up the typical cloverleaf structure and these may have a role akin to hairpins and cruciforms in the generation of length variation. The actual mechanism of generation of length variants has not yet been resolved but such structures have been suggested to cause replication slippage, although slippage mispairing may not be possible in mtDNA where each strand has a separate replication origin (Grivell, 1989). Recombination has also been suggested by Rand and Harrison (1989) as a length variation generating mechanism. However mtDNA seems to lack recombination except in the large mtDNAs of higher plants (Brown, 1990b), although Boulding *et al.* (1993) point out that DNA with high G + C content (as in some areas of the mtDNA) has been associated with high amounts of recombination. Buroker *et al.* (1990) put forward the "illegitimate elongation model" for the generation of length variation in sturgeon (*Acipenser transmontanus*) mtDNA. In this species up to 42% of individuals are heteroplasmic for up to 7 different mtDNA size classes (Brown *et al.*, 1992) although variant mtDNA molecules within an individual never differ by greater than 1 repeat unit length. There was no evidence of restriction site heteroplasmy, suggesting that length variation and heteroplasmy was due not to paternal inheritance, but to a high rate of recurrent mutation promoted by hairpin formation of the D-loop strand, working against a tendency for elimination of variants through rapid segregation. The model involved the conserved sequence blocks (CSB) a short, characteristic conserved sequence of the mtDNA and the termination associated sequences (TAS) found in many D-loop sequences. Displacement (D) loops are the origin of H strand replication and occur where a small piece of DNA (the D-loop DNA) complementary to the L strand has displaced the H strand. Buroker *et al.* (1990) noted that in sturgeon, the D-loop DNA could go from the CSB downstream to any one of the multiple TASs

resulting in multiple possible D-loop lengths. The resting state of the D-loop is triple stranded with the D-loop DNA and the H strand competing for base pairing with the L strand. It was hypothesised that the D-loop may occasionally be partially displaced by the H strand and subsequently reanneal in an misaligned fashion onto a different TAS. Then in order to compensate for this the H and L strand would be required to expand or contract in length. But although their model fitted their data very well it would be unable to generate the length variation seen in the cod, *Gadus morhua* (Árnason and Rand, 1992). It therefore seems possible that the generative mechanism is not universal.

One other unresolved issue relating to length variation is whether variants are selectively neutral and this has implications for the use of length variation in population subdivision (see below). It might be thought that repeated sequences in non-coding regions would be disadvantageous since usually no genes are multiplied and the only consequence is an increase in time and energy required for replication. The resulting "race for replication" would be won by smaller variants. This hypothesis is addressed by Rand (1993). One *pro* selection argument is that in cases where there are varying copy numbers of a repeated sequence, the most common number is not the highest or the lowest but an intermediate one suggesting a trade off between selection (for increased copy number) and replication time (Brown *et al.*, 1992). Zouros *et al.* (1992b) attempted to resolve this issue by comparing increasing copy number of one of the three repeated sequences in the mtDNA of *Placopecten magellanicus* with a measure of growth rate. Animals from the same cohort were grown up and the number of repeated sequences counted using RFLP. This was then compared against shell length to see if there was a relationship. They found a positive correlation between shell length and allozyme heterozygosity but no correlation with copy number or degree of heteroplasmy thus suggesting selective neutrality of length variants.

1.6.5.3 Rapid evolution of mtDNA

Because of the lack of "unimportant" non coding DNA within the mitochondrial genome and since the products of the genes play such a vital role (respiration) it would be expected that mutations of the mtDNA would be rapidly selected against, thus the rate of mtDNA evolution should be low. However early studies by Brown *et al.* (1979) using DNA-DNA hybridisation and RFLP cleavage maps found, unexpectedly,

that mtDNA evolves some 5-10 times faster in primates than does single copy (sc)nDNA, at a rate of 2% nucleotide differentiation per 10^6 years. Brown (1983) suggests a number of possible reasons for this including replication error, short turnover time with more rounds of replication and thus greater chance of error, exposure to mutagenic mitochondrial respiratory waste products (e.g. free radicals) and high mtDNA copy number allowing tolerance of mutations. The rate may actually be higher as a consequence of the fact that mtDNA does not code for genes involved directly in its own replication, transcription or translation and thus has a relaxed functional constraint. There have also been suggestions that scnDNA evolves slower than mtDNA because it is tightly bound by histones which are themselves constrained whilst mtDNA is naked. Replication error was demonstrated by Kunkel and Loeb (1981) who showed that DNA replication in the mitochondrion which is undertaken by a different enzyme to that involved in replication of nDNA is far less accurate. However if replication error was the only cause of elevated evolution it would be expected that all genes would evolve at similar rates and this apparently is not the case.

Brown *et al.'s* (1979) rate of DNA evolution has often been used as a molecular clock being applied to many taxa without evidence of its applicability. Brown (1983) stated, "the rate of mtDNA evolution could vary considerably among taxa". Vawter and Brown (1986) compared mtDNA and scnDNA evolutionary rates and found them to be equal in sea urchins, although this was believed to be due to an elevation of scnDNA evolution as opposed to a decrease in the mtDNA rate. It has now been demonstrated that mtDNA does evolve much slower (10x slower) in sharks (Martin *et al.*, 1992) testudinates (Bowen *et al.*, 1989) and possibly humpback whales (Baker *et al.*, 1990). Evidence that mtDNA evolution in poikolotherms is slower than ectotherms is reviewed by Rand (1994). It is therefore evident that a full reappraisal of mtDNA evolution is required in a variety of taxa if times of divergence *etc.* are to be estimated accurately from mtDNA sequence divergence data. Evolutionary rate also varies within the molecule with particular genes having their own particular rate of evolution (Cann *et al.*, 1984). This means that if individual regions of the mtDNA can be isolated then a section evolving at a rate applicable to a particular study can be chosen (Cracraft and Helm-Bychowski, 1989). For example if distantly related taxa are being compared then slowly evolving regions (e.g. the 16S rRNA gene)

can be picked in which saturation of mutations will likely not have occurred. For intraspecific population surveys rapidly evolving areas such as the D-loop or ATPase 6 can be used where mutations may have been able to accumulate to detectable levels. Such intramolecular variation in evolutionary rate is suggestive of the action of some form of gene dependent selective processes. Mitochondrial DNA evolution is reviewed by Brown (1983) and Moritz *et al.* (1987).

1.6.5.4 Maternal inheritance

Following initial studies it was believed that all mtDNA molecules in an organism were identical and passed solely from mother to offspring with no contribution from the father (maternal or clonal inheritance). Thus each mitochondrial clone which is effectively isolated from all others accumulates mutations independently and preserves its mutational history, enabling the use of mtDNA in deduction of phylogenies (Avise, 1986). In contrast, due to recombination and linkage, nDNA and hence electrophoretic alleles provide a more ambiguous picture of relatedness. A further consequence of this non-Mendelian inheritance is a lowering of the effective population size (N_e). Since mtDNA is haploid and transmitted only through the mother whilst nDNA is diploid and bisexually inherited then assuming a sex ratio of 1:1, N_e for mtDNA is ¼ of that of nDNA. The consequence is that given equal migration rates for both sexes, four times as much migration between populations is needed to prevent divergence in genetically similar populations and four times as much is required to erode any differences in mtDNA haplotype frequencies when compared to nDNA loci (Billington and Hebert, 1991). Indeed nuclear genes can be effectively panmictic whilst mitochondrial genes appear subdivided (Birky Jr. *et al.*, 1989). Bottlenecks will also be far more pronounced in the mtDNA than the nDNA and rare mtDNA variants may even become fixed during severe bottlenecks (Wilson *et al.*, 1985).

Maternal inheritance was first demonstrated by Hutchison *et al.* (1974). Then experiments by Lansman *et al.* (1983) were performed in which tobacco budworm (*Heliothis*) females were back crossed against males for 93 generations with the aim of amplifying any paternal contribution to a detectable level. These tests failed to detect any male mtDNA in tests that would have been able to pick up a 5% (after amplification) paternal contribution. Experiments using the polymerase chain reaction which allows for lower levels of detection were able to pick up a 0.01% input of

paternal DNA in mice (Gyllensten *et al.*, 1991). More recently Zouros *et al.* (1992a) have provided convincing evidence for high levels of paternal transmission of mtDNA in *Mytilus*. *Mytilus edulis* and *M.trossulus* (species with obviously different mtDNA RFLP patterns (allowing the differentiation of paternal from maternal mtDNA) were crossed and mixed probes (containing radiolabelled DNA from both species to hybridise with the studied mtDNA) used to detect fragments on a blot. Evidence of biparental inheritance was seen in 40% of the families used in the crosses, with the level of paternal contribution estimated at 10%, a value far higher than that seen previously in mice. Avise (1991) had previously noted that in instances where paternal inheritance has been demonstrated the experimental models have involved interspecific crosses. Although this was necessary in order that the mtDNA of the male was distinguishable from any low levels of heteroplasmic mtDNA that the female may contain it does raise the question as to whether foreign mtDNA escapes the usual exclusion mechanisms and that strict maternal inheritance is indeed the norm. However in *Mytilus edulis*, Skibinski *et al.* (1994) have demonstrated that females are homoplasmic for a genome transmitted to eggs whilst males are heteroplasmic for both this form of mtDNA and a second form found only in males and transmitted preferentially to sperm. This male only mtDNA (designated the M genome) must have a paternal transmission route since females are not heteroplasmic for this genome. At three protein coding loci these two mtDNA molecules exhibit over 20% sequence divergence suggesting that heteroplasmy has existed long term in *Mytilus*. There is also evidence for limited paternal transmission of mtDNA in anchovies (Magoulas and Zouros, 1993). The detection of paternal transmission has severe ramifications for interpretation of gene trees which have been based on clonal inheritance, as well as for the study of sex mediated gene flow. Effective population sizes for mtDNA would also require reevaluation

The inference from maternal inheritance is that all the mtDNA in any one animal is identical (homoplasmy) and until recently this was believed to be the case for all organisms. However there are increasing numbers of accounts of heteroplasmy for length variants in a range of organisms (de Giorgi and Saccone, 1989; Buroker *et al.*, 1990; Fisher and Skibinski, 1990; Mignotte *et al.*, 1990; Hoeh *et al.*, 1991; Wilkinson and Chapman, 1991; Árnason and Rand, 1992; Brown *et al.*, 1992;

Gjetvaj *et al.*, 1992; Magoulas and Zouros, 1993; Geller and Powers, 1994; Skibinski *et al.*, 1994; Terrett *et al.*, 1994; Geller, 1994; Berg *et al.*, 1995).

1.7 Advantages of DNA approaches

The application of DNA based methods has three main advantages over the use of alternatives such as allozyme electrophoresis. Firstly study of DNA directly should and does reveal more variation, including that at silent positions (Mitton, 1994) which is unobservable with allozyme methods. Secondly, as the methodology is not limited to studying variation of protein products on which selection may operate, there is no such limitation for the study of DNA and DNA regions which are widely believed to be neutral such as mtDNA, non-coding regions, VNTR polymorphisms etc. can be used. Thirdly where a PCR approach is taken, only minute quantities of tissue are required allowing non-destructive sampling and examination of small organisms such as larvae.

Many population studies utilising DNA have produced results in accord with those gained from allozyme methods. However there are also many that have produced dramatically different results when allozymes and DNA are compared, for example, in revealing information not suggested by allozyme methods (e.g. Avise, 1985; Moritz *et al.*, 1987; Harrison, 1989; Ovenden, 1990; Avise, 1994). Perhaps the most acclaimed of these concerns the American oyster *Crassostrea virginica*. Allozyme analyses on this species suggested high gene flow with little evidence for population subdivision (Buroker, 1983). In striking contrast, patterns of variation in mtDNA revealed a significant genetic break between Atlantic and Gulf areas (Karl and Avise, 1992) a pattern that has since shown to be repeated in many other species (Avise, 1992). Balancing selection at allozyme loci was implicated in the oyster study because patterns of variation at scnDNA loci revealed the same genetic break as did mtDNA. Thus where allozyme methods do not reveal stock structure for a species the analysis of DNA methods may.

1.8 The Scallop *Pecten maximus*

The family Pectinidae, more commonly known as scallops comprises some 400 extant species (Brand, 1991). Within the waters of the eastern North Atlantic, five species *Pecten maximus* (L.), *Pecten jacobaeus* (L.), the queen scallop *Aequipecten* (*Chlamys*) *opercularis* (L.), *Chlamys islandica* (O.F.Müller) and *Chlamys varia* (L.) support important fisheries. Of these the most valuable is that

for the commercial scallop *Pecten maximus* of which 17,722 mt were fished in 1992 (F.A.O., 1994). *P. maximus* has an extensive distribution throughout the coastal waters of northern Europe ranging from Norway, south to the Iberian Peninsula with an isolated population around the Canary Islands. The distribution extends slightly into the western Mediterranean where it overlaps with its congener *P. jacobaeus* (Ansell *et al.*, 1991; Brand, 1991; Roman, 1991; Figure 1.6). *P. jacobaeus* is morphologically similar to *P. maximus* although the shell has distinct distinguishing features including steeper, more rectangular ribs on the right valve, an acute angle between the posterior ears and hinge in *P. jacobaeus* compared to a right/obtuse angle in *P. maximus* and differences in development of the secondary costae (T.R.Waller, Department of Palaeobiology, Smithsonian Institution, Washington D.C., pers. comm.) and a finer microsculpture and more ribs on the shell (Wagner, 1991). Whilst *P. maximus* can be found at depths down to 183m it is most common between 20-45m on bottoms of sand, fine gravel or sandy-gravel. Such restrictions on suitable habitat result in a patchy distribution with dense aggregations occurring only where bottom type and depth are favourable. Many of these beds are temporally stable features where scallops occur in sufficient densities for fishery exploitation to be feasible (Figure 1.6).

As in many benthic bivalves *P. maximus* reproduces through external fertilisation to generate planktotrophic larvae. These larvae and the subsequent post-larvae have a considerable planktonic dispersal capability; the average length of larval life ranging from 78d at 9°C to 24d at 18°C (Beaumont and Barnes, 1992). Such an extensive dispersal capability would imply larval transfer among beds throughout the species range and therefore no self-recruiting populations. However using the NORSWAP model (North Sea water parcel following model) Dare *et al.* (1994) studied the likely extent of larval dispersal and identified potential hydrographic restrictions to gene flow among exploited populations in the English Channel and Celtic Sea. If larval dispersal is restricted then differences in population characteristics may reflect this and indeed there are a number of lines of evidence to reinforce this. Evidence ranges from differences in growth rate among populations (Mason, 1983) to breeding cycle disparities, the most celebrated example of which concerns the population in St. Brieuc Bay, Brittany which

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Figure 1.6: Principal fishing grounds for *P.maximus* and (inset) distribution. From Brand (1991).

Figure 1.7: Gonad index of St Brieuc *P. maximus* (open circles) and Bay of Brest *P. maximus* (solid circles). From Paulet *et al.* (1988).

possesses a markedly different spawning cycle from neighbouring populations the characteristics of which are maintained on transplantation (Paulet *et al.*, 1988; Cochard and Devauchelle, 1993; Mackie and Ansell, 1993).

In St. Brieuc individuals there is synchronous gonad maturation of the total population during the spring resulting in a massive spawning in early July. This contrasts with populations from elsewhere including neighbouring grounds where mature individuals are present year-round and synchrony of the spawning event is weak. The gonad never matures to the same extent as that of St. Brieuc scallops and spawning takes place from May to September, although typically with two peaks (see Figure 1.7). As these differences are maintained following transplantation between sites (Cochard and Devauchelle, 1993; Mackie and Ansell, 1993) it seems that they probably have a genetic basis. Evidence from larval drift simulations also indicate St. Brieuc Bay as a potentially hydrographically isolated area and indicate that other populations may also have larval retainment within gyres (Dare *et al.*, 1994).

The existence of temporally stable aggregations, discrepancies in population parameters and possible hydrographic isolation mechanisms questions whether populations do actually exchange larvae or whether they are reproductively isolated and self recruiting.

1.9 Application of molecular genetic techniques to scallop biology

Due to the commercial interests in scallop biology, fisheries and culture there have been a number of genetic studies on scallops (see Beaumont and Zouros, 1991). Studies range from the genetics of shell colour (Adamkewicz and Castagna, 1988) to the application of DNA techniques for forensic examination of fishery catches of dubious origin (Kenchington *et al.*, 1993). As with many studies in other species, some applications of allozyme electrophoresis have been concerned with examining variation within species (Nikiforov and Dolganov, 1983; Beaumont and Beveridge, 1984; Balakirev *et al.*, 1995). As important fishery resources, other studies have interpreted genetic variation from a population perspective (Beaumont, 1982a; Kijima *et al.*, 1984; Macleod *et al.*, 1985; Fevolden, 1989; Beaumont, 1991a; Galleguillos and Troncoso, 1991; Fevolden, 1992; Lewis and Thorpe, 1994; Igland and Nævdal, 1995). In northern Europe *A. opercularis* and *P. maximus* are the most important commercial species and have received limited attention from a population genetic perspective. Much of this has concentrated on searching for genetically isolated or partially isolated "stocks". Scallops are broadcast spawners, releasing eggs and sperm into the water column where fertilisation occurs and larvae develop which are then carried by currents for a number of days (20-30d in these species, Cragg and Crisp, 1991) although this varies with temperature (Beaumont and Barnes, 1992). This capacity for gene flow would suggest that there would be homogeneity of allele frequencies. However caution must be exercised when inferring gene flow from dispersal capacity alone as described above.

Mathers (1975) found significant differences in allele frequencies at the *Gpi* locus between *A. opercularis* samples from Galway and Dublin on the West and East coasts of Ireland respectively. In a subsequent study on the same species Beaumont (1982a) used data on variation at 3 loci in conjunction with information on current patterns to indicate the existence of 4 relatively genetically isolated

Figure 1.8: Geographic variation in allele frequencies at the *PT-A* locus of *A.opercularis* from Beaumont (1982a).

populations, namely North and West Scotland, Irish Sea, Celtic Sea (Brittany Coast) and English Channel (Figure 1.8). It is noteworthy that in this study the St.Brieuc population had allele frequencies indicative of a self recruiting population which were significantly different from those of queen scallops elsewhere. Lewis and Thorpe (1994) have subsequently provided evidence to confirm the population subdivision of queen scallop populations. The greatest contribution to the genetic differences in Beaumont (1982a) work came from a protein-A (*PT-A*) locus which was observed after staining with a general protein stain on polyacrylamide gels (Beaumont and Gruffydd, 1975). No function has been ascribed to this locus and it is possible therefore that it is more free from selection than enzyme coding loci may be and thus represents a truer picture of geographic structure than loci affected by selection may. Beaumont (1982b) and Lewis and Thorpe (1994) found allele frequencies in *A. opercularis* populations to be temporally stable although the proportions of heterozygotes and homozygotes in the population varied among year classes (Beaumont, 1982b). Selection was deemed to be responsible for this. Temporal shifts in allele frequencies were suggested to occur in *A. opercularis* populations by Macleod *et al.* (1985). Although the data in this study did not indicate any geographic variation in allele frequencies among samples taken from around the Isle of Man (Beaumont, 1982a, had suggested that Irish Sea populations were a single 'stock') the appearance of one allele (Pgi^4) in the 1979 year class only was taken as evidence to indicate an influx of larvae from another area where Pgi^4 was a more common allele. Although Pgi^4 was at very low frequency, its occurrence in every sample solely in the 1979 year class was sufficient to suggest that its appearance was not due only to random sampling variation. Thus in *A. opercularis* there is considerable evidence for at least 4 stocks around the U.K. within which allele frequencies are generally temporally stable although the appearance of rare alleles is suggestive of some temporal changes in recruitment and alterations to the frequencies of heterozygotes in the same year classes suggests the activity of selection.

1.9.1 Population genetic studies on *P. maximus*

P. maximus has a similar distribution (Brand, 1991) and life history to *A.opercularis* and thus hydrographic processes which are involved in the

generation of population sub-division in *A. opercularis* would be expected to be mirrored in *P. maximus* populations (Beaumont, 1991a). Indeed considerable evidence exists to suggest that the *P. maximus* population in the Bay of St. Brieuc (northern Brittany) is genetically distinct from neighbouring and U.K. populations (Huelvan, 1985; Lubet, 1986; Paulet *et al.*, 1986; Paulet *et al.*, 1988; Cochard and Devauchelle, 1993; Mackie and Ansell, 1993) and allozyme studies have focused on finding a genetic basis to underlie this hypothesis (Huelvan, 1985; Beaumont *et al.*, 1993). However these studies found no evidence to suggest that Bay of St. Brieuc scallops are genetically distinct from other populations nor that there is any population structure to *P. maximus*. The method may have been inappropriate, whether due to the action of balancing selection (Karl and Avise, 1992) keeping allele frequencies of populations similar in spite of gene flow, or to the inherent insensitivity of allozyme methods, or, as in *A. opercularis* where one locus (*PT-A*) proved instrumental in identifying the underlying stock structure, a single locus, as yet unidentified may be enough to indicate population structure in *P. maximus* (Beaumont, 1991a). To date there has been no population genetic study examining DNA variability within *P. maximus* for any loci.

1.9.2 Mitochondrial DNA in scallops

Studies at the DNA level on scallops have been limited. Most have focused on the unusually high levels of length variation uncovered in studies of mtDNA which in the family Pectinidae (scallops) are particularly pronounced. Gjetvaj *et al.* (1992) investigated size variation in a number of species prompted by the finding of length variation in *Placopecten magellanicus* (Snyder *et al.*, 1987; La Roche *et al.*, 1990; Fuller and Zouros, 1993). Indeed *P. magellanicus* has the largest animal mtDNA yet recorded at 41kb. One species within the family (*Argopecten irradians*) was found to have a typically (and invariantly) sized mtDNA whilst all others showed variation which, where investigated, have turned out to be due to tandemly repeated sequences or deletions, additions or insertions (Table 1.2). Different species did not share the repeated sequence since probes of the sequence from various species did not hybridise with other species' molecules even among species that are closely related. Thus either the repeated sequence has substantially diverged from an ancestral sequence or each has an independent origin. Rigaa *et al.*(1995) found similarities

between the large 1,442bp repeat of *P. magellanicus* and the 1,586bp repeat of *P. maximus* including the sequence of a hairpin, that suggests similar processes may be responsible for the generation of length variation in these species.

	MtDNA size and genotype data				
Species	Size of repeat unit	Copy number per molecule	Most frequent size	Size range	Genotype diversity
Argopecten irradians[a]	-	-	16.2	16.2	0.38
Pecten maximus[a,b,c]	1586bp	3-7	21.5	19.9-26.3	0.967
Crassodoma gigantea[a]	1.0kb	3-5	23.8	22.8-24.8	0.879
Aequipecten opercularis[a]	>0.4kb†	?	24.6	21-28.2	0.978
Chlamys hastata[a]	0.6kb†	?	25.5	23.9-27.2	0.894
Chlamys islandica[a]	1.2kb	1-3	23.6	22.5-25	0.796
Placopecten magellanicus[a,d]	1.45kb	2-8	35.5	31-41	0.990
Mizuhopecten yessoensis[e]	?	?	25.9±1	?	?
Swiftopecten swiftii[e]	?	?	25.3±0.4	?	?

Table 1.2: Mitochondrial DNA length variation in the Pectinidae. a: Gjetvaj *et al.*(1992). b: Rigaa *et al.*(1993). c: Rigaa *et al.*(1995). d: Fuller (1993). e: Repin and Brykov (1993). † accurate length not established.

Other scallops have since been found to exhibit length variation (Repin and Brykov, 1993). The presence of length variation raises important questions about their maintenance in the population since even if the mtDNA is a selectively neutral marker with regards to the genes for which it codes (Zouros *et al.*, 1992b), a large mtDNA must require more resources to replicate than a smaller molecule and thus

be at a potential selective disadvantage. If this is so then a high rate of mutation would be required to counterbalance this and keep the frequency of length variants at the frequencies seen within populations. Indeed there is evidence that the rate of creation of new length variants is exceptionally high. Cook and Zouros (1994) studied offspring of a known *P. magellanicus* pedigree and found numerous length variants not seen in either parent.

P. maximus mtDNA is variable in length due to varying numbers of a tandemly repeated 1.55kb element (Rigaa *et al.*, 1993; Rigaa *et al.*, 1995). Unlike *P. magellanicus* where there are 3 length variable loci within the mtDNA there appears to be only one within *P. maximus.* Despite the localised nature of this length variation any such repeated region can cause problems for RFLP studies as fragment pattern differences due to site mutations must be separated from those due to length differences.

Examples of the use of mtDNA in examining population structure are limited to *Argopecten.* Blake and Graves (1995) used restriction analysis of mtDNA to analyse population structure in *A. irradians* and *A. gibbus.* Evidence from these data suggested population subdivision in the former species but the populations of *A. gibbus* appeared to share a common gene pool in accord with allozyme analysis (Krause *et al.*, 1994). Boulding *et al.* (1993) have also used PCR-RFLP on 3 segments of mtDNA to measure inbreeding in scallop broodstock. Patwary *et al.* (1994) described the use of random amplified polymorphic DNA (RAPD) for studying stock structure in *P. magellanicus.*

1.10 Aims of this study

The purpose of this study was to further examine the population structure of the commercial European scallop, *Pecten maximus*, using the mitochondrial DNA molecule and compare the results with those obtained via the examination of allozyme and morphological data.

2.0 Shell shape variability in *P. maximus* and *P. jacobaeus*

2.1 Materials and Methods

2.1.1 Sampling

Samples of *Pecten maximus* were collected from 15 locations around the U.K., Eire and northern France (see Figure 2.1). Each population was given an abbreviated identifying code (Figure 2.1). All Isle of Man samples were dredged by the R.V. Rogan (Liverpool University, Port Erin Marine Laboratory). Polperro (offshore Looe/Polperro in the region of Middle Rocks) and Lyme Bay (from hauls on Lat. 50° 18″ between Long. 2° 56″ and 3° 12″) samples were dredged by the R.V. Corystes (M.A.F.F., Lowestoft). The Stonehaven sample was taken by a commercial fishing vessel as was the Anglesey/I.O.M. sample (27m NW of Puffin Island, Anglesey). Mull, Eire and French samples were all taken by SCUBA divers. Mulroy Bay animals were collected as wild spat settled on artificial spat collectors in Mulroy Bay, grown over several months to approximately 2cm length then transported to and ongrown in Kilkieran Bay in intermediate culture (seabed traps) until 5-6cm size when they were reseeded. There was no evidence of significant mortality after transplantation that would have been indicative of the action of selection (Dr Gavin Burnell, U.C. Cork, pers. comm.). *Pecten jacobaeus* were collected from 65-75m depth, 13 m off the Oropesa coast, Castellón, eastern Spain.

After tissue samples were taken for biochemical analysis (Chapters 3-6) shells were washed and cleared of fouling organisms. Measurements were then taken of clearly defined and easily measured characters considered to provide an adequate description of the animal's shape. Length, height and hinge length (Figure 2.2) were measured to 0.1cm using vernier callipers. Depth was measured by bringing a ruler mounted slide down onto the area of maximum height with the shell on a flat surface. Shell weight was taken to the nearest 0.1g. Using the underside of the upper (left) valve the number of ribs was taken by counting the number of distinct scalloped ridges around the edge. Raw data are in appendix A.

2.1.2 Statistical analysis

2.1.2.1 Meristic data

A one-way ANOVA was used to examine variability in rib counts using

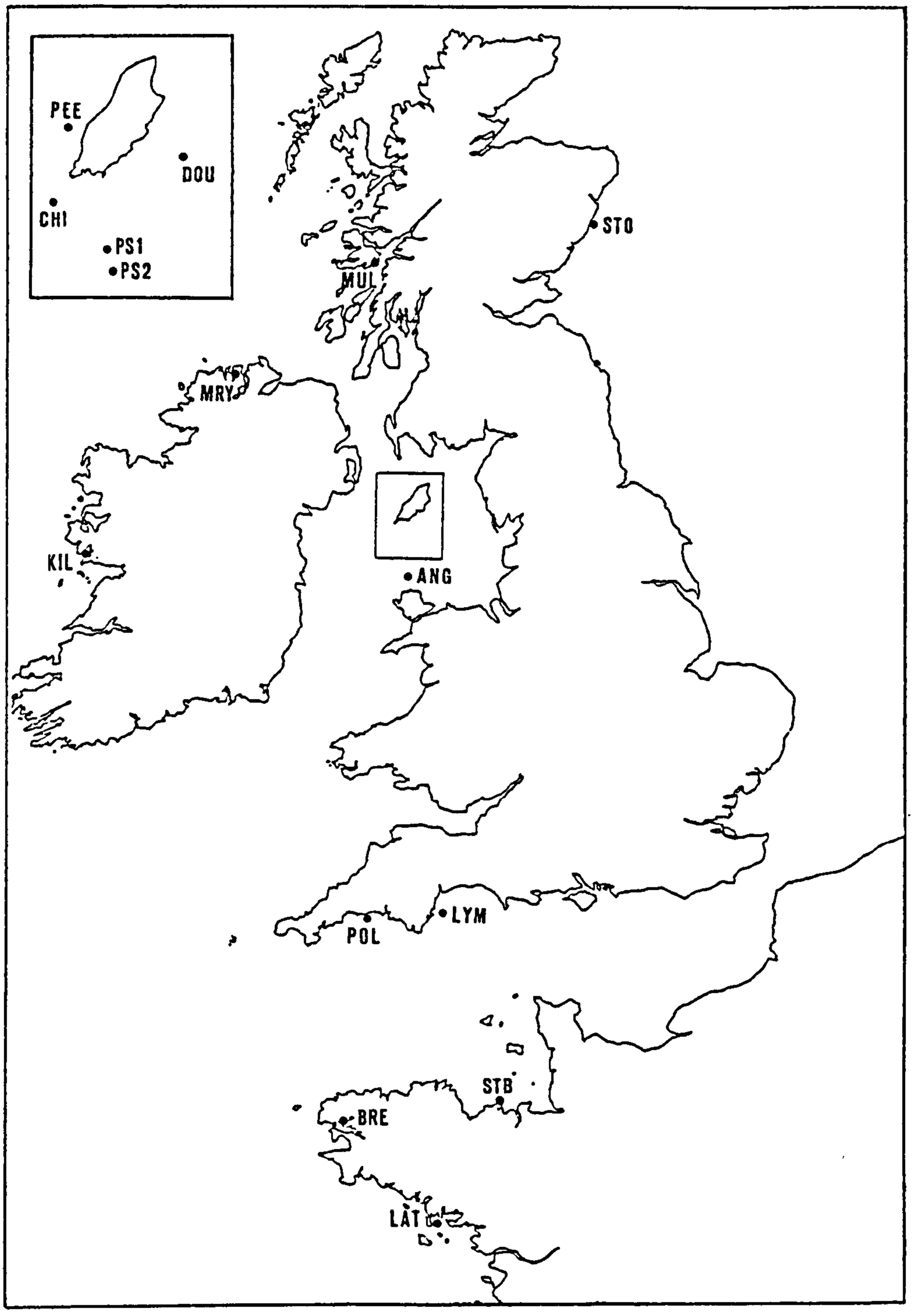


Figure 2.1. Collection sites of *P.maximus* samples. PS1: Port St. Mary (nearshore). PS2: Port St. Mary (offshore) DOU: Douglas. PEE: Peel. CHI: Chicken Rock. ANG: Anglesey/I.O.M. STO: Stonehaven. MUL: Mull. LYM: Lyme Bay. POL: Polperro. MRY: Mulroy Bay (transplanted and ongrown in Kilkieran Bay). KIL: Kilkieran Bay. STB: Nerput, St. Brieuc Bay. BRE: Tinduff, Daoulas Bay, Rade de Brest. LAT: La Trinité sur Mer.

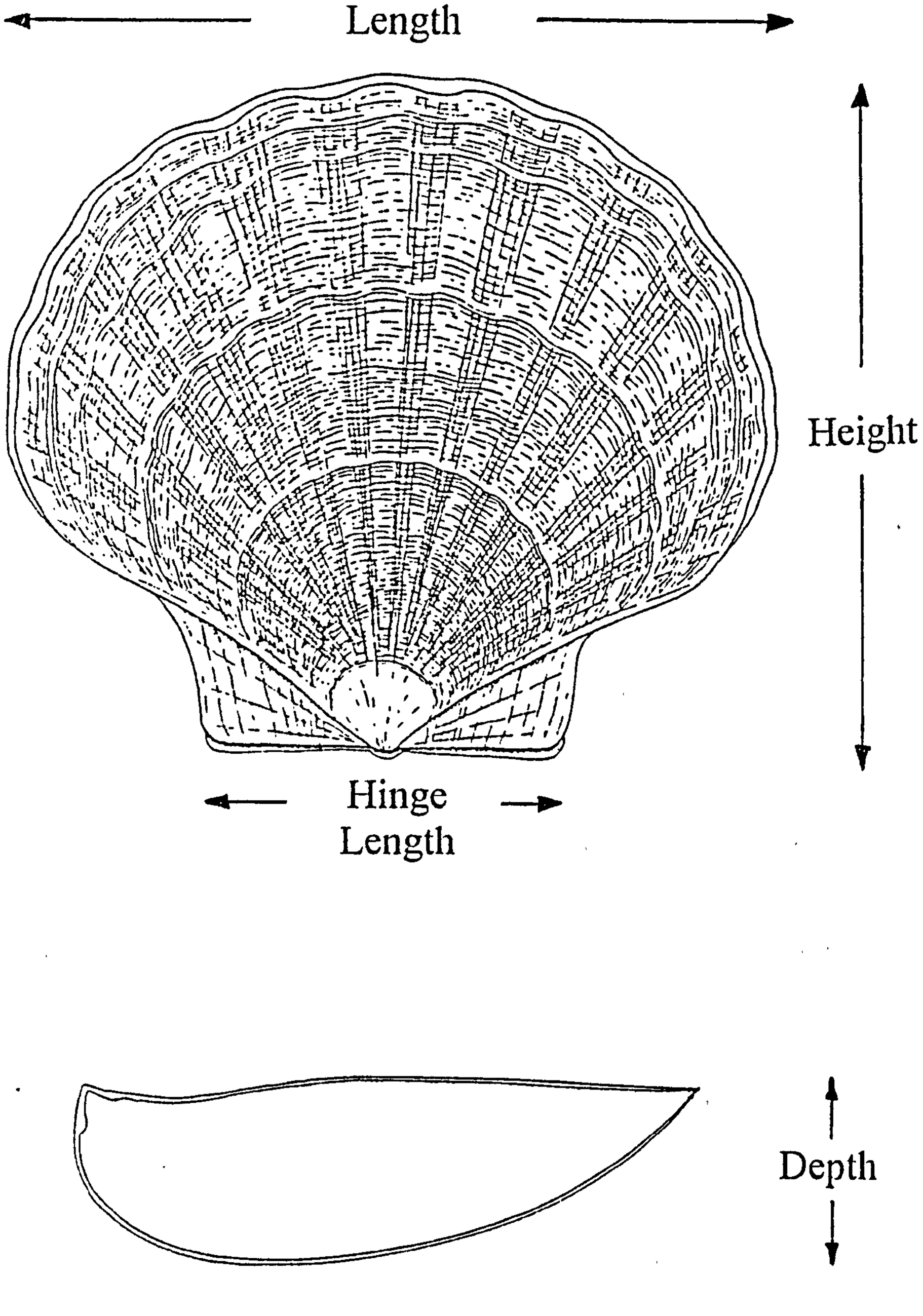


Figure 2.2. Shell dimensions measured for morphological analysis of *P. maximus* and *P. jacobaeus.*

MINITAB. Where significant differences among populations were indicated by the ANOVA, Scheffés test was utilised to detect which pairwise comparisons of populations had significant differences in numbers of ribs.

2.1.2.2 Allometry

The relative proportions of characters can change throughout an animal's life (allometry) such that there are ontogenetic shape differences. If two characters (x and y) are measured throughout the growth of an individual then this relationship can be described by $y = a.x^b$, or alternatively $\log(y) = \log(a) + b.\log(x)$ where the slope, b, describes the ratio of specific growth rates of structures x and y. If $b = 1$ then growth is said to be isometric with both characters growing at the same rate. If less than 1 then negative allometry occurs with y growing relatively less than x throughout life. If greater than 1 then there is positive allometry whereby y grows relatively faster than x. If allometry occurs then attempts to reveal any geographic differentiation of morphometric characters must account for among sample size variation in order to remove the effects of shape differences among samples due to allometry from the actual geographic differences which are being sought. Because the sampling strategy was not standardised, separate samples varied in representative size classes, particularly in the case of *P. jacobaeus* which were predominantly juveniles and, if growth is not isometric with respect to each character measured then any differences among groups could result from ontogenetic differences as opposed to geographic variation. Allometry was examined for every character (using $\log_{10}$ transformed data) by performing standard linear regression between length (as an indicator of overall size) and each continuously varying character (height, depth, weight and hinge length) to obtain a value for the intercept (a) and the slope (b). A true value of the allometric relationship can only really be taken from a full ontogenetic dataset (Shea, 1985) but an estimation can be made with a range of animals of different sizes, as here. If the variables being compared are both linear then should isometry hold, the expected value of the slope (β) is 1 whilst for weight β is expected to be 3. One sample T-tests were then used to compare b and β. Standard regression analysis performed on such data is strictly unsuitable since there is no independent variable and other methods of regression are required (Ricker, 1973; Clarke, 1980; Laws

and Archie, 1981; McArdle, 1988). However these approaches are not readily available in most statistical packages.

2.1.2.3 Multivariate analysis of continuous data

Even where a suitable method of regression is available, if a data set is composed of more than 2 variables, information would be lost and patterns missed by only using regression to analyse shape differences (Oxnard, 1978), although there are examples of the use of this in examining population level variation (Beukema and Meehan, 1985; Volckaert *et al.*, 1991; McShane *et al.*, 1994). Bivariate methods further suffer in that although size is accounted for, allometric effects can be recognised but not controlled. Finally, to fully analyse the complete range of combinations of variables versus length would not only be tedious but would be prone to type I errors in the assignment of significance levels.

The only truly suitable method for analysing multivariate data is through a multivariate method, usually either an ordination or cluster technique (Thorpe, 1976; Oxnard, 1978; Janson and Sundberg, 1983; James and McCulloch, 1990; Rohlf, 1990a). Ordination methods create new axes through data in multi-dimensional space in a manner that maintains as much variation as possible. One such method, the principal component analysis or PCA is used to reduce a multivariate data set to a more meaningful and manageable number of factors without losing information, by locating hidden axes through the observed variables. The axes which describe the original variables are rotated (through the eigenvectors) resulting in a new set of unrelated axes each successively describing a maximum of variation. The maximum amount of variance is expressed in the first principal component, with subsequent components being orthogonal and explaining progressively less of the remaining variability (Blackith and Reyment, 1971). Output consists of eigenvalues, eigenvectors and principal component scores. Eigenvalues express how much of the variability is explained by each of the principal components. Eigenvectors describe the rotation of the original axes necessary to define the new principal components (PCs) and thus indicate the "loading" (correlation of the original variable with one of the PCs constructed by the PCA) of each of the original measured variables on each PC. Scores are the projections of the original data points onto the new axes. The PCA is particularly

suited for this kind of study since not only is it useful for extracting and identifying variables of use in differentiating populations, but it is also possible to remove or at least identify the variation as a result of differential growth or allometry (Shea, 1985).

PCA was used to examine variation in all the continuous characters (meristic characters such as rib numbers are unsuitable for PCA analysis). Because of differing measurements (mm and g), all data were $\log_{10}$ transformed (to standardise the variables) and used directly as input for PCA analysis using the covariance matrix, in MINITAB. Principal component 1 is widely accepted as being largely influenced by size (with constituent allometric effects) and removal of this then leaves further PCs which explain shape unrelated to size. Indeed where there is a large difference in size among populations PC1 will be mainly a size related component (Krause *et al.*, 1994) and subsequent PCs will detail shape differences uncorrelated with size. Where PC scores exhibited evidence of geographic variation the character responsible for the major influence on the scores was traced from the factor loadings.

Other ways of controlling the effects of size include regression adjustment of characters (Thorpe, 1976; Shea, 1985) which was not an option for these data as some of the within group bivariate relationships were probably unrepresentative due to restricted size classes of samples and the use of ratios as input for the PCA (size can be controlled by using ratios of characters with length as the denominator). Although this is believed to remove the size component, it actually increases the correlation between the ratio variable and the variable being scaled (Atchley *et al.*, 1976) and would accentuate the allometrical effects. Also if ratios are used as input for a PCA then this can cause inflation of the first eigenvalue and result in changes in the magnitude and direction of coefficients of the other principal components (Atchley *et al.*, 1976). Control of size differences and allometric effects in the PCA was limited to elimination of PC1.

To evaluate the efficacy of PCA for separating geographically isolated populations of *P. maximus* on the basis of these measurements PC1 was disregarded and the remaining PC scores subjected to a quadratic discriminant analysis (this does not make the assumption that all groups have the same

covariance matrix) to assess if the remaining PC scores were useful for discriminating populations.

Mahalanobis distances (D^2) among all PC scores and, also the 4 non size related PC scores (-PC1) were calculated using a locally written function on Splus at Glasgow University (Dr Ken Johnson, Department of Geology and Applied Geology). Mahalanobis distances use both the centre and variation within groups to distinguish groups on the basis of overlap of variation. A pooled (combined) within group covariance matrix is included in the formula. With *m* characters (5 for the complete array of PC scores and 4 when PC1 was disregarded) where $\bar{x}_i$ and $\bar{y}_i$ are the means of the i^{th} character in populations X and Y respectively, with the variances (v_{ii}) and covariances (v_{ij}) of the characters assumed to be the same for the two populations then D^2 is given by:

$$D^2_{XY} = \sum_{i=1}^{m} \sum_{j=1}^{m} v^{ij} (\bar{x}_i - \bar{y}_i)(\bar{x}_j - \bar{y}_j)$$

D^2 values were then clustered using the SAHN option of NTSYS-PC (Rohlf, 1990b) and used to construct a dendrogram to graphically depict relatedness among populations.

2.2 Results

2.2.1 Allometry

Each character exhibited evidence of negative allometry relative to length (Table 2.1) that is, as the animal grows larger it gets relatively shorter, lower, lighter and also shorter in hinge length.

Character	b	s.e.b	β	N	t
Depth	0.85139	4.63×10^{-4}	1	699	321 ***
Height	0.97145	2.15×10^{-4}	1	700	132.7***
Hinge length	0.84002	6.13×10^{-4}	1	667	261.169***
Weight	2.5011	9.45×10^{-4}	3	699	527.8***

Table 2.1: Regression analysis of allometry in populations of *P. maximus* and *P. jacobaeus*. b=observed slope (s.e.=standard error). β=expected slope if growth is isometric. N=sample size. t=t value. ***=significant at 0.001 level.

Allometry was further implicated from the PCA output (below). Jolicouer (1963) suggested that using the eigenvectors of variable *i* (direction cosines, $\cos\theta i$), with *p* variables then if $\cos\theta i > 1/\sqrt{p}$ there is positive allometry and if $\cos\theta i < 1/\sqrt{p}$ then there is negative allometry. Here $1/\sqrt{p} = 0.447$ and since all the eigenvectors are negative (Table 2.3) then there is confirmation that each character exhibits negative allometry.

Since size (depicted as length) varied greatly across samples (Fig 2.3) these allometric relationships (Table 2.1) are likely to have an influence on the study of geographic variation and must be accounted for (by removal of PC1).

2.2.2 Variation in rib counts

Rib counts varied greatly among sample sites (Figure 2.4). ANOVA revealed at least one significant difference in rib numbers among samples ($F=7.22$, $p<0.001$). However two populations (PS2 and POL) were not normally distributed thus were removed from a repeat analysis in which there was also at least one significant difference detected ($F = 8.34$, $p<0.001$). Table 2.2 details the pairwise differences

	All Populations		excluding PS2 and POL	
Comparison	Lower C.I.	Upper C.I.	Lower C.I.	Upper C.I.
PS2-JAC	-1.907	-0.239		
DOU-STB	-1.870	-0.174	-1.804	-0.240
PEE-STB	0.119	1.441	0.171	1.389
CHI-JAC	-1.669	-0.071	-1.606	-0.134
PS1-JAC	-1.722	-0.096	-1.659	-0.159
ANG-JAC	-1.816	-0.036	-1.746	-0.106
LYM-JAC	-1.691	-0.044	-1.627	-0.109
POL-STB	0.023	1.283		
JAC-STB	0.593	2.283	0.659	2.217
JAC-LAT	0.401	2.270	0.474	2.196
JAC-BRE	0.158	2.012	0.230	1.940
KIL-STB			0.017	1.423

Table 2.2: Significant results of Scheffés comparison among populations following ANOVA performed on rib numbers.

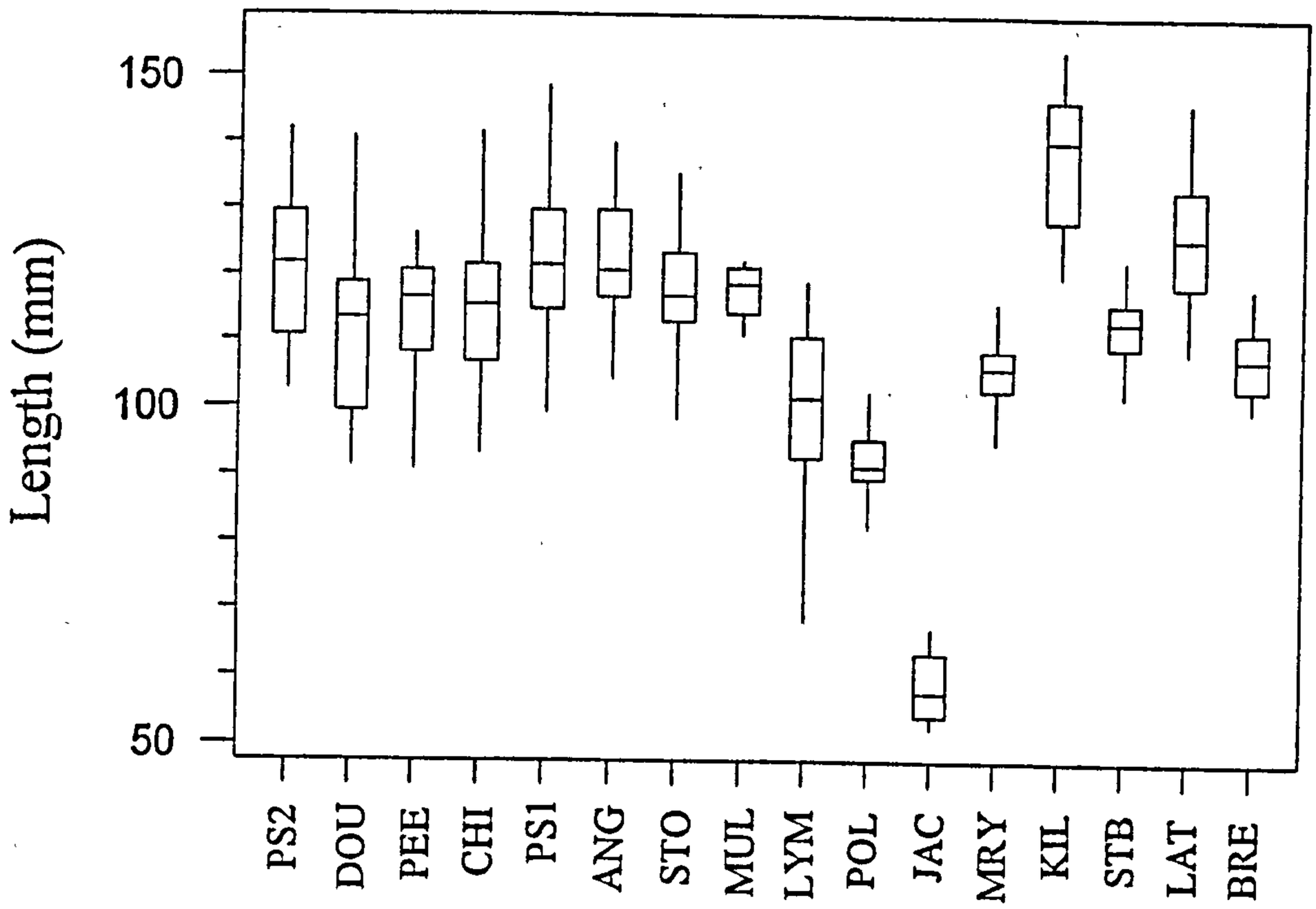


Figure 2.3. Box-and-whisker plot depicting variation in length in 15 populations of *P.maximus* and 1 sample of *P.jacobaeus*. Box shows first (Q1) and third quartile (Q3) around median line. Whiskers extend to highest and lowest value defined by region Q1±1.5(Q3-Q1).

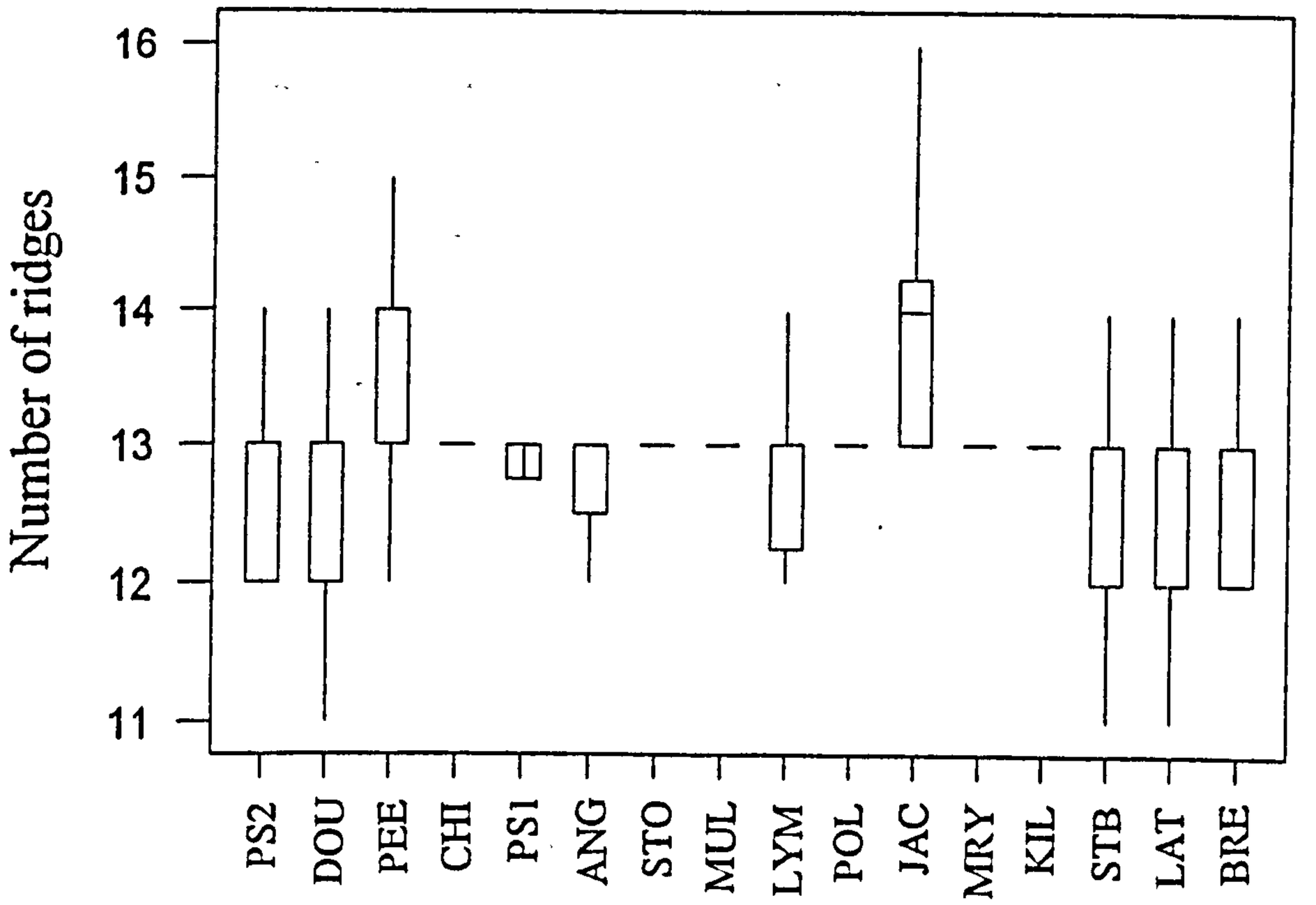


Figure 2.4. Box-and-whisker plot depicting variation in rib numbers in 15 populations of *P.maximus* and 1 sample of *P.jacobaeus*. Box shows first (Q1) and third quartile (Q3) around median line. Whiskers extend to highest and lowest value defined by region Q1±1.5(Q3-Q1).

detected using Scheffés test. Significant differences are suggested where the upper and lower confidence intervals do not span zero. Some significant differences in the analysis utilising all populations were not detected in the comparison without the Port St. Mary, offshore (PS2) and Polperro (POL) populations and, *vice versa*.

2.2.3 Principal components analysis of shell shape

PCA executed on the complete data matrix produced factor loadings for PC1 which were uniformly strongly negative, thus every measurement has an influence on PC1. This is therefore primarily a size component although it does provide data on allometry (see above) and as suggested by Shea (1985) should be more correctly labelled as an allometry vector. As a result of such a wide variation in sample lengths among groups, PC1 (overall size) accounted for 97% of the total variation (Table 2.3). Other PCs should then describe shape unrelated to size. The influence of the original measured characters on the other 4 PCs was traceable since the stronger the positive or negative loading of a character on a PC the greater the significance of that variable in accounting for the variation summarised in that factor.

	Eigenvector				
Character	PC1	PC2	PC3	PC4	PC5
$\log_{10}$(length)	-3.08	-0.103	-0.627	0.201	0.679
$\log_{10}$(depth)	-0.274	0.005	-0.243	-0.928	-0.072
$\log_{10}$(height)	-0.304	0.001	-0.544	0.289	-0.726
$\log_{10}$(weight)	-0.816	0.344	0.445	0.122	0.057
$\log_{10}$(hinge-l)	-0.269	-0.933	0.231	0.018	-0.055
Eigenvalue	0.056545	0.000890	0.000446	0.000346	0.000059
Proportion	0.97	0.015	0.008	0.006	0.001
Cumulative	0.97	0.985	0.993	0.999	1

Table 2.3: Summary of PCA performed on complete *P. maximus* and *P. jacobaeus* morphometric dataset using the covariance matrix.

Plots of PC1 and PC2 scores (Figure 2.5) suggest that samples appear to be distributed according to size for PC1 and this is in line with the differences in

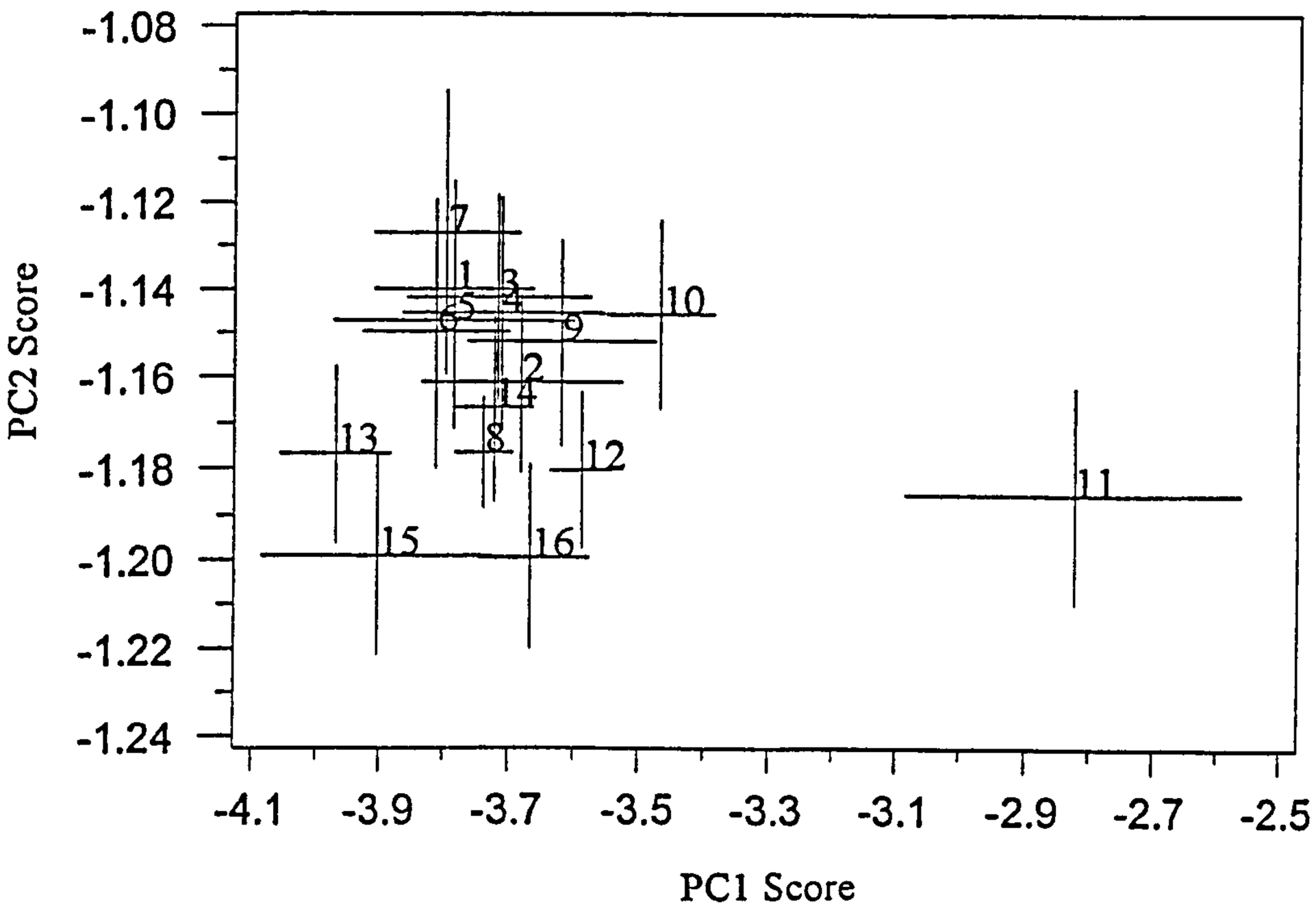


Figure 2.5. Plot of first and second principle component scores extracted from PCA, performed using covariance matrix on 15 populations of *P.maximus* and 1 sample of *P.jacobaeus*. Population PC scores are represented by 1 standard deviation either side of mean to avoid plotting a cloud of points. Population identifiers, 1:PS2 2:DOU 3:PEE 4:CHI 5:PS1 6:ANG 7:STO 8:MUL 9:LYM 10:POL 11:JAC 12:MRY 13:KIL 14:STB 15:LAT 16:BRE

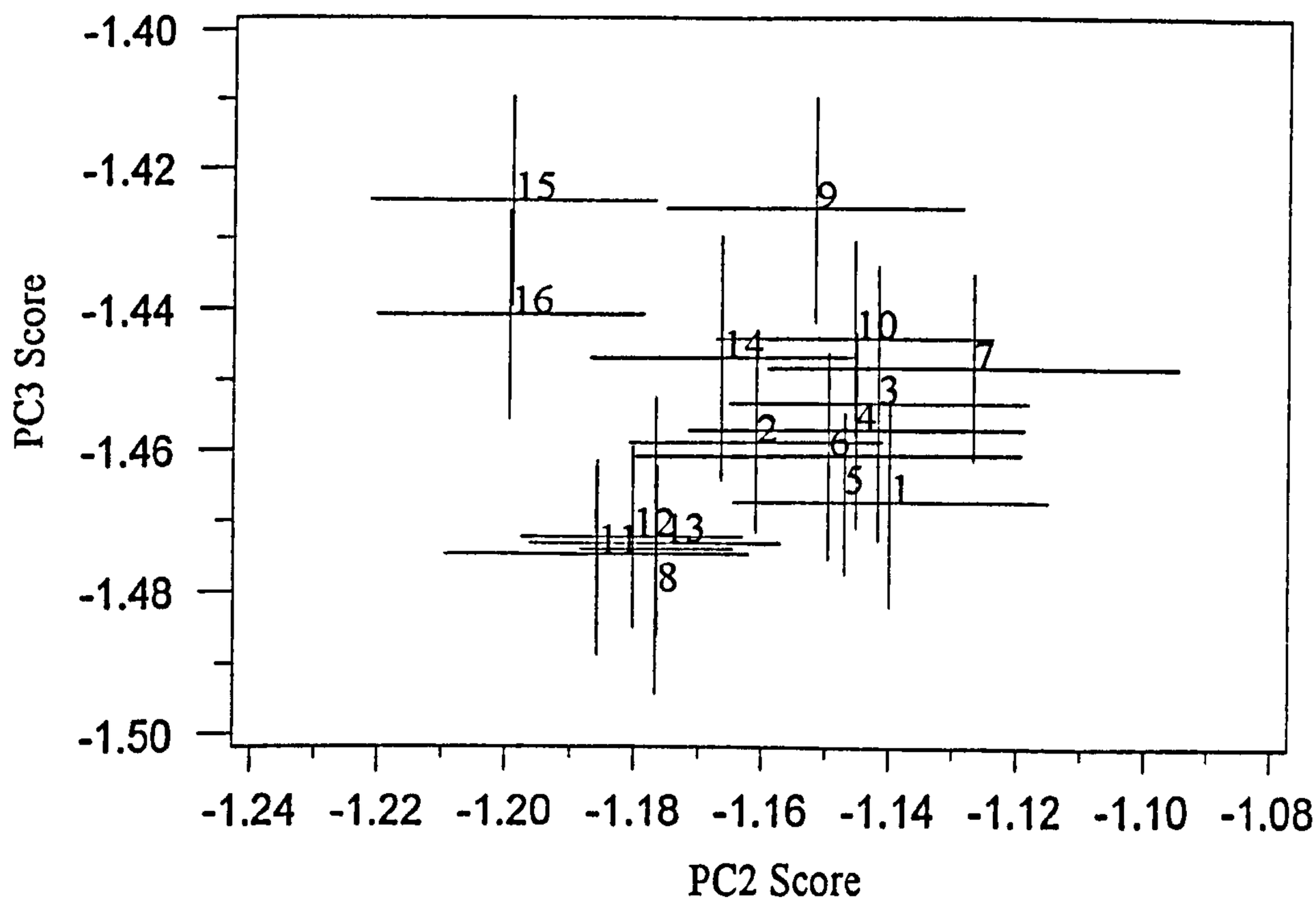


Figure 2.6. Plot of PC2 scores vs. PC3 scores. Details as for Figure 2.5.

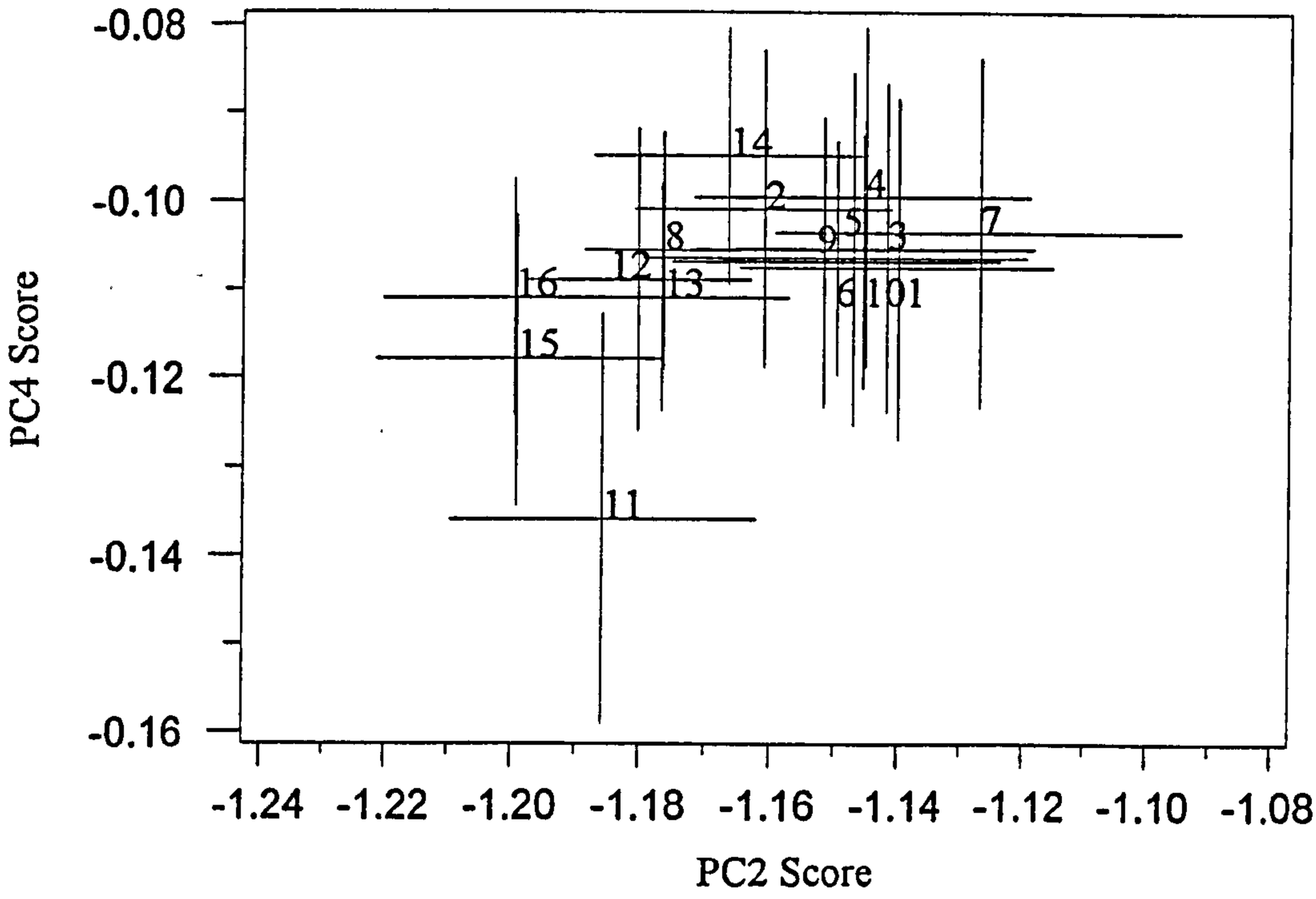


Figure 2.7. Plot of PC2 scores vs. PC4 scores. Details as for Figure 2.5.

size among samples (Figure 2.3). The second PC does not distribute according to overall size, individuals from most populations cluster together inseparably but La Trinité (LAT) and Brest (BRE) animals which are of average size have lower scores on PC2. The variable with overriding influence on PC2 is hinge length (Table 2.3). Hinge length has a strongly negative eigenvector for PC2 and the Brest and La Trinité population scores are more strongly negative than those of other populations (Figure 2.5), therefore it seems that animals from these vicinities have longer hinges than those of other populations. PC3 suggested some separation, with the La Trinité and Lyme Bay animals having higher values (Figure 2.6). There was some separation into two groups for the other populations but these were not distinct. No single factor had an overriding influence on PC3 and the variation explained by this factor is only half that of PC2 thus the differences are not particularly strong and the shape differences explained by this PC are difficult to visualise. For PC4 (Figure 2.7) which is mainly influenced by depth *P. jacobaeus* clustered away from other populations with more negative values. *P. jacobaeus* therefore appear to have deeper shells than *P. maximus* although since

PC4 explained only 0.6% of the total variability in shape this difference is very slight.

Because of such large variation in size among populations especially in the case of *P. jacobaeus* which were inordinately small in comparison to other samples a second PCA was performed with a restricted sample consisting of only animals between 110-130cm length (this severely reduced sample size in some cases and meant that no *P. jacobaeus* could be included in the analysis). This was undertaken because outliers can have dramatic effects on the PCA (James and McCulloch, 1990).

	Eigenvectors				
Character	PC1	PC2	PC3	PC4	PC5
$\log_{10}$(length)	-0.198	-0.021	0.604	0.274	0.721
$\log_{10}$(depth)	-0.317	0.109	-0.405	0.848	-0.067
$\log_{10}$(height)	-0.225	0.055	0.668	0.173	-0.686
$\log_{10}$(weight)	-0.848	0.286	-0.145	-0.418	0.055
$\log_{10}$(hinge-l)	-0.301	-0.95	-0.064	-0.025	-0.047
Eigenvalue	0.0054511	0.0009006	0.0002825	0.0002439	0.0000545
Proportion	0.786	0.13	0.041	0.035	0.008
Cumulative	0.786	0.916	0.957	0.992	1

Table 2.4: Summary of PCA performed on *P. maximus* morphometric data from animals of 110-130mm shell length using the covariance matrix.

The first PC from this analysis then explained only 78.6% of the variation (due to contraction of the size range and therefore less variation due to size), with PC2 (still accounted for by hinge length) explaining 13% and the other 3 PCs 8.4% (Table 2.4). The characters with strong eigenvectors are similar to those of the full data set (Table 2.3) and the distribution of PC scores was similar to that observed with the complete data matrix and plots the same (not shown).

Irrespective of whether the total or restricted sample size was analysed the PC scores suggested some limited evidence of inter-population variation (Figures 2.5, 2.6 and 2.7). Although the variance explained by some PCs was low they were

used in the subsequent discriminant analysis and calculation of Mahalanobis distances.

2.2.4 Discriminant analysis

The most obvious differentiation evident from these data was of the Brest and La Trinité samples on the basis of PC2. These two populations appeared to cluster closely and although discriminant analysis could only place 60.7% and 63.3% respectively in the correct populations, if they are considered pooled together then 84.5% were placed correctly (Table 2.5). This is much higher than correct placement for other populations suggesting that these 2 populations are indeed similar in shape but different from all others.

2.2.5 Mahalanobis distance

These results are reflected in the UPGMA dendrogram calculated from D^2 values. Mahalanobis distances calculated from the original data matrices cluster animals mainly on the basis of the large size differences (as expected where size itself was the main differentiator among populations) thus the much smaller *P. jacobaeus* cluster well away from the *P. maximus* (Figure 2.8a). After removal of PC1 and thus removal of the effect of size *per se*, any variation should be due to shape differences and consequently the clustering pattern changes, resembling to a greater extent what is expected on the basis of the PCA and discriminant analysis (Figure 2.6) in which the Brest and La Trinité populations appear to cluster away from the other populations (Figure 2.8b).

2.2.6 Shell colour

For most scallop shells the upper valve was brownish in colour and the lower pale cream. However colour variability was noted, in particular for St. Brieuc Bay scallops which were often coloured purple on the lower valve and marked with dark and white flecks on the upper valve (as in Figure 24 of Wagner, 1991). This population appeared obviously distinct from others in the observed numbers of coloured variants. Minchin (1991) suggests this is a phenomenon peculiar to Brittany scallops but this high frequency of coloured shells was not encountered in the La Trinité or Brest sample or indeed in any other *P. maximus* population or for *P. jacobaeus*.

Cain (1988b) provides a system for classifying colours but because the

Population																
	PS2	DOU	PEE	CHI	PS1	ANG	STO	MUL	LYM	POL	JAC	MRY	KIL	STB	LAT	BRE
PS2	13	2	2	4	5	2	0	0	0	1	0	1	1	0	0	0
DOU	3	10	2	3	3	1	1	0	1	3	0	1	1	3	0	0
PEE	2	2	4	5	0	0	2	0	2	3	1	0	0	1	0	0
CHI	1	0	2	6	2	0	2	0	0	0	0	0	0	0	0	0
PS1	9	2	7	8	18	7	1	0	0	1	0	1	2	4	0	0
ANG	4	3	6	5	15	5	0	2	1	4	0	1	1	3	0	0
STO	3	1	7	18	3	1	12	0	3	2	0	0	0	1	0	0
MUL	4	4	1	6	5	3	1	13	0	1	1	9	5	1	0	0
LYM	0	0	2	2	0	1	0	0	29	6	0	0	0	8	2	0
POL	8	8	5	8	3	3	3	0	12	25	0	1	2	7	0	1
JAC	2	2	0	1	4	0	0	1	0	0	15	3	1	0	1	2
MRY	2	4	1	2	3	3	0	7	0	0	1	9	4	5	0	0
KIL	1	2	1	1	0	0	0	4	1	0	1	3	9	1	0	1
STB	0	6	2	3	0	3	0	1	2	5	1	1	0	10	1	1
LAT	0	0	0	0	0	1	1	0	9	0	0	0	1	2	17	6
BRE	1	1	0	3	2	1	0	0	0	3	2	0	3	3	7	19
N	53	47	42	75	63	31	23	28	60	54	22	30	30	49	28	30
C	13	10	4	6	18	5	12	13	29	25	15	9	9	10	17	19
%	25	21	10	8	29	16	52	46	48	46	68	30	30	20	60	63

Table 2.5: Morphometric analysis of *P. maximus* and *P. jacobaeus*. Results of quadratic discriminant analysis on PC2-5 scores. N=sample size. C=number placed correctly (%=percent correct).

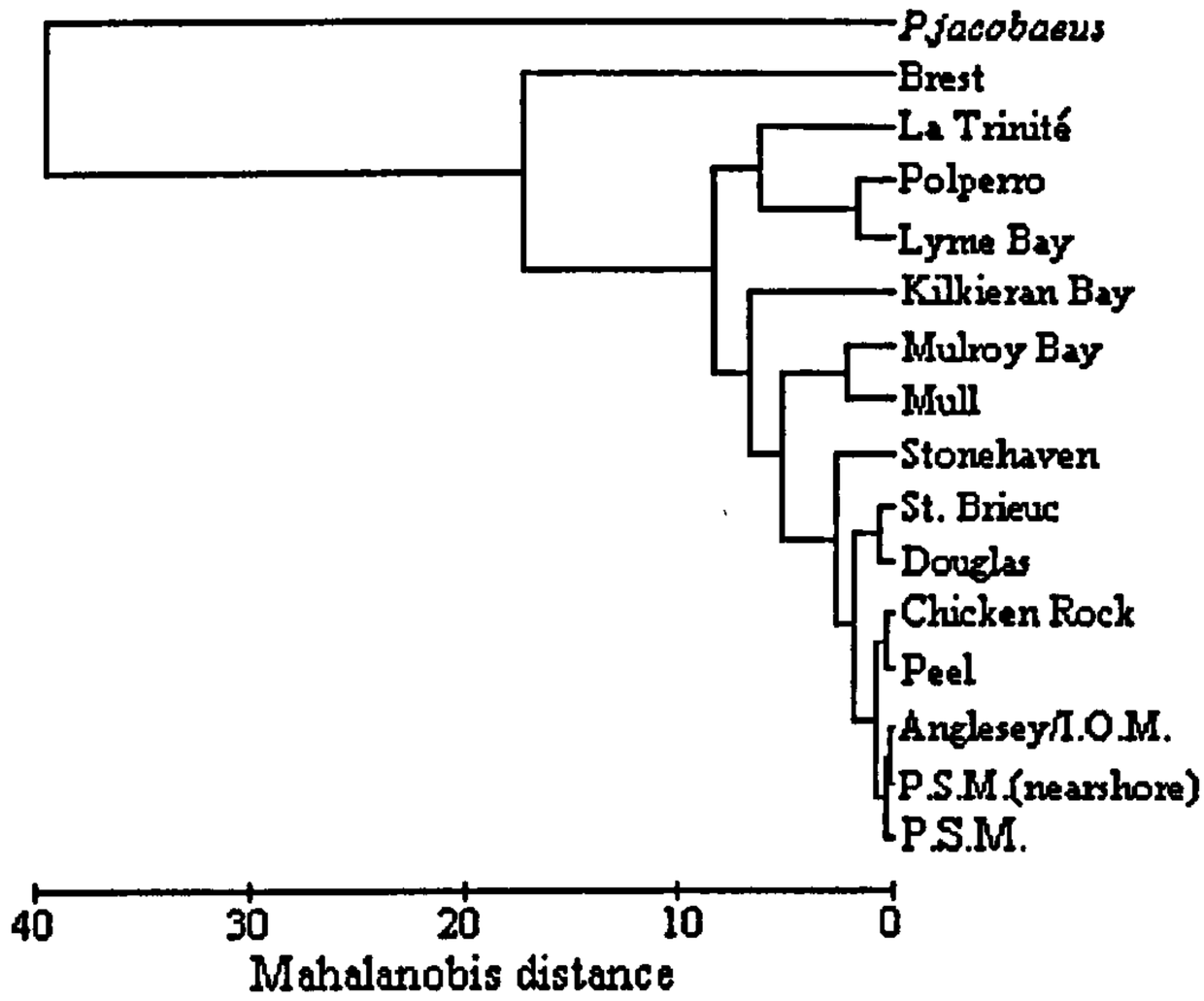


Figure 2.8. UPGMA dendrogram of Mahalanobis distance calculated from PC1-5 scores calculated using PCA with the covariance matrix from 5 shell characters in the morphometric analysis of *P. maximus* and *P. jacobaeus.*

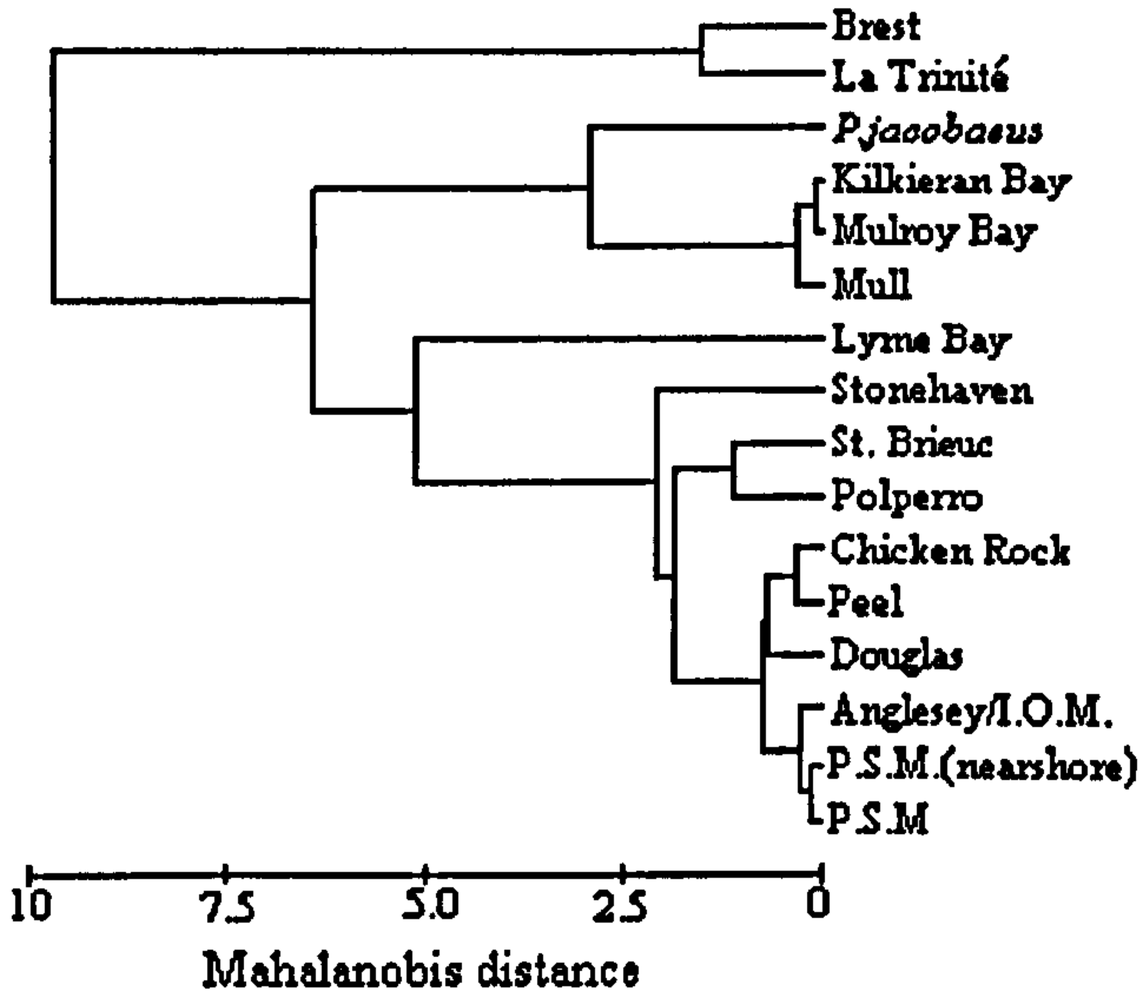


Figure 2.9. UPGMA dendrogram of Mahalanobis distance calculated from PC2-5 scores calculated using PCA with the covariance matrix from 5 shell characters in the morphometric analysis of *P. maximus* and *P. jacobaeus.*.

pigmentation did not cover the entire valve but usually ended after the first, second or third winter ring and did not colour the entire surface, the methods of classification were unsuitable in this instance.

2.3 Discussion

Although growth rates have been compared among geographically separated populations of *P. maximus*, there are no published studies comparing shell shape among populations.

In this study, inter-sample differences were observed for the three aspects of morphology examined; rib number, continuous measurements and colouration.

Univariate meristic data suggests that on the basis of ridge numbers *P. maximus* populations are inseparable but that *P. jacobaeus* have more ridges than *P. maximus* and indeed ridge number has previously been stated as different between the two species (Wagner, 1991). The St. Brieuc *P. maximus* population also appears different from the Peel populations and from the Polperro, Chicken Rock and Kilkieran Bay in the two analyses, but since the animals from St. Brieuc do not have consistently fewer ridges than scallops from other populations and because there is no detectable pattern to the observed significant differences this character seems unsuitable for discrimination of populations. Data on ridge number could not be included in the PCA analysis which was limited to mensural parameters. The nature of this data set, with wide variation in lengths (reflecting overall size or age) among sampling sites (Figure 2.3) required that growth be accounted for in the analysis. This would not be problematical if every character used grew isometrically resulting in no shape change throughout growth but since the very goal of the analysis was to attempt to uncover any differences in shape among geographically isolated populations, then should growth not be isometric and given that sizes of samples were different, then inter-population differences may be masked by or confused with ontogenetic differences. Although size differences among populations may be a useful discriminating factor they would firstly be influenced by environmental factors such as water temperature (Atkinson, 1994) and food availability and, secondly size data are of no use if scallop size classes were non randomly sampled, as in this case. Thus not only is the variation in sizes among samples a confusing factor as it may suggest differences among

populations which are solely a result of variable sampling practices, but also it creates possible confusion due to interplay with allometric effects.

The first principal component extracted from the covariance matrix is widely believed to be a size component (Blackith and Reyment, 1971; Atchley *et al.*, 1976; Oxnard, 1978; Dillon Jr., 1984; Cook *et al.*, 1985; Dillon Jr. and Manzi, 1989; James and McCulloch, 1990; Machado and Costa, 1994) but actually contains details of allometry (Shea, 1985) and this was shown for *P. maximus*. Since all characters had strong negative loadings on PC1 this verifies that it indeed represents a size related component. Once PC1 is removed, thus controlling the influence of size variation and allometry then any differences among groups in the remaining PCs should be a result of actual differences in shell shape among populations. Principal component analysis, in contrast to the discrimination on the basis of ridge number, provides little evidence that continuous measurements provide a means for discriminating between the two *Pecten* species although the fourth principal component extracted from the PCA provides some limited evidence for differentiation of the two species, mainly attributable to height differences. The variation described by this component was, however low. The paucity of useful discriminating characters is surprising given that the shells appear obviously different with a much more concave upper valve, steep sided, rectangular ridges (*P. maximus* ridges are rounded) and a finer pattern to the sculpture of the shell. Thus perhaps the choice of variables was poor or the analysis inadequate. Variables should be chosen to reflect the shape of the entire animal whilst in this case they measured only the maximum exterior dimensions. The interactions among such measurements (calculated through multivariate analysis) can be useful (Oxnard, 1978) but perhaps other measurements such as muscle scar dimensions, ligament length etc. may prove more effective. The number of measurements used was low since only easily measurable characters were used and a substantial increase may be beneficial (Oxnard, 1978). As stated by Rohlf (1990a) "the problem of selection of sets of variable is non-trivial because different, but seemingly equally reasonable sets of variables can yield very different results".

The inability of these variables to discriminate between the two species raises doubts over whether they will prove useful for discriminating conspecific populations. However there was some separation on the basis of continuous variables with the La Trinité and Brest populations having lower values of PC2 suggesting they had smaller hinges (Figure 2.5; 2.6). The La Trinité and Lyme Bay populations also showed some evidence of differences from other populations on the basis of PC3 to which no single character could be traced. Through discriminant analysis of all principal component scores except PC1 correct placement of shells from the La Trinité and Brest sites occurred 84.5% of the time into the pooled group suggesting these populations have distinctly different shaped shells. This was not the case for Lyme Bay animals

If body shape variation is heritable then the analysis of morphological variation among geographically isolated populations may provide an indication of genetic differentiation. However phenetic methods, such as PCA, used to measure the overall similarity among organisms require no suppositions about the genetic basis behind the characters used in its analyses but use only a variety of quantitative measurements to describe and compare organisms (Patterson, 1987), thus interpretation of the results of such an analysis must take into account not only a possible genetic viewpoint, but other potential causal mechanisms such as environmental influences, sex specific differences or ontogenetic variation. In this case *Pecten* are hermaphrodite and ontogenetic variation is hopefully controlled by elimination of PC1 leaving only genetic and environmental influences as possible effecters. The possible influence of the environment is the overriding problem with the use of morphometrics for delineating populations. There is no way of knowing to what extent it has influenced the morphology of individual animals in comparison to the effects of genetic differences. For instance substantial morphological differences seen between *Placopecten magellanicus* from deep and shallow water populations have been attributed to environmental effects (Volckaert *et al.*, 1991). Differences between onshore and offshore populations on the basis of morphology have also been seen for the American lobster *Homarus americanus* (Saila and Flowers, 1969). How much the differing environmental conditions in the various regions in this study have affected the shape of *P. maximus* cannot be

known so it is impossible to say whether significant differences in ridge number and hinge length indicate reduced gene flow among populations. However it is worth considering that on the basis of the continuous measurements that *P. jacobaeus* was not as different from its congener as the Brest and La Trinité populations were from other populations even given the differing environment (deep, Mediterranean water) from which it was taken, which to some extent argues against considerable environmental influence. Also in the intraspecific comparison the only useful differentiating variable was hinge length, a factor which is difficult to link to any facet of the environment.

Not only could the environment affect the shell shape but it could be related to year class. Kenchington and Full (1994) found that year class effects were greater than area differences in *P.magellanicus*, for which the hinge becomes more pronounced at about 10cm (Kenchington and Full, 1994). Whether this would have had an effect on the differentiation of the Brest and La Trinité populations is doubtful. It is unlikely that these 2 samples were represented by single year classes which may have had unrepresentative shapes, considering the distribution of lengths.

Colour variability was also noted to exhibit heterogeneity. Although *P. maximus* is known to show colour variation (Cain, 1988a) the most common shell colour is brown on the upper surface with a pale lower valve. In the St. Brieuc population there was however a substantial number of differently pigmented individuals, far many more than seen in any other population. Minchin (1991) has suggested that this is a common feature of Brittany scallops but the other two Brittany populations did not share this feature. The pigmentation of the lower valve which ended at one of the winter rings suggests that non-genetic factors may be responsible however if this were the case then the colouration may be expected to restart in later years or to end mid-year, dependent on the conditions, particularly if the colouration results from some factor in the food source. The alternative is that colouration has a genetic basis, as in *Argopecten* (Adamkewicz and Castagna, 1988). A prevalence of purple shells was seen in a hatchery rearing of *P. maximus* (referred to in Minchin, 1991) but since no record of parental colouration was kept, this cannot be taken as direct evidence of the heritability of

colouration. If the genetic basis of pigmentation were determined then the preponderance of variants in St. Brieuc Bay may be evidence for the genetic distinctness of these scallops.

3.0 Allozyme variation in *P. maximus* and *P. jacobaeus*

3.1 Materials and methods

3.1.1 Sampling

For details of sampling refer to Chapter 2. Allozyme variation was examined in 10 samples (excluding PS1, PS2, CHI, PEE, DOU and POL). In all cases except Lyme Bay, samples were received live (occasionally moribund) and samples of tissue (≈2mm^3 each of adductor muscle and digestive gland) were immediately placed in 1.5ml eppendorf tubes and frozen at -70°C. The Lyme Bay sample was received frozen then stored at -20°C with samples being taken from whole frozen scallops.

3.1.2 Sample preparation

Since enzymes are not uniformly distributed throughout tissues, for example, digestive enzymes such as esterases occur predominantly in the digestive gland whilst many enzymes involved in glycolysis/TCA cycle are abundant in the adductor muscle, initially a small sample of digestive gland was ground together with a portion of adductor muscle to ensure all enzymes should be present. However this drastically lowered the resolution of PGD allozymes which were clear from adductor muscle homogenates but smeared and uninterpretable when adductor muscle/digestive gland homogenates were used. To avoid this problem two samples from each specimen were prepared, one adductor muscle only (used in the gel to be stained for PGD/PGM, GPI and ODH) and one adductor muscle/digestive gland combination. Small portions (≈2mm^2) of tissue were ground, on ice, in 2 volumes of 0.1M Tris-HCl (pH 8.0) buffer in 1.5ml eppendorf tubes with a steel macerating rod, aided by a pinch of sterile sand. Samples were then centrifuged at 12,000 r.p.m. for 5 minutes before application to the gel. After application, which directly followed preparation, remaining sample was frozen at -20°C and saved for use as markers and for repeat runs to resolve inconsistencies if necessary. The resolution and intensity of GR, EST-D and LAP allozymes were severely affected by freezing homogenates whilst only the intensity of the other loci was affected.

3.1.3 Gel preparation

In order to obtain the best resolution for the loci under study a number of buffer systems were tested (see Table 3.1). Choice of buffer system can affect resolution of allozyme variants (Clayton and Tretiak, 1972; Schaal and Anderson, 1974; Dillon Jr., 1985; Aebersold *et al.*, 1987). In preliminary screenings, all resolvable enzymes

produced the clearest patterns on either a discontinuous tris-citrate (TC) pH6.3 (gel) and pH6.7 (electrode) system, used for resolution of GR (and LAP) allozymes and a tris-maleic-EDTA pH 7.4 (TME) system for the other loci. For buffer recipes see appendix B. Gels (12.5% w/v) were prepared by thoroughly mixing 68.75g starch (Sigma) in 550ml gel buffer, boiling (in a microwave) whilst maintaining complete suspension of the starch through periodic (every 30s) swirling. The starch mix was then de-gassed under vacuum to remove air bubbles and poured to excess into perspex frames (15 x 8.5 x 0.6cm) held onto glass plates with elastic bands. Following cooling (at room temperature for 1hr followed by cooling at 4°C for ≥1hr), excess set starch was sliced off to leave a 6mm thick gel with a flat, uniform surface. A slit was then cut parallel to the long (15cm) axis of the gel and 3cm from the edge for application of samples.

3.1.4 Gel loading and running

Samples were applied into the gel soaked onto Whatman Nº1 chromatographic paper strips (approximately 2 x 6mm) dipped into the supernatant, blotted then inserted into the sample slit in the gel ensuring that they covered the full height of the gel. Up to thirty samples were run per gel including 5 "markers" from previous runs to enable identification of homologous alleles, as well as a sample of bromophenol blue (which migrates quickly when an electrical field is applied and acts as a check that the gel is running correctly) and, a sample of horse spleen ferritin. Ferritin is a coloured protein that can be used to ensure equivalent protein migration across gels. TME gels were run at 80-90v overnight (16-18hrs) and TC gels run at 100-120v overnight, both at 4°C. See Figure 3.1 for details of gel running rig.

3.1.5 Staining of gels

After ensuring completion of electrophoresis (judged from distance moved by ferritin-approximately 50-70mm), gels were taken from the electrophoresis set-up and filter papers removed, then the gel was sliced horizontally to allow different stains to be applied to multiple slices of the same gel. By replacing the original 6mm former with one of 2mm the gel could be sliced using a taut wire, into a 4mm thick and a 2mm thick slice. The 4mm thick slice was subsequently sliced into 2 2mm thick portions. The 3

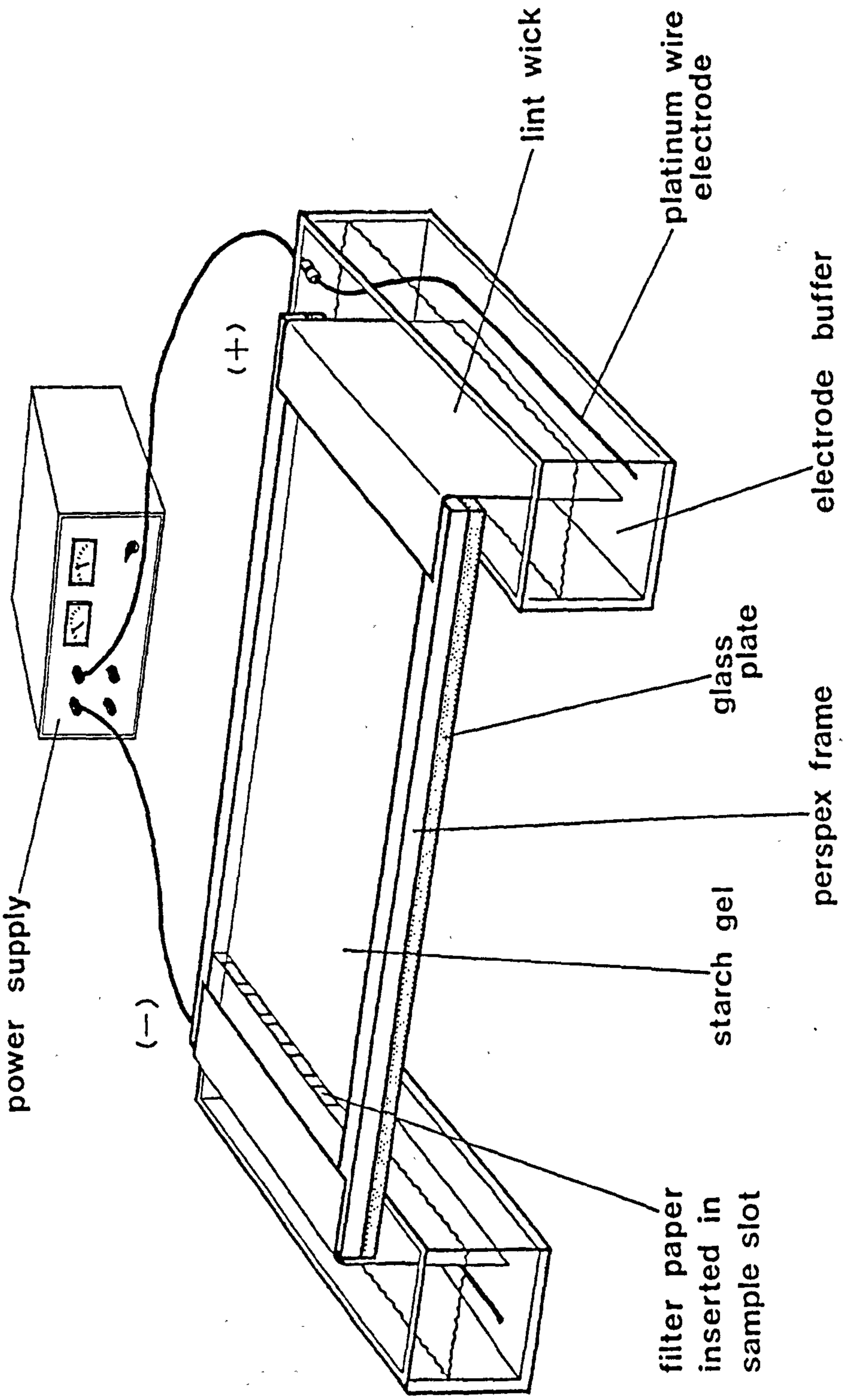


Figure 3.1. Apparatus for starch gel electrophoresis.

slices were placed on glass plates with a freshly cut face uppermost and enclosed by 6mm thick formers, creating 3 freshly sliced surfaces for application of histochemical stains. Stain recipes (see appendix B) were taken from Balakirev (1985), Balakirev and Zaykin (1990a,b), Beaumont *et al.* (1983), Dillon (1991b), Harris and Hopkinson (1976) and Schaal and Anderson (1974) however, whereas Harris and Hopkinson (1976) suggest the use of phenazine methosulphate (PMS) as a proton transfer agent within some stains, Meldola blue (0.8%) was used due to its lower carcinogenicity (Turner and Hopkinson, 1979). All stains except esterase were applied in agar solution to ensure the stain set on the surface to stabilise banding patterns. Agar was prepared by mixing 2g agar per 100ml distilled water and boiled in a microwave then cooled to 60°C and stored in a prewarmed oven until use. Stain ingredients were measured out and buffers made ready before gels were prepared for staining. After gel slicing these were dissolved in their respective buffers and mixed with agar before the mix was poured over one of the cut faces of the gels. After the agar had cooled and set the gels were incubated at 37°C in the dark until banding patterns appeared. Gels were then photographed and scored.

Variation at a number of loci (Table 3.1) was first screened in a sample of Isle of Man scallops. After the initial screening 2 polymorphic loci not previously studied in *P. maximus* (*Dia* Enzyme Commission Nº 1.6.2.2; and *Gr*; 1.6.4.2) were selected for continued screening along with *Est-D* (3.1.1.1) and the enzyme loci *Gpi* (5.3.1.9), *Odh* (1.5.1.11), *Pgd* (1.1.1.44) and *Pgm* (2.7.5.1) which had all been used in other studies (Beaumont and Beveridge, 1984; Huelvan, 1985; Beaumont *et al.*, 1993; Igland and Nævdal, 1995). The *Lap* (3.4.11.*) locus showed clear variation in early trials but could not be resolved consistently across samples during sample screening.

3.1.6 Interpretation of banding patterns

All loci for which banding patterns could be resolved sufficiently coded for either monomeric or dimeric enzymes as evidenced by the number of bands in heterozygotes. In both instances homozygotes display a single band on a stained gel. Heterozygotes for monomeric enzymes exhibit 2 bands whilst heterozygotes for dimeric enzymes have 3 bands (Figure 3.2). In all cases the subunit number matched those stated in Harris and Hopkinson (1976).

Gels were drawn and photographed and the distance from the origin (the slit in

Enzyme	EC	Locus	Buffer	Resolution	Polymorphism
Acid Phosphatase	3.1.3.2	*Acp*	1,2	nr	nr
Adenylate Kinase	2.7.4.3	*Ak*	2,7	nr	nr
Alcohol dehydrogenase	1.1.1.1	*Adh*	1	nr	nr
Aldehyde oxydase	1.2.3.1	*Ao*	1,7	g	m
Aldolase	4.1.2.13	*Ald*	1,5	nr	nr
Alkaline Phosphotase	3.1.3.1	*Akp*	5	nr	nr
Arginine Kinase	2.7.3.3	*Ark*	1	g	m
Aspartate aminotransferase	2.6.1.1	*Aat-1*	1,2,10	g	m
	2.6.1.1	*Aat-2*	1,2,10	p	v
Beta Galactosidase	3.2.1.23	*B-Gal*	1,6	p	v
Beta-N-Acetylglucosaminidase	3.2.1.30	*Hex*	1,2	g	m
Catalase	1.11.1.6	*Cat*	1,6,10	p	v
Carbonic Anhydrase	4.2.1.1	*Ca*	1,6	p	v
D-Amino Acid Oxidase	1.4.3.3	*Damox*	6	nr	nr
NADH Diaphorase	1.6.2.2	*Dia*	1,2,6,7	vg	p
Esterase	3.1.1.1	*EstD-1*	1,4	p	v
	3.1.1.1	*EstD-2*	1,4	g	p
Foramaldehyde Dehydrogenase	1.2.1.1	*Fdh*	1,2	e	m#
Fructose 1-6 Bisphosphatase	3.1.3.11	*Fbp*	1,2,9	p	v
Glycolate Oxidase	1.1.3.1	*Gox*	1	nr	nr
Glucose Phosphate Isomerase	5.3.1.9	*Gpi*	1	e	p
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	*G6pdh*	1,3,7	nr	nr
a-Glycerophosphate Dehydrogenase	1.1.1.8	*Gpd*	1,5	p	v
Glutamate Pyruvate Transaminase	2.6.1.2	*Gpt*	1	nr	nr
Glutathione Reductase	1.6.4.2	*Gr*	1,2,4,6	vg	p
Glutathione-S-Transferase	2.5.1.18	*Gst*	1,6,9,10	g	m
Guanylate Kinase	2.7.4.8	*Guk*	6	nr	nr
Hexokinase	2.7.1.1	*Hk*	1,4,6,7	qg	v
Inorganic Pyrophosphatase	3.6.1.1	*Ipp*	1,2,4,6	qg	m
Isocitrate dehydrogenase	1.1.1.42	*Icd-1*	1,3	e	m#
	1.1.1.42	*Icd-2*	1,3	e	m
Lactate Dehydrogenase	1.1.1.27	*Ldh*	1	nr	nr
Leucine Aminopeptidase	3.4.11.*	*Lap*	1,9	p-e	p/v
Malate Dehydrogenase	1.1.1.37	*Mdh*	1,8,9	g	m#
Malic Enzyme	1.1.1.40	*Me*	1	p	v
Mannose Phosphate Isomerase	5.3.1.8	*Mpi*	1,2,6,7,9,10	p	v
Octopine Dehydrogenase	1.5.1.11	*Odh*	1	e	p
Peptidase	3.4.11.*	*Pep*	1,6	g	v
Phosphoglucomutase	2.7.5.1	*Pgm*	1	e	p
6 Phosphogluconate Dehydrogenase	1.1.1.44	*Pgd*	1,2,6,7,9	e	p
Phosphofructokinase	2.7.1.11	*Pfk*	1,2	nr	nr
Phosphoglycerate Kinase	2.7.2.3	*Pgk*	1,2	nr	nr
Pyruvate Kinase	2.7.1.40	*Pk*	1,2	nr	nr
Sorbitol Dehydrogenase	1.1.1.14	*Sdh*	1,6	nr	nr
Superoxide Dismutase	1.15.1.1	*Sod*	1,2,6	qg	m
Tyrosinase	?	*Tyr*	1,6,8	nr	nr
General Protein	-	*Gp*	2	nr	nr

Table 3.1: Loci stained in preliminary screening of allozyme variation in *P. maximus*. E.C.=Enzyme Commision Number. Buffer: 1) TME pH7.4. 2) Tris-Citrate (buffer 3 of Saavedra *et al.*,1993). 3) N-(3-Aminopropyl)-morpholine (Clayton and Tretiak, 1972). 4) TME/Trisodium citrate. 5) Tris-Citrate (Ahmad *et al.* 1977). 6) TBE. 7) Tris-Citrate pH6.3/6.7. 8) Phosphate buffer XIV. 9) Tris-Citrate pH8. 10) Poulik. Resolution: nr=no result, p=poor, qg=quite good, g=good, vg=very good, e=excellent. Polymorphism: nr=no result, m=monomorphic, m#=≥2 alleles (polymorphism < 95%), v=variable but unresolved, p=clear polymorphism.

the gel) to the centre of each band measured. The commonest allele in the preliminary screening was designated the 100 allele and other alleles numbered relative to this, for example, if there was a band that migrated 90% as far as the commonest allele it would be designated the 90 allele. Such relative mobilities were calculated for each allele.

Figure 3.2: Banding patterns revealed by staining allozyme loci. a: 2 codominant alleles, monomeric protein. b) 3 codominant alleles, monomeric protein. c: 2 codominant alleles, dimeric protein. d: 2 codominant alleles, tetrameric protein. e: 1 normal and 1 null allele. From Ferguson, 1980.

3.1.7 Nomenclature of loci and alleles

Nomenclature of alleles and loci followed the standard system for invertebrates with enzymes designated by capitals (e.g. GPI) and, loci in italics with the initial letter uppercase (e.g. *Gpi*). Multiple loci are designated in the order of increasing mobility (e.g. *Gpi-1*, *Gpi-2* etc.). Allele relative mobility is indicated by superscript text.

3.1.8 Statistical analysis

The Biosys program v1.7 (Swofford and Selander, 1981) was used for most statistical analyses with genotype frequencies as input. For the *Gpi* locus there were more observed genotypes than the program is capable of analysing therefore alleles were pooled to 5 (Gpi^{38}-Gpi^{78}, Gpi^{87}, Gpi^{93} & Gpi^{100}, Gpi^{105}-Gpi^{115}, Gpi^{119}-Gpi^{136}). Where calculations were performed manually ($N_{(eff)}$, H(exp), gene diversity) the allele frequencies used for *Gpi* were calculated from unpooled genotypes. Pooled *Gpi* genotype or allele frequencies were used in Hardy-Weinberg comparisons, F statistics, genetic identities and distances and dendrogram production (all performed within Biosys).

3.1.8.1 Allele frequencies

Allele frequencies for each locus are determined as follows:

$$Frequency = \frac{(2N_{hom} + N_{het})}{2N}$$

Where N_{hom} is the number of individuals homozygous for the allele, N_{het} is the number of individuals heterozygous for the allele and N is the number of individuals examined for that locus.

3.1.8.2 Variability

A number of measures of variability can be calculated from genotype frequency data but as only loci that were polymorphic were used in this study measures such as proportion of polymorphic loci are meaningless.

One suitable measure of genetic variation at a locus is the number of alleles seen yet this takes no account of their respective frequencies. The effective number of alleles (N_{eff}) does account for this:

$$N_{eff} = \frac{1}{\sum x_i^2}$$

where x_i is the frequency of the i^{th} allele.

A further indicator of variation in the sample is the observed heterozygosity (proportion of heterozygous individuals):

$$H_o = \frac{N_{het}}{N}$$

3.1.8.3 Population structure

In order to establish if the population is in Hardy-Weinberg equilibrium at each locus, χ^2 values comparing observed genotype frequencies with those expected under Hardy-Weinberg equilibrium given the actual allele frequencies, were calculated:

$$\chi^2 = \frac{(O-E)^2}{E}$$

where O is the observed number of occurrences of a genotype in the sample and E is the number of occurrences expected if the population were in Hardy-Weinberg equilibrium determined as follows. For a 2 allele system with alleles A and B segregating with frequencies of p and q respectively, the number of occurrences of the genotypes AA, AB and BB will be Np^2, 2Npq and Nq^2 where N is the sample size. Since this is a χ^2 test, no expected value should be less than 1 and if this proves to be the case then alleles must be pooled to produce synthetic genotypes and the χ^2 recalculated. The calculated χ^2 value can then be compared to the χ^2 distribution with $\frac{1}{2}(n^2-n)$d.f. where n is the number of alleles (after pooling if performed). Deviation from Hardy-Weinberg expectations was considered significant at $p<0.05$. Where multiple alleles are observed the expected genotype frequencies can be calculated through expansion of the relevant polynomial, for example, where there are 3 alleles A, B and C at frequencies p, q and r respectively genotype frequencies would be expected in the proportions $(p + q + r)^2$. Thus genotypes AA, BB, CC, AB, AC, BC would be expected in the proportions Np^2, Nq^2, Nr^2, 2Npq, 2Npr and 2Nqr. Because performance of multiple tests, essentially testing the same hypothesis was required, significance levels were adjusted according to the method of Hochberg (1988). This procedure orders tests according to probability. The highest value is tested against the significance level α (0.05). If $p<\alpha$ then all tests are significant. If $p\geq\alpha$ this test is insignificant and the next highest p value is tested against a modified significance level

$\alpha'_{i+1}=\alpha/(1+i)$, where I is the number of tests already performed. When a test is significant, testing stops and it and all subsequent tests are deemed significant.

Wright's F statistics were also used to detect deviations from Hardy-Weinberg equilibrium. The genetical structure of populations can be described with the aid of Wright's three F-statistics, F_{IS}, F_{IT} and F_{ST} whose interrelationship can be written in the form $(1 - F_{IT}) = (1 - F_{ST}).(1 - F_{IS})$. F_{IS} provides information on the structure of individual populations, F_{IT} on the structure of the population as a whole and F_{ST} on population subdivision (F_{ST} was not calculated, although its equivalent G_{ST} was-see below).

Wright defined F_{IS} as the correlation between homologous alleles within individuals with reference to the local population. There are a number of formulae for calculation of F_{IS} but Nei (1987) described a method using only heterozygosity levels:

$$F_{IS} = \frac{h_s - h_O}{h_O}$$

where h_S (sample heterozygosity) is the average over all populations of $(1-\sum x_i^2$; expected heterozygosity under Hardy-Weinberg equilibrium within the population) and, h_O is the observed heterozygosity thus it is a measure of deviation of a sample from Hardy-Weinberg equilibrium within a sub-population where positive values indicate a deficiency of heterozygotes. It is calculated for every allele and every locus of each population and a mean taken for the average value $\overline{F}_{IS}$. The significance of F_{IS} was tested with the formula of Waples (1987) where the null hypothesis $F_{IS} = 0$ is evaluated with a χ^2 test:

$$\chi^2 = F_{IS}^2 N(k - 1); \; df = [k(k - 1)]/2$$

where k is the number of alleles at the locus and N the sample size. Significance levels for multiple tests were adjusted according to Hochberg (1988).

F_{IT} is the corresponding allelic correlation with reference to the total population regardless of population sub-structure and is calculated using gene and genotype frequencies from the total population.

$$F_{IT} = \frac{(h_T - h_O)}{h_T}$$

where h_T (total heterozygosity) and h_O are the expected and observed heterozygosities of the total population. If $F_{IT}\sqrt{N}>1.96$, F_{IT} is deemed significantly different from 0 at the

5% level (Skibinski *et al.*, 1983). Significance levels for multiple tests were adjusted according to Hochberg (1988).

3.1.8.4 Geographic variation

In order to quantify population subdivision a number of approaches were taken. The first was to check for differences in allele frequencies among populations with χ^2 contingency tests performed for each locus between each pair of populations. Due to small sample sizes and high variability, standard χ^2 would be inappropriate without substantial pooling of alleles as many cells would contain expected values less than 1 (Lessios, 1992). As this would lose information χ^2 tests were performed with a Monte-Carlo method (Roff and Bentzen, 1989) using the MONTE option in REAP (McElroy *et al.*, 1992) with 1000 randomisations. In this way the probability of obtaining by chance a χ^2 value higher than that calculated from the data matrix is determined by randomising the matrix (keeping row and column totals equal) and re-determining the χ^2 from randomised data. This is repeated 1000 times and p is taken as the number of randomised values higher than the original. Again, because of multiple tests, significance levels for tests at each locus were adjusted (Hochberg, 1988).

The second method to quantify any differences among populations was via Nei's genetic identity (Nei, 1972) calculated for each locus as follows:

$$I = \frac{\Sigma x_i y_i}{\sqrt{(\Sigma x_i^2 \Sigma y_i^2)}}$$

Where x_i and y_i are the frequencies of the i^{th} allele in populations x and y. Nei's I can be averaged over all loci and then becomes:

$$\bar{I} = \frac{I_{XY}}{\sqrt{(I_X I_Y)}}$$

Where I_{XY}, I_X and I_Y are the arithmetic means over all loci of $\Sigma x_i y_i$, Σx_i^2 and Σy_i^2 respectively.

Nei's genetic distance is calculated from this simply by the following equation:

$$D = - \ln.I$$

In order to graphically represent differences among populations a dendrogram was created from Nei's D using the UPGMA method, described by Ferguson (1980) to sequentially cluster the most identical pairs. This creates a graphical representation of

the relationships among sub-populations on the basis of data from this particular set of loci.

Genetic diversity can also be analysed using Nei's (1973) gene diversity analysis. Gene diversity statistics are related to F statistics and indeed G_{ST}, the coefficient of gene differentiation among populations can be used to estimate F_{ST} (Slatkin and Barton, 1989). Gene diversity analysis is also used to ascertain the relative contribution of the within sample and among sample heterozygosity to the total heterozygosity.

The sample gene diversity H_S was calculated for each locus as follows:

$$H_S = 1 - J_S$$

where J_S is the sample gene identity:

$$J_S \equiv \sum \overline{x_i^2}$$

where x_i^2 is the squared frequency of the i^{th} allele averaged across S subpopulations. The total gene diversity H_T is then:

$$H_T = 1 - J_T$$

and

$$J_T = \sum_i \bar{x}_i^2$$

where $\bar{x}_i$ is the average allele frequency over all populations ($=(\sum x_{ik})/s$).

From these, D_{ST}, the gene diversity among populations is calculated as:

$$D_{ST} = H_T - H_S$$

D_{ST} includes comparisons of populations with themselves. To exclude these the absolute measure of gene differentiation $\overline{D}_m$ is calculated as:

$$\overline{D}_m = \frac{s.D_{ST}}{(s-1)}$$

Gene differentiation relative to the total population (G_{ST}) is then given by:

$$G_{ST} = \frac{D_{ST}}{H_T}$$

Since this is effectively F_{ST}, significance was attached as for F_{ST}. To test the null hypothesis $G_{ST} = 0$ for single locus G_{ST} values a χ^2 test was used (Waples, 1987):

$$\chi^2 = 2NG_{ST}(k - 1); \text{ d.f.} = (k - 1)(s - 1)$$

where N is the total number of individuals examined, k is the number of alleles and s the number of populations. Significance levels were adjusted for multiple tests.

If the studied populations are at equilibrium (for example not recovering from bottlenecks) then the calculated G_{ST} values can be used to estimate the effective number of migrants (N_em) among areas on the basis of these data.

$$N_em \approx \frac{1}{4}\left(\frac{1}{G_{ST}} - 1\right)$$

3.2 Results

3.2.1 Within sample variation

Preliminary screenings identified enzyme loci as monomorphic, polymorphic or variable but unresolvable with the conditions employed. Some produced no visible result (Table 3.1). Of those loci that did give clear patterns only those with over 95% polymorphism were screened across all samples. These were NADH-diaphorase (*Dia*) esterase-D (*Est-D*), glucose phosphate isomerase (*Gpi*), glutathione reductase (*Gr*), leucine amino peptidase (*Lap*), octopine dehydrogenase (*Odh*), phosphogluconate dehydrogenase (*Pgd*) and phosphoglucomutase (*Pgm*). However *Lap* proved to be inconsistently resolvable and so was not used on most samples. Thus 7 isozyme loci were scored on samples of *P. maximus* from 8 sites around the U.K. and France and 1 sample of *P. jacobaeus* from the Mediterranean. The *Dia*, *Est-D* and *Lap* loci were unresolvable in samples from Lyme Bay possibly due to the storage regime of the Channel samples which probably went through multiple, partial freeze-thaw cycles. Table 3.2 summarises the allele frequencies for the various populations. No alleles were fixed in particular populations nor did there appear to be significant shifts in frequencies among populations. Although all loci were chosen for their high degree of polymorphism, levels varied among loci. Polymorphism and heterozygosity were lowest for *Pgd* or *Pgm* depending on the population examined and, highest for *Gpi* where a total of 16 alleles were present and on average 77% of individuals were heterozygous (see Table 3.3). Heterozygosity estimates were not dramatically different among populations.

3.2.2 Hardy-Weinberg equilibrium

χ^2 comparisons of genotype frequencies with Hardy-Weinberg expected levels produced only 5 significant outcomes from unpooled data (Table 3.3). These were the *Est-D* and *Odh* loci at St. Brieuc, the *Pgd* and *Pgm* loci of individuals from Kilkieran

Locus	R.M.	BRE	STB	KIL	MRY	LAT	PEE	STO	MUL	LYM	JAC
Dia	94	0.000	0.033	0.000	0.017	0.087	0.016	0.021	0.017		0.075
	100	0.638	0.750	0.667	0.700	0.609	0.742	0.729	0.683		0.500
	105	0.310	0.200	0.300	0.233	0.283	0.242	0.229	0.217		0.425
	110	0.034	0.017	0.033	0.050	0.022	0.000	0.021	0.083		0.000
	116	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
	N	29	30	30	30	23	31	24	30		20
Est-D	64	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000		0.000
	75	0.017	0.000	0.000	0.000	0.000	0.017	0.000	0.000		0.000
	84	0.000	0.017	0.017	0.017	0.017	0.050	0.000	0.000		0.000
	100	0.569	0.550	0.500	0.533	0.586	0.500	0.667	0.595		0.675
	108	0.069	0.067	0.052	0.000	0.052	0.017	0.000	0.000		0.175
	119	0.328	0.317	0.431	0.433	0.328	0.367	0.292	0.310		0.125
	125	0.017	0.033	0.000	0.017	0.017	0.033	0.042	0.071		0.025
	140	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.024		0.000
	N	29	30	29	30	29	30	24	21		20
Gpi	38	0.033	0.000	0.017	0.017	0.000	0.000	0.042	0.000	0.017	0.000
	50	0.000	0.000	0.017	0.000	0.017	0.000	0.000	0.033	0.000	0.000
	64	0.083	0.033	0.100	0.017	0.017	0.065	0.083	0.050	0.083	0.000
	74	0.067	0.083	0.050	0.217	0.086	0.081	0.104	0.067	0.100	0.119
	78	0.033	0.017	0.017	0.000	0.017	0.000	0.000	0.017	0.017	0.000
	87	0.217	0.183	0.200	0.217	0.190	0.210	0.167	0.167	0.183	0.071
	93	0.033	0.033	0.017	0.017	0.000	0.000	0.021	0.033	0.000	0.095
	100	0.200	0.250	0.167	0.366	0.207	0.306	0.313	0.367	0.067	0.429
	105	0.000	0.033	0.017	0.000	0.000	0.000	0.000	0.017	0.000	0.000
	112	0.217	0.233	0.150	0.050	0.103	0.145	0.083	0.117	0.317	0.048
	114	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024
	115	0.017	0.000	0.050	0.017	0.017	0.000	0.042	0.017	0.033	0.024
	119	0.033	0.000	0.017	0.017	0.069	0.081	0.021	0.067	0.017	0.166
	124	0.033	0.100	0.167	0.067	0.190	0.097	0.104	0.017	0.150	0.000
	130	0.000	0.017	0.016	0.000	0.017	0.000	0.000	0.033	0.000	0.000
	136	0.033	0.017	0.000	0.000	0.069	0.016	0.021	0.000	0.017	0.024
	N	30	30	30	30	29	31	24	30	30	21
Gr	62	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000
	75	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000
	85	0.100	0.067	0.017	0.067	0.077	0.016	0.021	0.050	0.000	0.100
	96	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000
	100	0.500	0.500	0.500	0.567	0.692	0.500	0.500	0.500	0.519	0.475
	110	0.033	0.000	0.033	0.017	0.019	0.016	0.021	0.017	0.019	0.000
	119	0.300	0.417	0.450	0.300	0.212	0.419	0.438	0.417	0.423	0.375
	125	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.050
	130	0.017	0.000	0.000	0.017	0.000	0.000	0.021	0.000	0.019	0.000
	140	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000
	N	30	30	30	30	26	31	24	30	26	20
Odh	78	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	87	0.000	0.000	0.033	0.017	0.017	0.016	0.000	0.000	0.017	0.024
	90	0.333	0.283	0.217	0.133	0.259	0.226	0.417	0.433	0.217	0.143
	100	0.633	0.683	0.733	0.833	0.707	0.726	0.583	0.567	0.733	0.810
	108	0.033	0.033	0.000	0.017	0.017	0.032	0.000	0.000	0.033	0.024
	N	30	30	30	30	29	31	24	30	30	21
Pgd	52	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000
	75	0.133	0.067	0.100	0.100	0.069	0.065	0.188	0.067	0.121	0.071
	100	0.867	0.917	0.867	0.817	0.914	0.919	0.813	0.933	0.879	0.929
	125	0.000	0.017	0.033	0.017	0.017	0.016	0.000	0.000	0.000	0.000
	N	30	30	30	30	29	31	24	30	29	21
Pgm	71	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000
	77	0.017	0.000	0.000	0.000	0.017	0.032	0.021	0.033	0.033	0.024
	89	0.050	0.017	0.050	0.000	0.034	0.065	0.042	0.033	0.067	0.071
	100	0.883	0.933	0.917	0.967	0.914	0.839	0.896	0.833	0.850	0.881
	114	0.050	0.050	0.033	0.033	0.034	0.065	0.021	0.083	0.050	0.024
	124	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000
	N	30	30	30	30	29	31	24	30	30	21

Table 3.2: Allele frequencies at 7 loci in 9 populations of *P. maximus* and 1 sample of *P. jacobaeus*.

R.M.=relative mobility.

POPULATION	LOCUS	N	N(o)	N(e)	H(obs)	H(exp)	Chi-sq	df	p
Rade de Brest	*Dia*	29	4	1.98	0.379	0.495	4.547	6	0.603
	Est-D	29	5	2.29	0.379	0.564	12.512	10	0.252
	Gpi	30	12	6.56	0.833	0.848	5.345	10	0.867
	Gr	30	6	2.83	0.733	0.646	8.726	15	0.891
	Odh	30	3	1.95	0.4	0.487	2.597	3	0.458
	Pgd	30	2	1.30	0.266	0.231	0.710	1	0.399
	Pgm	30	4	1 27	0.233	0.214	0.523	6	0 998
St. Brieuc	*Dia*	30	4	1.66	0.3	0.396	10.059	6	0.122
	Est-D	30	6	2.44	0.467	0.591	0.466	1*	0.495
	Gpi	30	11	5.84	0.8	0.828	10.719	10	0.380
	Gr	30	4	2.33	0.667	0.572	3.372	6	0.761
	Odh	30	3	1.83	0.5	0.452	0.724	1*	0.395
	Pgd	30	3	1.18	0.1	0.155	6.498	3	0.090
	Pgm	30	3	1 15	0.133	0.126	0.153	3	0 985
Kilkieran Bay	*Dia*	30	3	1.87	0.433	0.464	0.533	3	0.912
	Est-D	29	4	2.28	0.414	0.561	4.611	6	0.595
	Gpi	30	14	7.39	0.767	0.865	10.609	10	0.389
	Gr	30	4	2.20	0.567	0.546	3.200	6	0.783
	Odh	30	4	1.71	0.4	0.414	1.295	6	0.972
	Pgd	30	3	1.31	0.2	0.238	0.544	1*	0.461
	Pgm	30	3	1.18	0.133	0.156	3 580	1*	0 058
Mulroy Bay	*Dia*	30	4	1.83	0.533	0.453	2.517	6	0.867
	Est-D	30	4	2.12	0.3	0.527	8.970	6	0.175
	Gpi	30	10	4.23	0.6	0.763	10.070	10	0.434
	Gr	30	7	2.40	0.6	0.583	4.977	21	1.000
	Odh	30	4	1.40	0.333	0.287	1.200	6	0.977
	Pgd	30	4	1.47	0.267	0.318	3.690	6	0.718
	Pgm	30	2	1.07	0.067	0.064	0 036	1	0 850
La Trinite sur Mer	*Dia*	23	4	2.18	0.435	0.542	4.913	6	0.555
	Est-D	29	5	2.20	0.586	0.546	1.653	10	0.998
	Gpi	29	12	6.94	0.828	0.856	7.206	10	0.706
	Gr	26	4	1.89	0.384	0.47	6.823	6	0.338
	Odh	29	4	1.76	0.448	0.433	0.828	6	0.991
	Pgd	29	3	1.19	0.103	0.16	6.249	3	0.100
	Pgm	29	4	1.19	0 172	0 162	0 258	6	1 000
Peel	*Dia*	31	3	1.64	0.323	0.391	1.573	3	0.666
	Est-D	30	7	2.57	0.5	0.611	9.210	21	0.987
	Gpi	31	8	5.38	0.677	0.812	16.086	10	0.097
	Gr	31	5	2.33	0.613	0.571	2.831	10	0.985
	Odh	31	4	1.73	0.419	0.421	1.181	6	0.978
	Pgd	31	3	1.78	0.161	0.15	0.239	3	0.971
	Pgm	31	4	1.40	0 29	0.287	2 820	6	0.831
Stonehaven	*Dia*	24	4	1.71	0.417	0.415	3.752	6	0.710
	Est-D	24	3	1.88	0.417	0.469	1.609	3	0.657
	Gpi	24	11	6.02	0.875	0.834	17.722	10	0.060
	Gr	24	5	2.26	0.667	0.557	4.245	10	0.936
	Odh	24	2	1.95	0.667	0.486	3.311	1	0.069
	Pgd	24	2	1.44	0.375	0.305	1.278	1	0.258
	Pgm	24	5	1.24	0 208	0.194	0 324	10	1 000
Mull	*Dia*	30	4	1.92	0.467	0.479	1.106	6	0.981
	Est-D	21	4	2.19	0.619	0.544	3.014	6	0.807
	Gpi	30	13	5.20	0.867	0.808	6.613	10	0.761
	Gr	30	5	2.34	0.667	0.573	3.739	10	0.958
	Odh	30	2	1.97	0.4	0.491	1.033	1	0.310
	Pgd	30	2	1.14	0.133	0.124	0.153	1	0.696
	Pgm	30	5	1.42	0.3	0.296	11.808	10	0.298
Lyme Bay	*Gpi*	30	12	5.55	0.833	0.82	11.684	10	0.307
	Gr	26	5	2.23	0.5	0.55	3.258	10	0.975
	Odh	30	4	1.71	0.3	0.414	4.603	6	0.596
	Pgd	29	2	1.27	0.241	0.212	0.546	1	0.460
	Pgm	30	4	1.37	0.3	0.269	0 934	6	0.988
P.jacobaeus	*Dia*	20	3	2.29	0.7	0.564	2.346	3	0.504
	Est-D	20	4	1.99	0.35	0.498	0.818	1*	0.366
	Gpi	21	9	4.10	0.571	0.756	14.322	10	0.159
	Gr	20	4	2.64	0.7	0.621	4.687	6	0.585
	Odh	21	4	1.48	0.381	0.323	1.163	6	0.979
	Pgd	21	2	1.15	0.143	0.133	0.124	1	0.724
	Pgm	21	4	1 28	0.238	0.218	0 383	6	0 999

Table 3.3: Measures of variability and deviation from Hardy-Weinberg equilibrium at 7 loci, in 9 populations of *P. maximus* and 1 sample of *P. jacobaeus*. N=sample size. N(o)=observed number of alleles. $N_{(eff)}$ =effective number of alleles. H(obs)=observed heterozygosity. H(exp)=expected heterozygosity. Chi-sq=χ^2 estimate of deviation from Hardy-Weinberg equlibrium, df=degrees of freedom and probability of rejection of null hypothesis. * df after pooling.

POPULATION	*Dia*	*Est-D*	*Gpi*	*Gr*	*Odh*	*Pgd*	*Pgm*
Rade de Brest	0.234	0.327	0.065	-0.135	0.178	-0.154	-0.088
St. Brieuc	0.243	0.211	-0.028	-0.166	-0.107	0.355	-0.057
Kilkieran Bay	0.067	0.263	0.083	-0.038	0.034	0.159	0.146
Mulroy Bay	-0.178	0.431	0.181	-0.029	-0.161	0.162	-0.034
La Trinite	0.197	-0.074	0.013	0.05	-0.036	0.353	-0.062
Peel	0.174	0.182	0.176	-0.073	0.004	-0.073	-0.011
Stonehaven	-0.004	0.111	-0.08	-0.196	-0.371	-0.231	-0.071
Mull	0.026	-0.137	-0.114	-0.163	0.186	-0.071	-0.013
Lyme Bay	-	-	-	0.091	0.275	-0.137	-0.113
P.jacobaeus	-0.242	0.296	0.136	-0.127	-0.179	-0.077	-0.094
Mean Fis	0.05	0.179*	0.046	-0.083	-0.01	0.018	-0.044
Fit	0.073	0.2	0.075	-0.061	0.029	0.036	-0.03

Table 3.4: Summary of F statistics for 7 loci in 9 populations of *P. maximus* and 1 sample of *P. jacobaeus*. *<0.05.

Bay and *Est-D* of *P. jacobaeus*. Due to the presence of expected values less than 1 these may have been type I errors (Lessios, 1992), therefore alleles were pooled and calculations repeated. In order to get all expected values > 1 it was necessary to pool to 2 alleles (the most common plus all others). From the pooled data there was no evidence of significant deviation from equilibrium. F_{IS} values, for which calculation did not require pooling were not significant for these loci and indeed produced only 1 significant value, for *Est-D* in Mulroy Bay where there appeared to be a significant deficiency of heterozygotes (this was not suggested by the initial χ^2 test) but after correction for multiple testing (Hochberg, 1988) this was deemed insignificant. For *Est-D* the mean F_{IS} was also significant probably due to the extreme value of the F_{IS} value for the Mulroy Bay population.

Although after correction of significance levels there were no significant values of F_{IS} in particular populations, the F_{IS} values for *Gpi* and *Est-D* (although insignificant) were mostly positive suggesting a tendency for heterozygote deficiency. Equally, values

a)

Dia	Brest	St. Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	*P.jacobaeus*
Brest	-	5.28	1.08	2.98	6.08	5.12	3.16	4.26	8.06
St. Brieuc	0.231	-	3.83	1.59	2.87	1.65	3	3.23	8
Kilkieran Bay	0.935	0.251	-	1.75	5.5	3.66	2.05	3.1	8.02
Mulroy Bay	0 63	0.629	0.658	-	3.86	3.18	0.67	0.55	8.43
La Trinite	0.165	0.459	0.134	0.312	-	5.06	2.7	5.16	2.6
Peel	0.2	0.701	0.277	0.38	0.128	-	1.35	5.4	6.95
Stonehaven	0.54	0.915	0.522	0 872	0.488	0.802	-	2	6.7
Mull	0.291	0.371	0.377	0.884	0.176	0.125	0.635	-	10.17
P.jacobaeus	0.047	0.022	0.042	0 034	0.497	0.031	0 053	0 013	-

b)

Est-D	Brest	St Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	*P.jacobaeus*
Brest	-	3.3	4.22	7.07	2.16	6.46	5.21	6.84	7.53
St. Brieuc	0.922	-	4.19	6.44	1.46	6.16	5.51	4.39	8.25
Kilkieran Bay	0.567	0.606	-	4.13	2.22	6.18	8.38	9.78	14.18
Mulroy Bay	0.168	0.248	0.419	-	4.12	4.73	3.65	5.18	19.43
La Trinite	0.963	0.985	0.748	0.438	-	4.77	4.25	6.09	8.55
Peel	0.419	0.557	0.383	0.606	0.601	-	6.59	7.01	17.39
Stonehaven	0.267	0.35	0.043	0.303	0.402	0.355	-	1.7	11.39
Mull	0.175	0.538	0.031	0.224	0.261	0.407	0.701	-	12.59
P.jacobaeus	0 07	0.094	0 003	0***	0 039	0.001*	0 002	0 004	-

c)

Gpi	Brest	St. Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	Lyme Bay	*P.jacobaeus*
Brest	-	12.6	13.85	22.29	19.39	13.12	9.57	10.96	13.81	29.14
St. Brieuc	0.512	-	13.3	21.23	17.11	13.36	14.97	16.25	18.1	33.37
Kilkieran Bay	0.495	0.519	-	24.93	14.71	17.37	10.62	18.24	12.53	41.04
Mulroy Bay	0.007	0.015	0.007	-	21.7	15.61	8.56	18.69	34.77	22.22
La Trinite	0.082	0.176	0.398	0.02	-	11.41	14.37	20.15	20.62	28.56
Peel	0.271	0.236	0.164	0.082	0.398	-	9.72	14.77	20.96	24.11
Stonehaven	0.469	0.282	0 81	0.634	0.346	0.468	-	15.61	19.21	21.54
Mull	0.363	0.202	0.121	0.074	0.06	0.311	0.329	-	36.48	17.92
Lyme Bay	0.229	0.082	0.624	0***	0.027	0.01	0.035	0***	-	51.6
P.jacobaeus	0.001*	0***	0***	0 007	0***	0.004	0.009	0.156	0***	-

d)

Gr	Brest	St Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	Lyme Bay	*P.jacobaeus*
Brest	-	8.54	9.37	5.98	6.43	12.35	6.55	7.47	7.36	7.44
St. Brieuc	0.139	-	4.88	6.39	7.46	6.81	4.54	1.14	7 82	1.36
Kilkieran Bay	0.053	0.292	-	7.18	8.89	3.34	1.43	2.41	3.45	8.02
Mulroy Bay	0.607	0.491	0.252	-	4.2	6.39	4.48	5.53	7.67	6.79
La Trinite	0.24	0.067	0.021	0.752	-	10.46	8.18	6.39	10.95	7.43
Peel	0.032	0.23	0.556	0.364	0.016	-	3.7	5	5.78	9.32
Stonehaven	0.245	0.507	0.896	0.712	0.056	0.686	-	2.71	2.04	6.71
Mull	0.26	0.977	0.701	0.726	0.113	0.409	0 887	-	5.81	2.53
Lyme Bay	0.173	0.176	0 81	0.362	0.015	0.494	0.953	0.511	-	10.33
P.jacobaeus	0 287	0 748	0 07	0 477	0 083	0 057	0 189	0 722	0 038	-

e)

Odh	Brest	St. Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	Lyme Bay	*P.jacobaeus*
Brest	-	0.36	6.92	8.11	2.13	2.62	2.21	3	2.92	6.11
St. Brieuc	0.767	-	5.64	5.46	1.42	1.44	3.4	4.54	1.64	4.2
Kilkieran Bay	0.091	0.171	-	3.91	2.55	3.35	6.79	8.62	3.33	3.11
Mulroy Bay	0.007	0.07	0.349	-	2.99	2.2	12.16	14.58	1.91	0.16
La Trinite	0.494	0.849	0.715	0.449	-	0.42	4.26	5.97	0.55	2
Peel	0.401	0.823	0.647	0.661	0.991	-	6.34	8.1	0.02	1.27
Stonehaven	0.392	0.151	0.036	0.002	0.158	0.05	-	0.03	6.79	9.76
Mull	0.143	0 048	0.01	0 ***	0.053	0.019	0.84	-	8.62	11.69
Lyme Bay	0.365	0.763	0.642	0.668	0.843	0.965	0.042	0.011	-	1.05
P.jacobaeus	0 071	0.189	0 568	0.94	0 616	0.754	0 001*	0 ***	0.777	-

f)

Pgd	Brest	St. Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	Lyme Bay	*P.jacobaeus*
Brest	-	2.42	2.29	5.37	2.31	2.53	0.59	1.48	0.042	0.984
St. Brieuc	0.238	-	0.82	4.75	0	0	4.37	1.01	1.94	0.71
Kilkieran Bay	0.337	0.488	-	4.42	0.71	0.93	3.16	2.55	2.05	1.73
Mulroy Bay	0.094	0.204	0.244	-	4.52	4.97	5.47	5.87	5.08	4.09
La Trinite	0.315	0.861	0.624	0.221	-	0.01	4.15	1.05	1.86	0.73
Peel	0.183	0.847	0.487	0.13	0.875	-	4.59	0.98	2.02	0.7
Stonehaven	0 445	0.047	0.222	0.097	0.065	0.049	-	3.68	0.91	2.61
Mull	0 124	0.753	0.184	0.069	0.725	0.856	0.03	-	1.02	0.01
Lyme Bay	0.753	0.29	0.443	0.178	0.361	0.295	0.279	0.178	-	0.66
P.jacobaeus	0 187	0 789	0.415	0 243	0 837	0 825	0.062	0.711	0.279	-

g)

Pgm	Brest	St. Brieuc	Kilkieran Bay	Mulroy Bay	La Trinite	Peel	Stonehaven	Mull	Lyme Bay	*P.jacobaeus*
Brest	-	2.08	1.24	4.43	0.37	0.6	1.93	2.12	0.51	0.69
St. Brieuc	0.552	-	1.21	1.24	1.58	4.06	3.75	4.17	4.03	3.82
Kilkieran Bay	0.803	0.6	-	3.08	1.2	2.86	2.7	4.72	2.49	1.73
Mulroy Bay	0.136	0.335	0.254	-	3.19	6.96	5.29	6.88	6.65	3.99
La Trinite	0.947	0.566	0.819	0.383	-	1.55	1.44	2.67	1.21	0.84
Peel	0.882	0.229	0.462	0.045	0.638	-	2.92	1.78	0.12	0.99
Stonehaven	0.844	0.421	0.606	0.153	0.926	0.599	-	4.25	2.38	1.36
Mull	0.667	0.325	0.295	0.065	0.647	0.867	0.564	-	2.18	3.08
Lyme Bay	0 877	0.26	0.459	0.062	0.733	0.968	0.719	0.81	-	0.54
P.jacobaeus	0.842	0 258	0 647	0.05	0 82	0 786	0 865	0 614	0 915	-

Table 3.5a-g: Results of Monte-Carlo simulations of χ^2 tests for comparison of allele frequencies. Above diagonal=calculated χ^2 value, below=estimated *p* level. *=significant at 0.05 level after adjustment via the sequential method of Hochberg (1988). ***<0.001

for *Gr* and *Pgm* were mostly negative suggesting a tendency for an excess of heterozygotes in comparison with expectations. Over all loci and populations there is no evidence for general heterozygote deficiency or excess (Sign test. 36-ve values, 31+ve. Z = -0.6114; NS).

3.2.3 Among sample variation

Allele frequencies appeared to be relatively homogeneous across sampling sites (Table 3.2) with no allele appearing at high frequency in any area except for *Pgd* ^{52} which appeared four times in heterozygous combination in the Mulroy Bay sample and was not seen elsewhere. Other alleles *Dia*^{116}, *Est-D*^{64}, *Gpi*^{114}, *Gr*^{62}, *Gr*^{75}, *Gr*^{140}, *Odh*^{78}, *Pgm*^{71}, *Pgm*^{124} were also found in only one population but were at very low frequency and with an increase in sample size may well be seen in the other populations. From χ^2 tests no population appears distinct from any other although 3 tests between *P. maximus* populations were significant following adjustment of significance levels, two involving *Gpi* (LYM-MRY, LYM-MUL) and 1 for *Odh* (MRY-MUL). Nine other tests suggested significant differences in genotype frequency all involving interspecific comparisons mainly at the *Gpi* locus.

The results of the gene diversity analysis (Table 3.6) show that gene diversity (1-heterozygosity) is high within populations but not highly partitioned among populations such that within sample diversity (H_S) is nearly as great as the total diversity (H_T). Indeed from the H_S:H_T estimates it can be seen that 98% of the heterozygosity is within *P. maximus* populations and only 2% partitioned among populations. When calculations account for *P. jacobaeus* this rises slightly. These gene diversity measures appear to suggest a very low level of population subdivision, as has been indicated by χ^2 tests. Nevertheless, significance tests on the G_{ST} estimates (after correction for multiple tests) were significant for *Gpi*, *Gr* and *Odh* among just *P. maximus* populations and, significant for *Est-D*, *Gpi* and *Odh* over all samples (including *P. jacobaeus*). This appears to suggest that allele frequencies are not homogeneous across the species' range.

3.2.4 Genetic distances

Unbiased genetic distances were calculated for all loci over 8 *P. maximus* populations and 1 sample of *P. jacobaeus* (Table 3.7). Distances and identities between pairs involving Lyme Bay utilised data from only 5 loci (through two separate runs of

a)

LOCUS	H_T	H_S	D_{ST}	$\overline{D}_m$	G_{ST}	mN_e
Dia	0.4592	0.4543	0.0049	0.0056	0.0107 NS	23.11
Est-D	0.5584	0.5515	0.0069	0.0080	0.0124 NS	19.91
Gpi	0.8496	0.8260	0.0236	0.0265	0.0277***	8.68
Gr	0.5748	0.5632	0.0116	0.0131	0.0202 NS	12.13
Odh	0.4470	0.4318	0.0152	0.0171	0.0340***	7.10
Pgd	0.2140	0.2102	0.0038	0.0043	0.0178 NS	13.80
Pgm	0.1995	0.1967	0.0028	0.0032	0.0142 NS	17.36
Mean over all loci	0.4718	0.4620	0.0098	0.0111	0.0196	12.51

b)

LOCUS	H_T	H_S	D_{ST}	$\overline{D}_m$	G_{ST}	mN_e
Dia	0.4778	0.4665	0.0113	0.0127	0.0236 NS	10.34
Est-D	0.56	0.5456	0.0145	0.0163	0.0259***	9.40
Gpi	0.85	0.8192	0.0307	0.0341	0.0361***	6.68
Gr	0.5805	0.569	0.0115	0.0128	0.0199 NS	12.31
Odh	0.4376	0.4209	0.0167	0.0186	0.0382***	6.3
Pgd	0.2061	0.2024	0.0037	0.0042	0.0182 NS	13.49
Pgm	0.2015	0.1988	0.0027	0.003	0.0134 NS	18.41
Mean over all loci	0.4733	0.4603	0.013	0.0145	0.025	9.75

Table 3.6: Gene diversity (Nei, 1973, 1987) analysis of a) 9 populations of *Pecten maximus* (*P. jacobaeus* omitted). Calculations for *Dia* and *Est-D* were based on only 8 populations (excluding Lyme Bay, see text). b) 9 populations of *P. maximus* and 1 sample of *P. jacobaeus*. Calculations for *Dia* and *Est-D* were based on only 9 populations (excluding Lyme Bay, see text). Gene diversities: H_T = total diversity; H_S = within populations; D_{ST} = among populations; $\overline{D}_m$ = absolute differentiation among populations. Coefficient of gene differentiation: G_{ST} = among populations. *** < 0.001. NS = not significant. N_em = effective number of migrants.

BIOSYS and incorporation of the Lyme Bay D values into the matrix calculated for all other populations using 7 loci). Intraspecific distances were between 0.006-0.045 whilst when *P. jacobaeus* was included the maximum value increased to 0.05. The higher distances involved comparisons with Mulroy Bay, La Trinité sur Mer and Mull. The dendrogram from these data is shown in Figure 3.3 and depicts those populations with the higher D values as most divergent. No distinct clusters are apparent from this, although as expected *P. jacobaeus* branches off first.

BRE	-	0.991	0.988	0.977	0.98	0.985	0.986	0.985	0.982	0.957
STB	0.009	-	0.99	0.978	0.976	0.993	0.985	0.988	0.982	0.958
KIL	0.012	0.010	-	0.981	0.978	0.992	0.977	0.974	0.992	0.951
MRY	0.023	0.022	0.019	-	0.972	0.984	0.969	0.967	0.956	0.956
LAT	0.020	0.024	0.022	0.028	-	0.979	0.967	0.967	0.966	0.957
PEE	0.015	0.007	0.008	0.016	0.022	-	0.981	0.984	0.979	0.96
STO	0.014	0.015	0.023	0.031	0.034	0.019	-	0.992	0.963	0.951
MUL	0.015	0.013	0.027	0.034	0.034	0.016	0.008	-	0.956	0.955
LYM*	0.019	0.018	0.008	0.045	0.034	0.021	0.037	0.045	-	0.944
JAC	0.044	0.043	0.050	0.045	0.044	0.041	0.050	0.047	0.058	-
	BRE	STB	KIL	MRY	LAT	PEE	STO	MUL	LYM	JAC

Table 3.7: Nei's genetic identity (I) above diagonal and, distance (D) below diagonal calculated from data for 7 loci in 9 populations of *P. maximus* and 1 sample of *P. jacobaeus* (*only 5 loci for Lyme Bay).

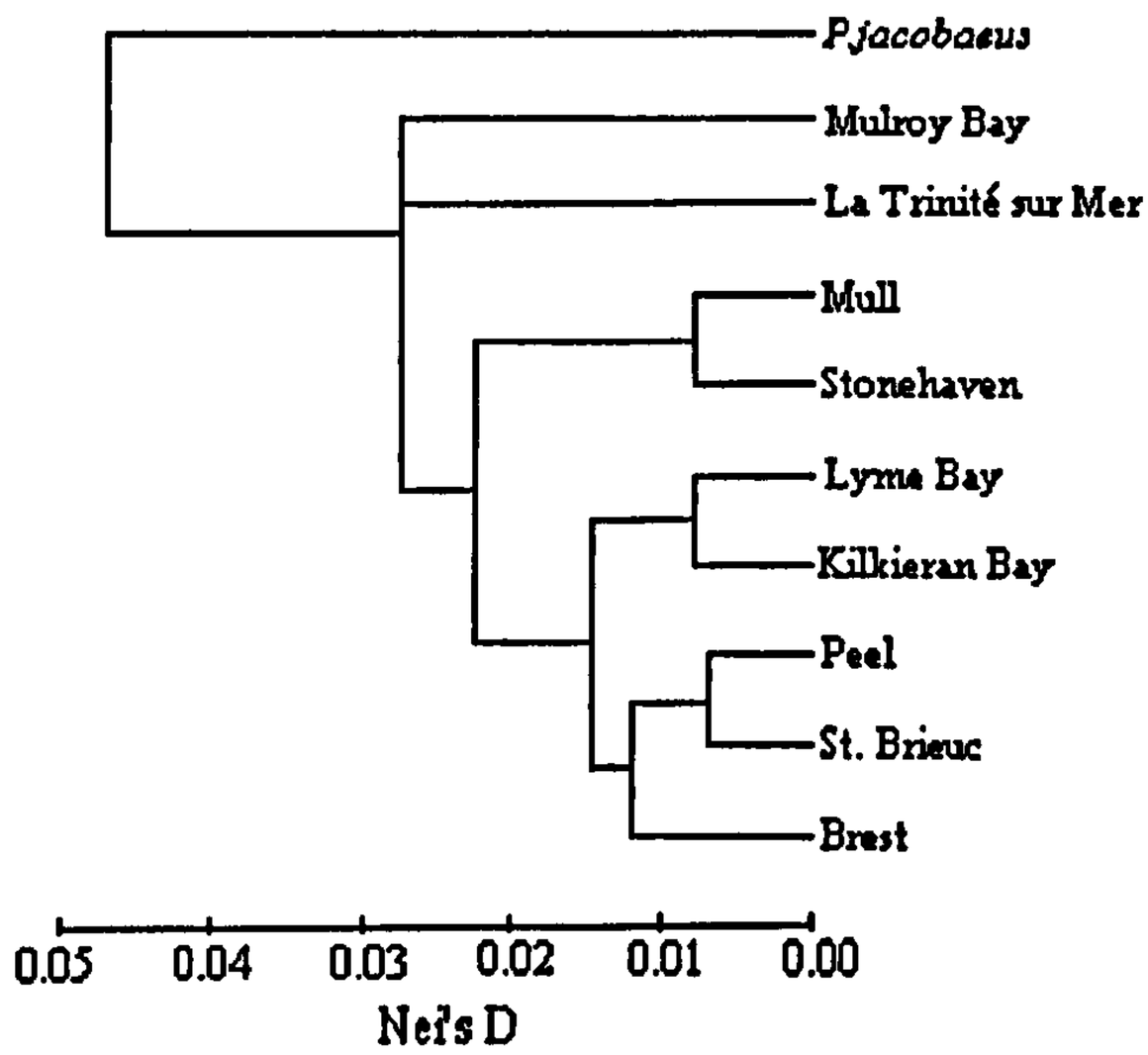


Figure 3.3. UPGMA dendrogram based on Nei's genetic distance for 9 populations of *P. maximus* and 1 sample of *P. jacobaeus*.

3.2.5 Gene flow among populations

The G_{ST} estimates were used to calculate the expected number of migrants among populations (N_em). Although some G_{ST} values were not significantly different from zero the figures for each locus (Table 3.6) do show that at every locus the numbers of migrants are greater than the levels required to prevent fixation of alleles, but not necessarily to preserve similar allele frequencies (Allendorf and Phelps, 1981). The lowest per locus estimate was for *Odh* for which the estimate was 7.103 for all *P. maximus* populations and 6.295 when allele frequencies for *P. jacobaeus* were included. The exceptionally low frequencies of alleles confined to single populations meant that these N_em estimates could not be confirmed via Slatkin's (1985) private allele method.

3.3 Discussion

3.3.1 Allozyme variation in *Pecten maximus*

Allozyme electrophoresis has proved useful for detecting geographic heterogeneity in allele frequencies in scallop populations (Beaumont, 1982a; Kijima *et al.*, 1984; Fevolden, 1989; Fevolden, 1992; Krause *et al.*, 1994; Lewis and Thorpe, 1994) but not in *P. maximus* (Beaumont *et al.*, 1993). In this study, genetic variation at seven polymorphic allozyme loci was quantified in 9 populations of *P. maximus* from the U.K., France and Eire and 1 population of *P. jacobaeus*. Variability at the studied loci was high (Tables 3.2 and 3.3) but provided little convincing evidence for geographic structure to the genetic variation. Estimates of variation [N(e) and H(o)] matched closely those of Beaumont *et al.* (1993) for the loci included in both studies although *Pgm* was slightly less variable in this study. Allele frequencies for *Odh* and *Pgd* were not directly comparable due to quite different relative mobilities, probably due to differing electrophoretic conditions in the two studies. Frequencies and relative mobilities at the *Pgm* locus were concordant. Data on allozyme variability for *P. jacobaeus* (Peña *et al.*, 1995) including *Odh*, *Pgd* and *Pgm* are not directly comparable since allele mobilities do not correspond. Heterozygosity levels for *Odh* are similar in this study to those of Peña *et al.* (1995) but the values in this study for *Pgd* and *Pgm* are higher.

In contrast to the majority of studies of allozyme variation in bivalves there is little evidence for deficiencies of heterozygotes. The *Dia*, *Est-D* and *Gpi* loci had a

majority of positive values of F_{IS} but none were significant. *Gpi* and *Est-D* have previously been noted to exhibit evidence of heterozygote deficiency by Beaumont and Beveridge (1984) and it was suggested that for *Gpi* any deviation from Hardy-Weinberg equlibrium could be due to mis-scoring for this highly variable locus. *Est-D* has been suggested not to be totally under direct genetic control and this may explain the deficiency of heterozygotes observed by Beaumont and Beveridge (1984) and the particularly high mean F_{IS} value in this study. Alternatively, deficiency of heterozygotes could be attributed to the presence of null alleles. Hoare and Beaumont (1995) have provided evidence that *Odh* null alleles in *M.edulis* are not necessarily deleterious to the organism. Since null alleles may not be selected out, their frequency in a population may be sufficient to cause an apparent deficiency of heterozygotes.

Overall there is little evidence for any pattern of population subdivision for *P. maximus* apparent from these allozyme data. Allele frequencies appeared relatively homogeneous across sites and although three χ^2 were significant suggesting differences among sites, two of these were at the highly polymorphic *Gpi* locus. This apparent heterogeneity may be attributable to inadequate sample sizes such that the actual allele frequencies of each population are not accurately depicted. If sample sizes had been greater, to ensure the sample allele frequencies equated to the population allele frequencies for this very variable locus (assuming that they did not where only 20-30 animals were scored) then the χ^2 tests may have been insignificant.

Although variability was high (only polymorphic loci were studied) the variability appeared only slightly partitioned among populations, most (98%) being within populations (Table 3.6). This low level of among sample variation does not appear to reveal anything concerning population structure. As *P. maximus* appears to have an extensive dispersal capability such a result is not surprising and is in agreement with previous studies that have failed to detect significant differences in allele frequencies among *P. maximus* populations (Huelvan, 1985; Beaumont *et al.*, 1993). High variability within populations seems typical of bivalves. The proportion of variability within populations of *Mytilus galloprovincialis*, 97.1% (Quesada, Zapata and Alvares, 1995) and the oysters *Crassostrea virginica* 96.1%, *C.gigas*, 96.1% and *Saccostrea commercialis* 97.7% (Saavedra *et al.*, 1995) are all of similar levels to the partitioning of variability for *P. maximus*. In *Ostrea edulis* which broods its

larvae and where limited evidence of population differentiation is shown (Saavedra *et al.*, 1993) only 91.2% of the variation is within populations (Saavedra *et al.*, 1995).

Despite this lack of inter-sample variation and insignificant χ^2 values, G_{ST} values suggested some population heterogeneity with significant G_{ST} values within *P. maximus* populations for the *Gpi* and *Odh* loci. The excessive variation at the *Gpi* locus with many alleles at low frequency, the frequencies of which may not be accurate due to the relatively low sample sizes, is the likely cause of the inflated G_{ST} estimate at this locus. Had larger sample sizes been analysed for this locus ensuring representative allele frequencies, the level of G_{ST} may have been reduced and the significance removed. The significant G_{ST} estimate for *Odh* cannot be explained in this way since there were far fewer alleles. In a case such as this where a single locus exhibits differences not seen for other loci then this may be attributable to selection, since if stochastic forces are responsible for any observed population differentiation G_{ST} values should be roughly equivalent across loci (Lewontin and Krakauer, 1973). Lewontin and Krakauer (1973) developed a test for identification of this but because the assumptions behind their proposed test to show this are flawed, such that factors other than selection can produce significant outcomes (Lewontin and Krakauer, 1975; Nei and Maruyama, 1975; Robertson, 1975) the test was not applied to the data presented here. *Odh* was less variable and the allele frequencies are probably representative, thus if this is the only locus contributing to heterogeneous G_{ST} estimates (presuming the value for *Gpi* is a result of sampling error) then selection may be responsible. The major contributor to the elevated G_{ST} for *Odh* estimate comes from the Mulroy Bay population and indeed the *Odh* Nei's I values were lower for intraspecific comparisons involving Mulroy Bay than those excluding this population (0.951 and 0.977). Octopine dehydrogenase allozymes have been hypothesised to be subject to selective pressures in *Placopecten magellanicus* where deviations from Hardy-Weinberg equilibrium at the *Odh* locus, due to deficiency of heterozygotes, were argued to be a result of associative overdominance (Volckaert and Zouros, 1989). If selection can operate at the *Odh* locus in scallops then this may explain the differences seen for *Odh* in *P. maximus.* Selection against particular genotypes in Mulroy Bay would result in differences between Mulroy Bay and other areas which would elevate G_{ST} (of course *Odh* allele frequencies in other populations will affect the G_{ST} but the differences of these from the

mean are not as great as in Mulroy Bay). If selection operates it would be expected that F_{IS} values would reflect this, since conformation to Hardy-Weinberg equilibrium is limited evidence that selection is not operating, however they are non-significant. However heterozygote deficiencies decreased with age for *P.magellanicus* (Volckaert and Zouros, 1989) and given the large size of adult scallops in this study (see Figure 2.3) may have become insignificant despite the previous action of selection. Since Mulroy Bay is a very different environment from other studied areas, being enclosed, exceptionally sheltered (Minchin, 1981, 1983) and having large populations of predatory starfish (Minchin, 1981, 1992) the escape response to predators linked with *Odh* variation by Volckaert and Zouros (1989) may produce different selective pressures from other environments and hence be responsible for the differences detected.

Nei's D values were relatively high for intraspecific studies (Table 3.7) but this is in large part due to the use of only highly variable loci in this study. Previous studies on *P. maximus* where genetic distances were lower (Beaumont *et al.*, 1993; maximum D = 0.034) and (Huelvan, 1985; maximum D = 0.0132) incorporated data from other less polymorphic loci such as *Icd* and *Mdh* which had they been included in this study would likely have lowered the mean D. Although high, the genetic distances between populations provided no evidence to suggest any population was reproductively isolated from any other, confirming that the few significant values are of little value in suggesting population structure. This is reinforced by the dendrogram based on Nei's D where there is no clear clustering of a population or group of populations. Even *P. jacobaeus* does not appear to be highly divergent from the *P. maximus* samples.

It seems that data from allozyme electrophoresis has been of only limited value in detecting population subdivision in *P. maximus*, results being inconclusive and vague. The failing of allozyme methods to reveal population substructure (assuming substructure exists) is not due to lack of variation. The loci studied here were highly polymorphic but little variation was partitioned among populations. Of course such subdivision may not actually occur and thus the inferred high gene flow may be typical of *P. maximus* populations. With allozyme methods for the loci currently detectable and scorable there is no way of disproving this hypothesis although inclusion of data from further loci may change this picture (Beaumont, 1991a). The significant

differences noted in some comparisons involving, for instance Mulroy Bay and the resultant Nei's D values which were almost as high as for pairwise comparisons involving the congeneric *P. jacobaeus* provide only limited evidence that a search for such loci may prove fruitful.

3.3.2 Interspecific differentiation

There were only limited differences evident between the two species on the basis of allozyme data. Heterozygosity levels within the two species were not drastically different although they were slightly lower for *P. jacobaeus.* No loci had diagnostic alleles for differentiation between *P. maximus* and *P. jacobaeus* and using χ^2 tests, allele frequencies were not significantly different for most pairwise comparisons of *P. maximus* populations with *P. jacobaeus*. Most tests that were significant involved the *Gpi* and *Est-D* loci which are highly variable and therefore sampling error may explain the heterogeneity. Differences between species would be expected in interspecific comparisons but in greater numbers than seen here. Indeed although congeneric species would be expected to have identical allele frequencies at some loci and overlapping frequencies at others, some loci should exhibit fixed differences (Thorpe and Solé-Cava, 1994). This is not seen at any loci in the interspecific comparisons undertaken in this study. Of course the loci studied here may not be truly representative of the interspecific relationships. When *P. maximus* and *P. jacobaeus* were considered together then the within sample variation in allele frequencies explained 97.5% of the total (Table 3.6) once again suggesting that differentiation is limited. Indeed this estimate is much higher than the value for *Ostrea edulis* (Saavedra *et al.*, 1995) in which population subdivision is seen and a much lower value would be expected in this study.

Estimates of G_{ST} involving *P. jacobaeus* would be expected to be significant and this proved to be the case for *Est-D, Gpi* and *Odh* (these were perhaps expected due to heterogeneity suggested by χ^2 values). However *Gpi* and *Odh* G_{ST} estimates were significant for *P. maximus* alone and the significance seen when *P. jacobaeus* data is included may not be due to the inclusion of these data.

Although no gene flow is expected between these species, from the G_{ST} levels effective numbers of migrants were estimated to be over 6 for all loci (Table 3.6) which would be expected to prevent fixation of alleles but not necessarily divergence

(Allendorf and Phelps, 1981). Gene flow between true species would not be expected and indeed would violate the biological species concept. If the N_em values are accurate then the specific status of *P. jacobaeus* is brought into question. The N_em values will however be inaccurate if the G_{ST} values are erroneous, if the loci are affected by selection, or if the populations are not at equilibrium.

Nei's I values were, overall, lower in interspecific comparisons (0.954) than those involving conspecifics (0.979) although a value of 0.954 is still higher than the value expected for comparison of congeneric species. Thorpe and Solé-Cava (1994) show empirically derived frequency distributions for I values within genera, species and populations. The value reported here falls within the range expected for populations not species. As an example, I values among congeneric oysters (*Crassostrea*) are 0.483-0.584 as compared to interspecific values of 0.926-0.966 (Hedgecock and Okazaki, 1984) values which are considerably lower than those calculated in this study. Figure 3.4 depicts the dendrogram drawn from Nei's D values and although *P. jacobaeus* splits first, it is not highly divergent from other populations. Overall there is no evidence of substantial genetic divergence between the two *Pecten* species. Indeed they are capable of hybridisation in the laboratory (Huelvan, 1985) which would likely not be possible in highly divergent species. This is not to say that interbreeding occurs in the wild if the distributions slightly overlap. The two species are morphologically differentiable (Chapter 2) and there is no evidence for genetic introgression although there are no published accounts of population genetics over the Meditteranean ranges of these species which would aid in understanding their true status. Although genetic distance measures provide "guidelines, not absolute yardsticks for making taxonomic decisions" (Rosenblatt and Waples, 1986) they do show the close similarity of the two studied species and overall the results of this study calls into question the specific status of *P. jacobaeus* and perhaps argues for a lowering to subspecific status.

4.0 Mitochondrial DNA length variation

4.1 Materials and methods

4.1.1 Isolation of mitochondria

In order to extract mtDNA the mitochondria must first be released. This is apparently easiest with soft tissues such as gonads (Chapman and Powers, 1984) but gonads are not available all year in contrast to adductor muscle, therefore the method of Snyder *et al.* (1987) with the modifications of Boulding *et al.* (1993) and other minor refinements was employed to extract mtDNA from adductor muscle. Approximately 5g of tissue was used, fresh where possible, or after thawing of frozen samples. Tissue was minced with a sharp scalpel, added to 10ml of homogenising buffer (see appendix B) in a 50ml polycarbonate tube and macerated using an Ultra-Turrax tissue homogeniser. This required operation at approximately 75% full speed for over 30 seconds and was judged complete when no flakes of tissue were visible. Homogenisation of adductor muscle with teflon in glass pestle and motar systems was impossible as the tissue could not be adequately macerated. Immediately following homogenisation 2ml protease type XXIII (Sigma, 200mg ml^{-1} in homogenising buffer) was added, mixed by inversion then incubated at 37°C with shaking, for approximately 3 hours in a water bath. The mixture changed from a pale-brown viscous "soup" to a dark-brown watery liquid. Protease XXIII digests the muscle filaments but does not break down mitochondrial walls, thus mitochondria are left in a non-viscous suspension along with other cell components and debris which were removed by centrifugation for 10 mins at 2,000g in a Heraeus Sepatech centrifugation, with a fixed angle rotor. This slow speed spin removed nuclei and cell debris but was not fast enough to pellet mitochondria. Following centrifugation the supernatant was poured into a clean tube then centrifuged once again, for 30 mins at 40,000g to pellet the mitochondria. Following this second high speed spin which pelleted the mitochondria, the supernatant was aspirated from the mitochondrial pellet which was then resuspended in 1-2ml of homogenising buffer.

Once isolated, this crude mitochondrial preparation was cleaned on a sucrose gradient (Lansman *et al.*, 1981), prepared by underlaying 7.5ml of 1.0M sucrose (see appendix B) with 2.5ml 1.5M sucrose in a 16.5ml polycarbonate tube.

The mitochondrial suspension (from step 6) was then pipetted on top, the tubes balanced and spun in an MSE Superspeed 65 centriuge for 45 mins (time at speed) at 25,000r.p.m. (≈100,000g) with a swing-out rotor. Use of fixed angle heads was inadequate for sucrose-gradient centrifugation. On completion of this step the intact mitochondria banded at the 1.0M/1.5M sucrose interface, appearing as a white-pale brown band and could be removed with a Pasteur pipette, taking care not to disturb the dark pellet at the bottom of the tube, deemed to contain cell debris, nuclei and other cellular components which had escaped the slow speed isolation. After addition to a new tube with approximately 10ml of homogenising buffer the mitochondria were pelleted for 20 mins at 40,000g in the Heraeus centrifuge. At certain times of the year the supernatant remained cloudy after this centrifugation step and the pellet was unconsolidated. When this occurred, the supernatant was aspirated off and 10ml homogenisation buffer added to the mitochondria which were then repelleted.

4.1.2 Extraction of mitochondrial DNA

Following isolation of mitochondria, mitochondrial DNA was released through lysis of the mitochondria using the non-ionic detergent NP-40. This lyses mitochondrial walls but if used at low concentration will not lyse nuclear membranes (Chapman and Powers, 1984; Edwards and Skibinski, 1987) and therefore if any escape removal, nuclear DNA should not be released. The supernatant from above the mitochondrial pellet was removed by aspiration taking care to remove as much liquid as possible and the pellet resuspended in STE (see appendix B) buffer to make a final volume of 600µl (this required addition of less than 600µl STE accounting for the pellet's volume, approximately 530µl is usually sufficient) and added to a 1.5ml micro-centrifuge tube. Then 80µl 10% (v/v in water) Nonidet P-40 was added to lyse the mitochondria, mixed by inverting the tubes repeatedly and kept on ice for 15 mins. Following release of mtDNA, mitochondrial debris was removed through spinning for 3 mins at full speed in a micro-centrifuge. After removal of the supernatant (now containing mitochondrial contents including mtDNA) and transferal to a new tube, 100µl 5M NaCl and 80µl CTAB (see appendix B) were added to the suspension. Note that the volumes (600:100:80) are critical and the ratio must be maintained (add more STE to bring

mitochondrial suspension to 600µl if necessary). This was then incubated at 65°C in a water bath for 15 minutes to precipitate polysaccharides which appear as pale flocculent suspensions. After cooling to room temperature, 600µl chloroform: isoamyl alcohol (24:1; C:IAA) were added and mixed by inversion for 1-2 mins then spun at 13,000g in microfuge for 3 mins. All C:IAA and subsequent phenol extractions were performed in a fume hood. Following removal of the aqueous supernatant which was transferred to a new tube an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1; P:C:IAA) was added, mixed by inversion, then spun at 13,000g for 3 mins. Phenol extraction removed proteins, including DNAases from the preparation and was judged successful when no precipitate was observable at the interface. Extraction was repeated until the interface was clear. After these cleansing steps the supernatant was added to a new tube using a micropippete, noting the volume. In order to precipitate the mtDNA, 1 vol 3M sodium acetate pH 5.2 and 0.6 vol isopropanol were added and the tube incubated overnight at -20°C. Once precipitation had occurred the mtDNA was then pelleted at 13,000g for 30 mins and the residual alcohol poured off and replaced with approximately 0.5ml 70% ethanol. By "washing" the DNA in 70% ethanol excess salt precipitated with the DNA was removed, whilst the DNA remained as a precipitate. Dislodgement of the pellet ensured salt could be washed from all surfaces of the pellet. If not adequately removed it can affect restriction digestion and electrophoresis. The DNA pellet was dislodged from the tube wall by "finger flicking" the tube. Once washed, the pellet was spun again for 10 mins at 13,000g then the supernatant discarded and excess ethanol removed by touching the tube walls with tissue. The pellet was then left to air dry until it went clear. The DNA was then resuspended in 50µl dH_2O and stored at -20°C.

4.1.3 Restriction digestion of mtDNA

8µl mtDNA solution, 10µl dH_2O, 2µl buffer and 3 units (U) enzyme were added to a 0.5ml microfuge tube and mixed by "finger flicking" the bottom of the tube then incubated in a water bath or thermal cycler at relevant temperature (usually 37°C) for 2 hours. Fifteen minutes prior to the end of the digestion period RNAase was added to 50µg ml^{-1} (1µl of 1mg ml^{-1} stock to 20µl). The extraction procedure for

mtDNA also released substantial quantities of RNA (as judged by agarose electrophoresis) and RNAase treatment prevented this from causing problems for assessing RFLPs. *Bam*HI, *Eco*RI, *Hin*dIII, *Xho*I and *Eco*RI-*Hin*dIII digests were undertaken. Double digests used 3U each enzyme, undertaken in multi-core (Promega) buffer. On completion of digestion the reaction was stopped by addition of 1-2µl gel loading dye (50mM EDTA, 30% glycerol, 0.25% [w/v] bromophenol blue).

4.1.4 Agarose gel electrophoresis

Electrophoresis was undertaken in a standard "submarine" electrophoresis rig in which gels were both cast and run. In order to cast gels, baffles were placed at the end of the base plate, a glass plate laid on the base plate and the comb positioned approximately 1cm from the baffle at the cathodal end and 1mm above the glass plate.

Agarose electrophoresis was undertaken in either tris-borate or tris-acetate buffers (Sambrook *et al.*,1989; see appendix B). TBE could be used more times without losing buffering capacity but could only be stored in a 5x concentration whereas although TAE required replacement after 2-3 runs, it could be stored at 50x concentration and therefore did not require frequent preparation. No differences were noted on the performance of the buffers if regularly replaced. Gels were made by boiling 0.8g agarose in 100ml of either 0.5xTBE or 1xTAE buffer, then cooled to approximately 60°C. The joints of the gel rig were then sealed by running a bead of agarose around the edge and bottom of each baffle. Once set then the remaining agarose solution was poured into the gel mould, the comb positioned and the gel left to set for 1 hour. Following setting, baffles were carefully removed and 1300ml buffer (0.5xTBE or 1xTAE) poured into the tank to cover the gel and the comb removed.

After restriction digestion of samples was complete and gel loading dye had been added, these were pipetted into individual sample wells of the gel under the buffer surface. When restriction digestion was undertaken in 20µl or less, 20 samples could be electrophoresed per gel. Larger samples required larger wells and hence fewer samples could be examined. Following gel loading, gels were run at either 70V for 3-5 hours or 20V overnight (Figure 4.1). Longer, lower voltage

runs result in sharper and straighter bands and reduce the likelihood of "smiling" effects. Electophoresis was judged complete when the bromophenol blue migrated to within 2-3cm of the gel edge. At 0.8% agarose concentration this represents the distance migrated by an approximately 500bp DNA fragment (Sambrook *et al*, 1989). On completion gels were removed and stained in ethidium bromide solution (1mg ml^{-1} in gel buffer) for 30-45 minutes. Excess stain was removed with water and the gel examined under UV light then photographed.

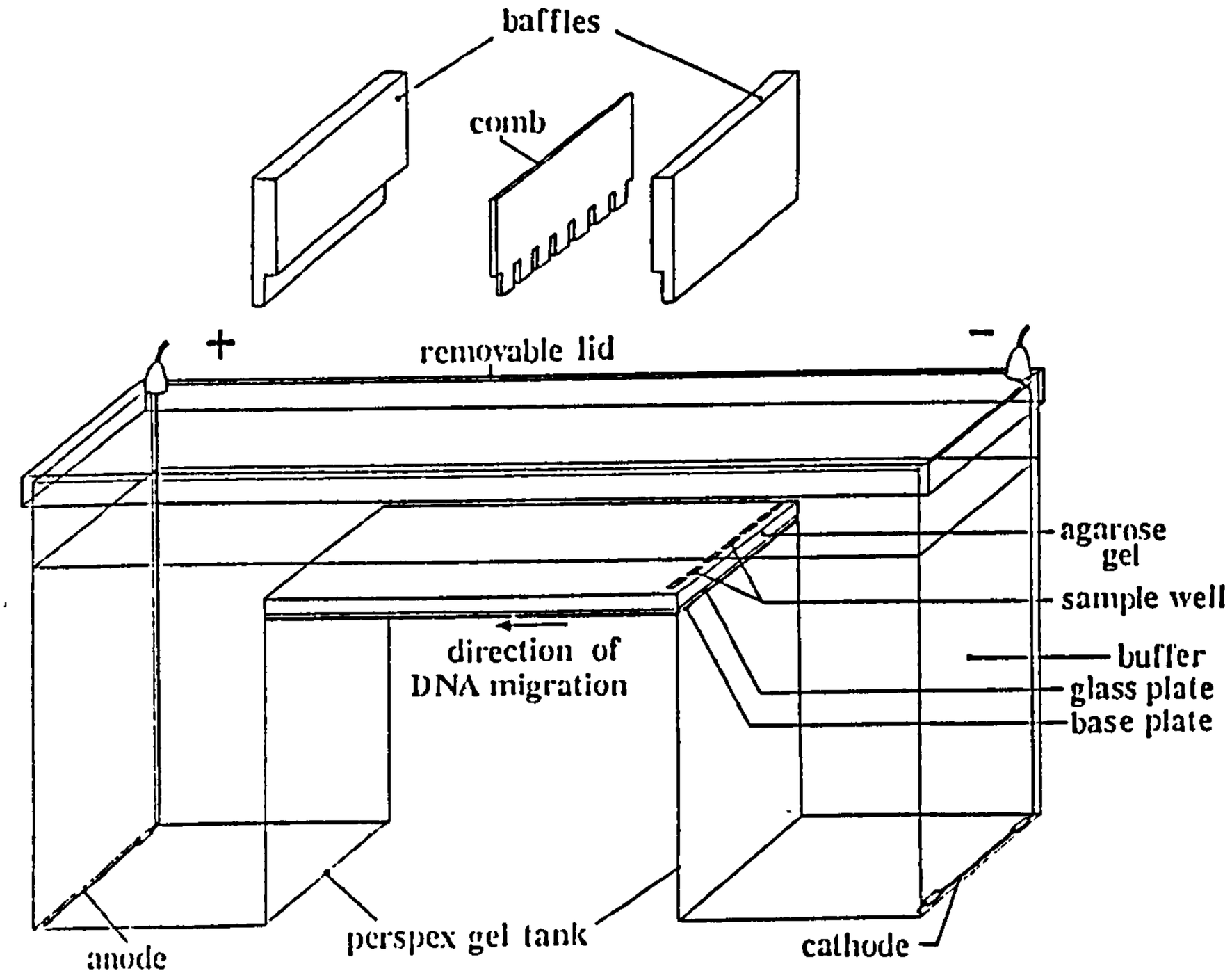


Figure 4.1. Agarose electrophoresis gel rig.

During certain times of the year a white substance was detected during electrophoresis, migrating backwards from the wells towards the anode. Whether this slightly retarded migration of the DNA is unknown. It could not be identified but showed no reaction with Coomassie blue or glycogen tests (however this may have reacted with the agarose).

4.1.5 Interpretation of banding patterns on an agarose gel

If two related DNA sequences are examined by restriction analysis and no differences are seen in the number and patterns of fragments then the mtDNAs share the same restriction sites for that enzyme and have identical nucleotide sequences at the recognition sites of that enzyme. Should the patterns vary in the number or position of fragments then differences seen in banding patterns (restriction fragment length polymorphisms) can be attributed to site loss/gain due to point mutations, or by the rarer events of insertions, deletions or rearrangements (See Figure 1.2).

4.2 Results

4.2.1 Length variation

Evidence of length variation was seen in the RFLP profiles of all enzymes for which numerous individuals were screened; *Eco*RI, *Bam*HI, *Hin*dIII and *Xho*I. Typical *Eco*RI patterns are depicted in Figure 4.2(a) and examples of RFLPs for *Bam*HI and *Hin*dIII are depicted in Figure 4.2(b).

For *Eco*RI all individuals possess bands of 6.3kb, 2.95kb and 0.5kb whilst other bands differ in either size or presence/absence. The size variation is evident when individuals with otherwise identical profiles are compared, for example individuals 4, 2 and 6 of Figure 4.2(a) each possess bands of 6.3kb, 3.4kb, 2.95kb and 0.5kb but the largest band varies in size, estimated at 8.2kb, 9.8kb and 13kb respectively in these animals. The differences in size between individuals 4 and 2 and, 4 and 6, at 1.6kb and 4.8kb could be explained by 1 and 3 (respectively) additional copies of the 1.6kb repeat sequence (Rigaa *et al.*,1993). This size variable band is seen in all animals but comparisons of profiles is made difficult by both length variation and restriction site variability (see below).

The size variable band was the largest in the *Eco*RI RFLP profile in all but two cases. In individual 3 of Figure 4.2(a) no large band is evident, but the intensity and thickness of the 6.3kb band suggests the presence of a doublet (2 bands of near identical size). A 6.6kb band would perhaps be indistinguishable from 6.3kb and this size would differ from animal 4 by 1.6kb (i.e one repeat unit). Also in Figure 4.2(c) for animal 4, the 4.5kb band may contain the repeat region but be smaller than other bands due to restriction site variability (see below).

The mitochondrial DNA of *P. maximus* proved to be considerably variable in length, estimated to vary between approximately 20-26kb, although a precise figure could not be placed on the size as a result of the difficulties in accurately sizing the largest band of each restriction digest, containing the repeat region. Since larger fragments do not migrate far and also do not migrate linearly a slight (1-2kb) difference in the size of a 8-13kb band was hard to differentiate.

Comparisons of patterns among lanes to differentiate length classes of mtDNA, although often possible in adjacent lanes was difficult between non-adjacent lanes and often impossible between gels. Major variation in length was therefore recognised but not quantified and the distribution of length variation among populations could not be ascertained.

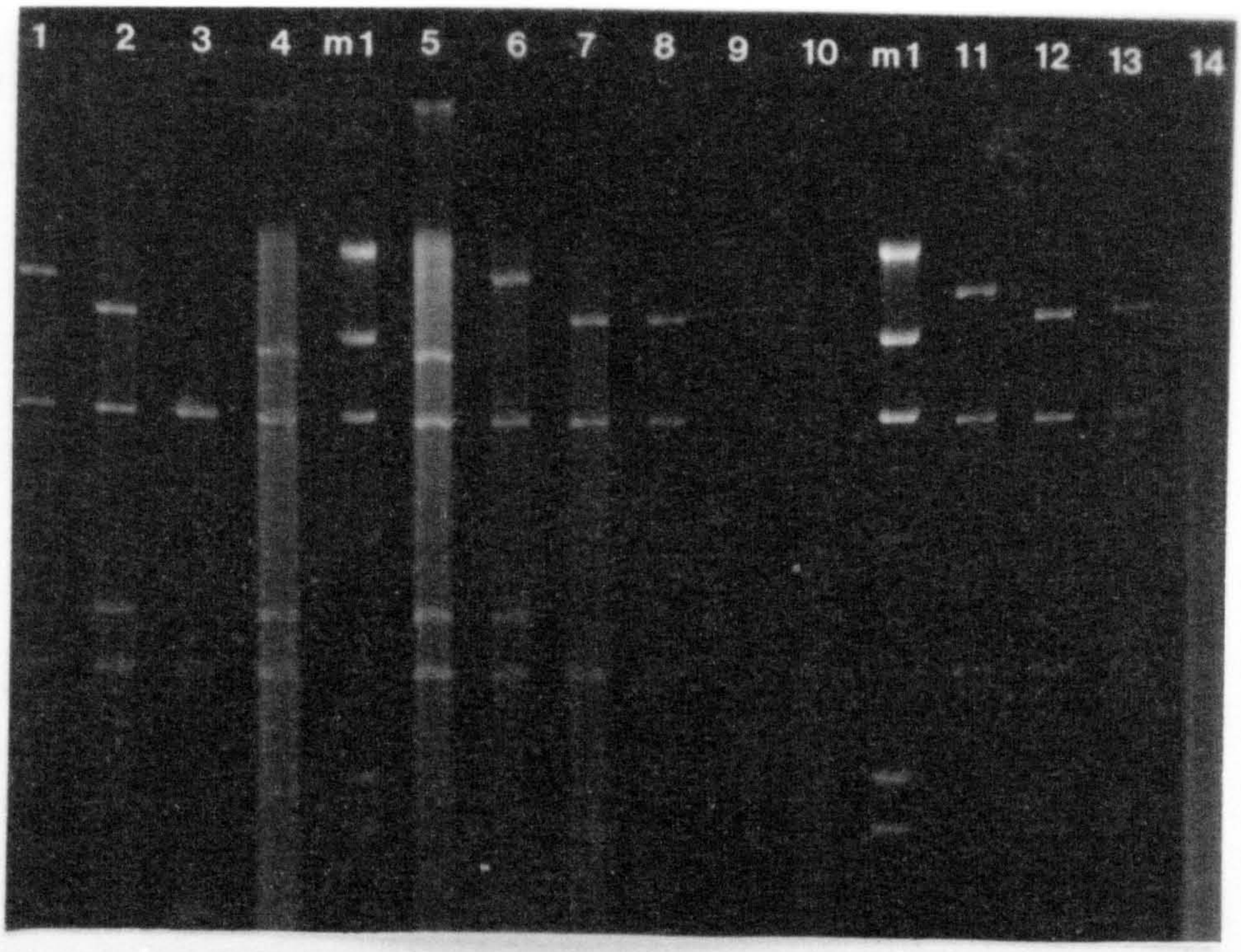
Heteroplasmy (the existance of two different types of mtDNA in the same individual) was seen at a very low level, recognised by equally intense but different sized large bands in *Eco*RI profiles [see Figure 4.2(d) and (e)]. Only 2 of 143 animals cut with *Eco*RI exhibited such evidence of heteroplasmy.

4.2.2 Restriction site variation

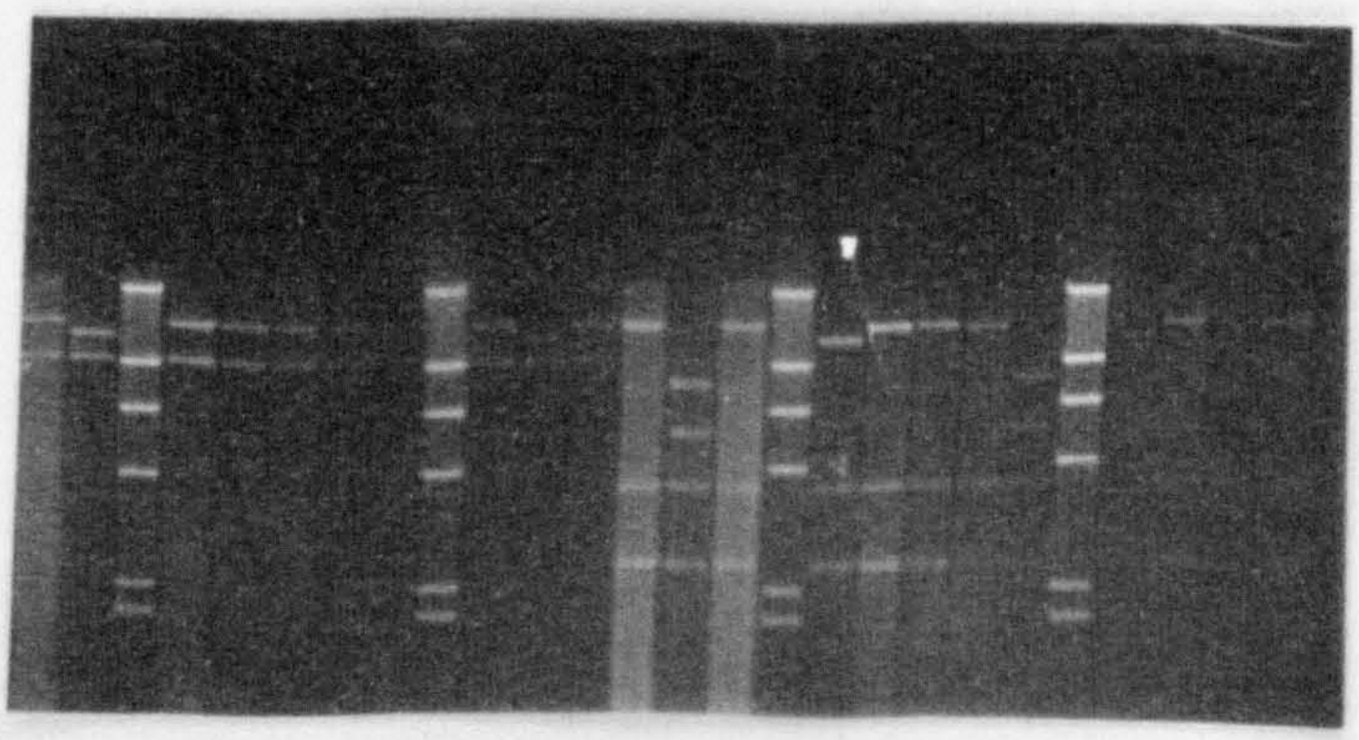
The presence of fragments additional to the length variable band and 3 size invariable bands suggests restriction site variation. For instance, individuals 2, 4, 5 and 6 of Figure 4.2(a) all possess a 3.4 kb not seen in other scallops, although for animal 3 this band may have been cleaved to yield 2 fragments, estimated to be of 2.4kb and 1kb. The other individuals depicted in Figure 4.2(a) do not possess either the 3.4kb, 2.4kb or 1kb band, which suggests that these bands must originate within the size variable band, either from within the repeat region or the sequence between the repeats and the *Eco*RI site. Without mapping restriction sites of *Eco*RI and other enzymes this cannot be confirmed. Thus the length variation complicates the analysis of restriction site variation since it is difficult to ascertain whether the restriction site occurred within one of the repeats or in the sequence of the length variable fragment flanking the repeats. As can be seen from the restriction map formulated by Rigaa *et al.*(1993; see Figure 4.2) the length variable band in restriction digests is not solely made up of varying numbers of the repeated sequence but has additional non length variable flanking sequence between the repeats and the recognition site which could contain a polymorphic restriction site.

Figure 4.2. (a) *Eco*RI digestions of *P. maximus* mtDNA showing length and restriction site variation for 14 individuals. m1=λ-*Hin*dIII size marker. (b) *Bam*HI (Left side of gel) and *Hin*dIII (Right side of gel) digestion of mtDNA.(c) Length variation and restriction site variation of *Eco*RI profiles in 5 samples. m2=1kb ladder (Life Technologies). (d),(e) Heteroplasmy of *Eco*RI variants. * indicates heteroplasmic individuals. (f) Possible heteroplasmy coupled with restriction site variability. †=unusual sized band (see text).

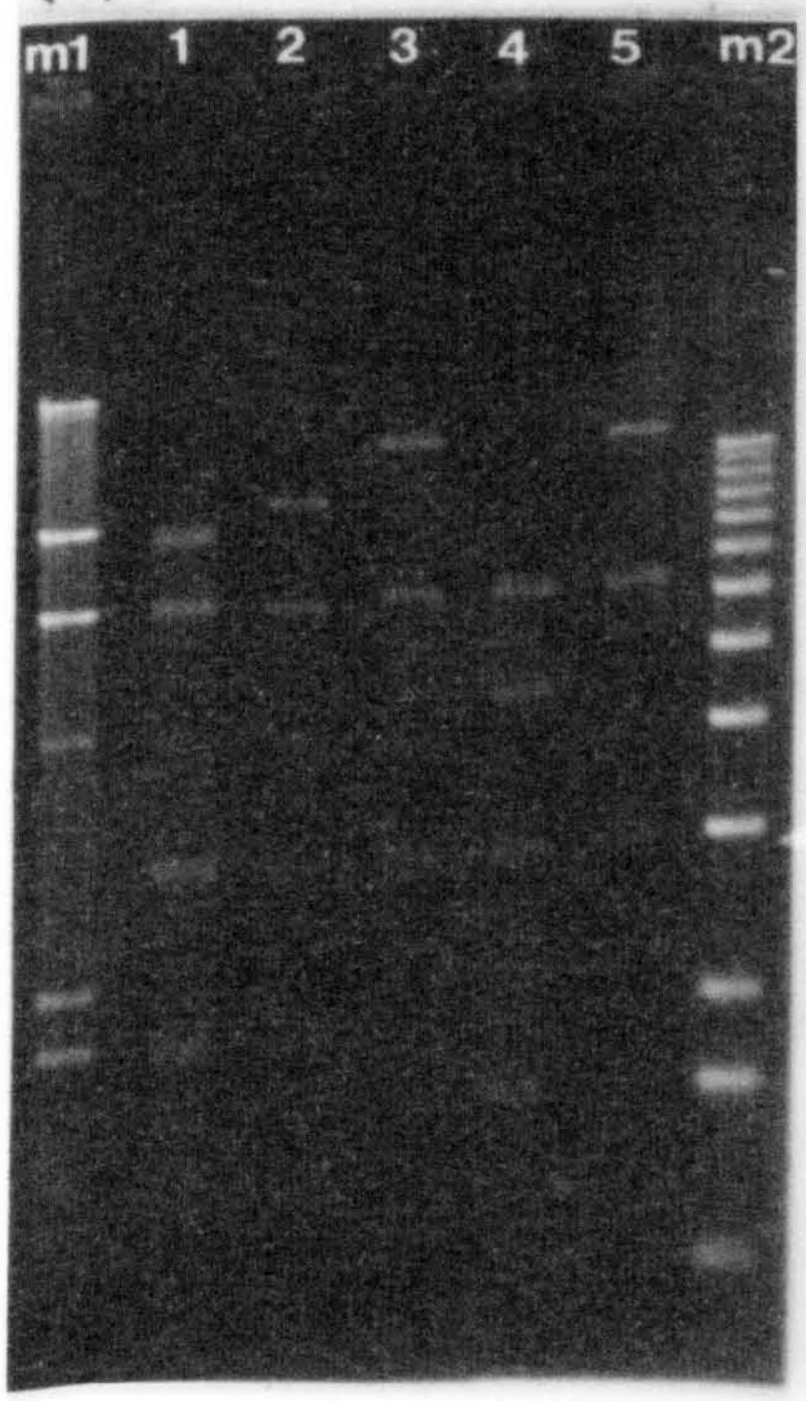
(a).



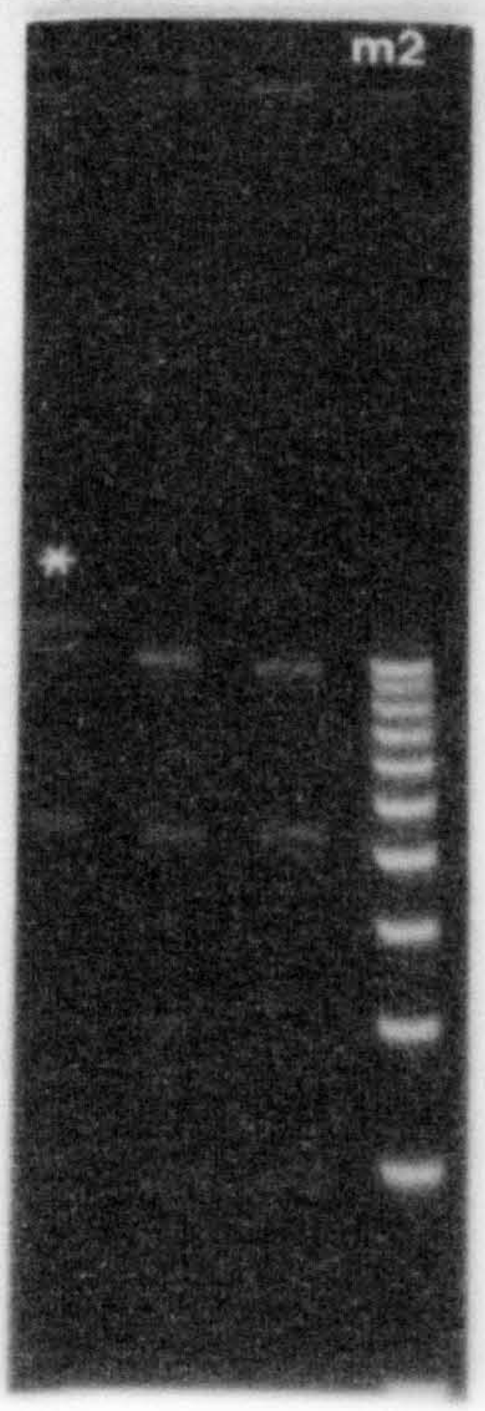
(b).



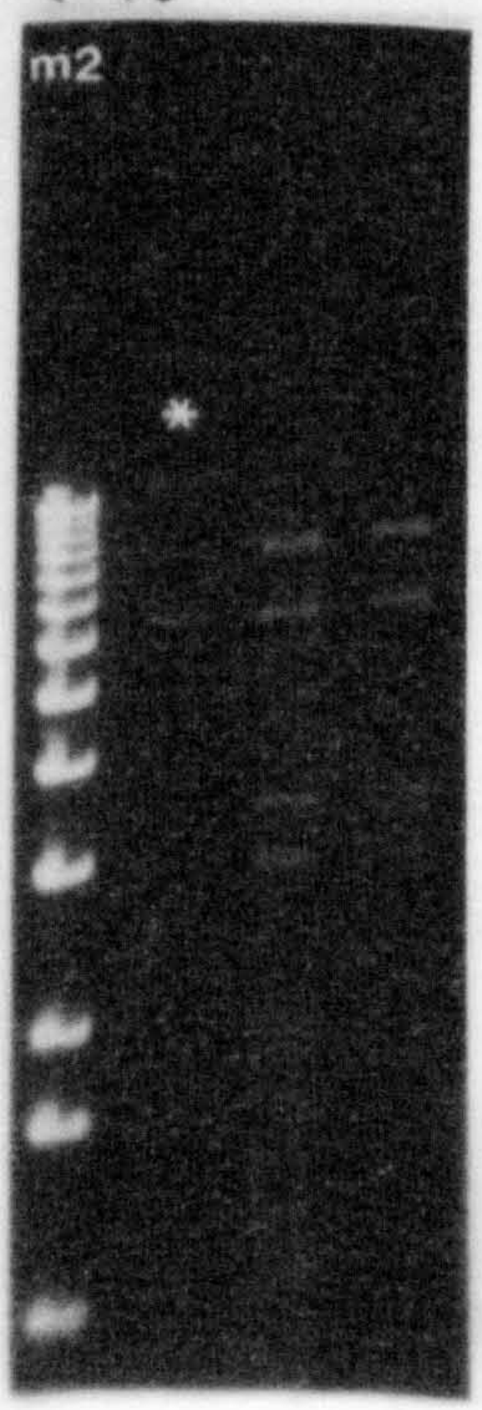
(c).



(d).



(e).



(f).

A 2kb band is also seen in lanes 7, 8, 10, 12 and 13 of Figure 4.2(a). There is no evidence to suggest this band is derived from cleavage within the 3.4kb band since no smaller bands were evident (the 2kb band is also seen in Figure 2B of Rigaa *et al.*,1993 for which fragments were visualised by autoradiography and thus any smaller fragments would be expected to be seen). This 2kb fragment is thus also likely to be derived from within or close to the variable domain but again mapping is required for confirmation of this.

Additional bands not recognised in other specimens were noted in individual 4 of Figure 4.2(c). For this animal the length variable band is not the largest of the digest and the intensity of the 6.3kb band does not suggest the presence of a doublet, thus the band containing the repeat region must be smaller than 6.3kb, but larger than 3.2kb (since individuals contain at least 2 copies of the repeat, Rigaa *et al.*,1993). The size of the second largest band (4.5kb) cannot be explained solely by variation in the number of repeats since this length does not differ from the standard lengths by multiples of 1.6kb. Once again this animal must contain a restriction site within a repeat or flanking sequence of the repeat region. This individual also possesses 3 additional bands of 2.2kb, 2kb and 1.6kb thus must contain multiple restriction sites.

One pattern that displays unique variation is depited in Figure 4.2(f). The 5.6kb band marked † is in addition to the 3 non-length variable bands (accounting for the 0.5kb band which is not visible) and a 9.9kb length variable band. For these 2 large bands to result from cleavage within a repeat unit would require the existance of at least 6 repeats. The maximum number seen by Rigaa *et al.*,(1993) was 5 (although 7 were detected by Gjetvaj *et al*, 1992). If not due to this then the pattern could be explainable by heteroplasmy, with one mtDNA containing a restriction site within, or close to the variable domain yielding a length not explainable solely by differences in repeat number.

Restriction site variation was not seen for *Bam*HI [Figure 4.2(b)] or *Xho*I but was recognised for *Hin*dIII [Figure 4.2(b)]. Only 1 pattern (seen in 2 of 20 individuals) contained the variable pattern for *Hin*dIII.

4.2.3 Double-digestion

*Eco*RI-*Hin*dIII double digestion was successful (Figure 4.3). It is apparent that the *Eco*RI sites closest to the length variable domain must be situated in close proximity to *Hin*dIII sites since the fragment size of the largest band in single and double digests is undifferentiable. Double digest does result in fragments between 2-3kb which have both an *Eco*RI and a *Hin*dIII end.

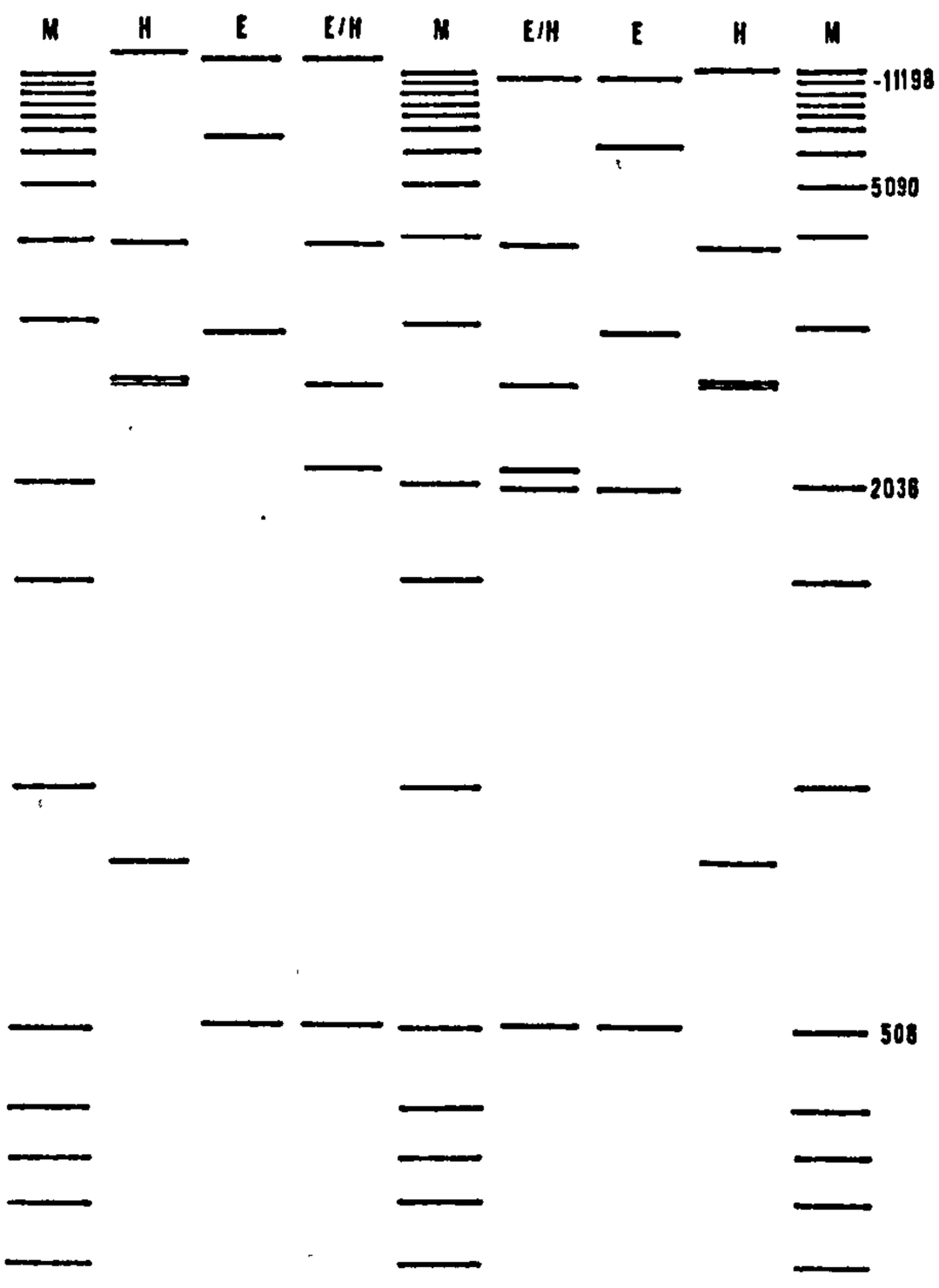


Figure 4.3: Fragments seen in *Eco*RI-*Hin*dIII double digests (E/H) in comparison with single *Eco*RI (E) and *Hin*dIII (H) digests. RFLPs of two individuals are shown to either side of marker lane (M). Marker is a 1kb ladder (Life Technologies), marker fragment sizes in bp.

4.3 Discussion

If mtDNA variation is to be assessed accurately through restriction enzyme analysis then site variation must be separable from length variation. This can be a problem in species where there is extensive mtDNA length variation (Moritz *et al*, 1987; Boulding *et al.*,1993).

In the manner in which this small scale study was conducted, RFLPs of *P. maximus* mtDNA proved unsuitable for the measurement of genetic variation as a direct result of the large amount of length variability and low level of restriction site polymorphism (using enzymes with a 6bp recognition site) in the molecule. The estimated length of the molecule closely matched the estimates of Gjetvaj *et al.* (1992) and Rigaa *et al.* (1993) but the separation of restriction site variation from length variation proved difficult using only single enzyme digests. Through restriction mapping this is likely to be possible but since restriction site polymorphism proved to be so low the effort may not be justified.

Restriction site variability was shown to occur and included *Eco*RI site polymorphisms and a single polymorphism for *Hin*dIII, but no restriction site variability for *Bam*HI or *Xho*I was detected. Site variation measured with 6bp cutters was however, only slight and there did not appear to be enough variability to measure variation among populations. Since 6bp cutters exhibited such a low level of polymorphism the mtDNA would need to be cut with a substantial battery in order to reveal sufficient variability for population analysis (Ovenden, 1990). Also if restriction site variation were to be accurately assessed, a more elaborate approach would be needed utilising polyacrylamide gel electrophoresis and possibly end-labelling or hybridisation in order that multiple enzymes could be undertaken (when ethidium bromide staining is used there is not enough mtDNA for more than 5 or 6 digests). This would also ensure small fragments are not missed and therefore will allow the length variation to be localised with double digests.

As a consquence of these difficulties the effort required for a RFLP study does not seem justified on the basis of the limited number of restriction site polymorphisms identified and the difficulties in accounting for length variation. A method permitting the use of 4bp recognition sequence restriction enzymes could likely prove more valuable but although 4bp cutters would likely reveal more variation throughout the molecule they would also be more likely to cut within the repeat region thus compounding the difficulties of examining RFLP profiles. Ideally a technique for targetting areas of the mtDNA away from the repeat region

and permitting the use of 4bp cutters is required if restriction site variation is to be accurately estimated.

The levels of heteroplasmy are low and should not cause difficulties for subsequent use of mtDNA as a population marker.

5.0 PCR primer design

5.1 Materials and methods

5.1.1 Cloning of mtDNA

The use of mtDNA RFLPs had proven impractical in *P. maximus* due to the extensive length variation (see Chapter 4) so a method using RFLPs of sections of mtDNA, situated away from the length variable section and amplified using the polymerase chain reaction (PCR) was developed. Standard PCR protocols and reagents allow for amplification of DNA segments up to 5kb in size (Jeffreys *et al.*, 1988; Saiki *et al.*, 1988) and, with the mtDNA of *P. maximus* containing approximately 16.8kb of sequence distinct from the problematical length variable section (Gjetvaj *et al.*, 1992; Rigaa *et al.*, 1993) it was first necessary to choose suitable sections of the mtDNA from this for amplification by the PCR and subsequent RFLP analysis. Areas of scallop mtDNA that would be potentially useful would have to fulfil the following three criteria:

- Be situated away from the length variable region of the mtDNA.
- Be long enough that there would be a reasonable probability that they contain a number of restriction sites for various endonucleases. The longer the fragment, the higher the probability that it will contain a restriction site for a given restriction enzyme under the assumption of random distribution of nucleotides.
- Not be so long as to require specialist procedures to ensure consistent amplification (Kainz *et al.*, 1992; Cheng *et al.*, 1994).

The length variable domain in the mtDNA of *P. maximus* had been localised to a single fragment in single enzyme digests involving *Bam*HI, *Eco*RI, *Hin*dIII and *Xho*I and, double *Eco*RI-*Hin*dIII digests (Chapter 4) thus any other fragment in a digest, of an appropriate size should be suitable. To enable primer design such fragment(s) then required cloning into a suitable vector to enable sequencing of the ends. In this process DNA is inserted into the vector by ligation of compatible ends in which restriction enzymes are utilised to cut both the vector and future insert DNA such that the ends of the vector DNA are able to complement and join with the ends of the insert. This can be acheived by cutting both insert and vector with the same enzyme or cutting both with different enzymes that leave compatible ends, whether these are sticky ends (overhanging

ends with the same sequence) or blunt ends (all blunt ends are compatible with each other). The ends can then be joined using the enzyme DNA ligase, resulting in a contiguous sequence of vector and insert which the bacteria accepts, takes up and amplifies during its growth.

5.1.1.1 Mitochondrial DNA isolation

In order to reduce to a minimum any contaminating nuclear DNA (nDNA) that may have ligated instead of the mtDNA and, be subsequently cloned and sequenced under the false impression that it was mtDNA, mtDNA preparations had to be essentially free of nDNA. A single attempt at purification of mtDNA on a CsCl gradient (see Lansman *et al.*, 1981; appendix C) was made but the resultant bands on the gradient were diffuse instead of sharp and well defined and, when the DNA was checked by agarose electrophoresis after dialysis, precipitation and resuspension, the DNA appeared smeared indicating mitochondrial DNA had not been separated adequately from the nuclear DNA. Due to the difficulties of ultracentrifugation and the inherently protracted protocol it was decided not to pursue this method further. Instead mtDNA was extracted as normal (see Chapter 4) from a single individual dredged from Chicken Rock (see Figure 2.1), except that the mitochondria aspirated off the 1.0M/1.5M sucrose interface were subjected to a second bout of sucrose gradient centrifugation. This should not only separate out any nuclei that were accidentally aspirated off with the mitochondria after the first centrifugation step but also aid in diluting out glycogen and other contaminants that may interfere with subsequent ligation steps. Following this treatment, the mitochondria were lysed and the mtDNA purified, precipitated and resuspended in 20µl of dH_2O following the method given in Chapter 4. An estimation of mtDNA concentration relative to that of the vector was then made after agarose electrophoresis, on the basis of relative ethidium bromide fluorescence (Sambrook *et al.*, 1989).

5.1.1.2 Cloning vector and host cell

Cloning as defined here involves inserting a section of foreign DNA (the desired fragment) into a cloning vector (bacterial plasmid or phage DNA) and then utilising the bacteria or phage to replicate both the vector and the insert, thus creating large quantities of the insert-containing vector (Sambrook *et al.*, 1989; Brown, 1990a).

The general all-purpose cloning vector, pBluescript II SK$^+$ (Stratagene) a 2.96kb phagemid derived from pUC19 was chosen for cloning. Since the vector contains the *lacZ* gene, when used in conjunction with a suitable host cell (cells containing the *lacI^{q}ZΔM15* gene on their F′ episome) blue-white colour selection can be used for detection of recombinant colonies (Sambrook *et al.*, 1989). If no insert is ligated and re-ligation of the digested vector occurs then the *lacZ* gene remains intact and can produce a functional α-peptide that complements the product of the *lacI^{q}ZΔM15* to produce a functional β-galactosidase protein. When cells containing such DNA are plated on media containing Isopropyl-β-D-thiogalactopyranosid (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-GAL) the colonies are blue due to metabolism of the X-GAL by the enzyme. If a fragment has been successfully ligated into the vector so disrupting the *lacZ* gene then the colonies cannot produce β-galactosidase, so cannot metabolise the X-GAL and are white. pBluescript II SK$^+$ contains the *amp^r* gene for amplicillin resistance allowing for screening of recombinant bacteria. Cells lacking the phagemid are ampicillin sensitive and thus can be selected against through plating on media containing ampicillin.

XL1-Blue cells (Bullock *et al.*, 1987) were chosen as the host cell. These contain the F′ episome which codes not only for the *lacI^{q}ZΔM15* gene but also the gene for tetracycline resistance thus through plating on tetracycline containing media the prescence of the F′ episome is ensured.

5.1.1.3 Choice of insert

Ligation is easiest when insert ends are non-compatible (Sambrook *et al.*,1989) since re-ligation of linearised vector cannot occur. Double digested *Eco*RI-*Hin*dIII *P. maximus* mtDNA had been seen to contain at least two potentially suitable fragments including a 2kb section with *Eco*RI-*Hin*dIII ends (Figure 4.3). This and the 3.85kb *Hin*dIII fragment [see Figure 4.2(b)] were targeted for ligation. Although for the latter this would require ligation into a vector with compatible ends, no fragment of this size with suitable non-compatible ends had been identified in the *Eco*RI-*Hin*dIII double digests. It was desirable to have a range of

different sized fragments (non-length variable among individuals) for cloning in order to decide which size was the most suitable for PCR-RFLP.

5.1.1.4 Preparation of insert and vector

10µl of mtDNA solution (section 5.1.1.1) was treated with *Hin*dIII and 10µl with *Eco*RI-*Hin*dIII in 20µl total volume with 1 x restriction enzyme buffer (multi-core buffer for double digest) and 3U enzyme(s). Restriction enzyme was subsequently removed by phenol chloroform treatment and the cleaved mtDNA precipitated with 0.1 vol 3M sodium acetate and 3vols ethanol, pelleted, then resuspended in 5µl dH_2O.

0.5-1µg pBluescript II SK^+ was digested for 2h at 37°C with either *Hin*dIII, or *Hin*dIII and *Eco*RI double digestion (3 units of each enzyme in 20µl volume, double digests performed in Promega multi-core buffer). After digestion with *Hin*dIII alone the vector was left with compatible ends which would be able to re-ligate (Sambrook *et al.*, 1989). To reduce the possibility of this occurring the cut vector was treated with alkaline phosphatase (Promega). Following restriction digestion, 2µl alkaline phosphatase buffer and 1U alkaline phosphatase were added and incubation continued for 30 mins at 37°C. The enzyme was then inactivated by heating at 65°C for 30 minutes then removed by phenol/chloroform extraction. Since *Eco*RI-*Hin*dIII cut vector had incompatible ends there was no need for alkaline phosphatase treatment. Vector digestion products (from both treatments) were run on a 0.8% agarose gel at 70V for 2-3h then stained with 0.002% methylene blue in 0.1% TAE for approximately 2 hours, following which the required band was excised with a scalpel. Methylene blue (MtB) is suitable as a DNA stain but is much less sensitive than EtBr. To overcome this, large quantities of plasmid (0.5-1µg) were digested and electrophoresed. Since MtB stained DNA is visible without UV light then the DNA will not suffer from UV induced damage in the same way that DNA stained with EtBr may do (Pope and Pierce, 1995) and is therefore in better condition for further manipulations such as ligation (Flores *et al.*, 1992). Once excised, the desired band required separation from the agarose, so was placed inside a 0.5ml microfuge tube, the bottom of which had been pierced using a hot Nº16 hypodermic needle and plugged with siliconized glasswool. This

small tube was then placed inside a 1.5ml microfuge tube and spun for 10 minutes at 6,000g. This forced buffer containing the DNA through into the larger tube whilst retaining the agarose and MtB in the small tube. The DNA was then precipitated from this solution at -20°C for 30 minutes using 0.1vol sodium acetate and 3vols ethanol, then centrifuged at 13,000g for 15 minutes. The pellet was then washed with 70% ethanol and given a final 10 minute 13,000g spin. The vector DNA was resuspended in 10µl dH_2O and its concentration checked by electrophoresis of an aliquot on a 0.8% agarose gel. Gel extraction was necessary to remove the suitably treated vector away from any uncut vector (which because of its small size relative to any vector containing insert would transform cells more efficiently) and also in the case of the double digested vector away from the excised small fragment of multiple cloning site (MCS) which may subsequently preferentially re-ligate back into the vector ahead of the desired insert.

5.1.1.5 Ligation

For ligations the ideal ratio of vector:insert is 1:3 (Sambrook *et al.*, 1989) although this depends ultimately on the nature of both types of DNA. After comparison of vector and insert concentration on the basis of EtBr fluorescence (by eye), an attempt was made to adjust the quantities to the appropriate ratio. As both vector and insert were resuspended in small volumes, the ligations could also be performed in a small volume which increases the ligation success. Ligation was performed in 10µl volume (made up with dH_2O) containing vector at two different concentrations relative to the insert (1/3 x insert concentration and 2/3 x insert concentrations were used), insert (half of the DNA from restriction digestion of mtDNA obtained from 5g adductor muscle), 1µl T4 DNA ligase buffer and 1U T4 DNA ligase (Promega). Ligations were performed overnight at 16°C.

A variety of control DNAs were also prepared for use in the ligations. These included the following treatments; "cut vector alone" which will contain an inactive *lacZ* gene and thus when transformed, the resultant cells should all appear blue, "cut vector treated with ligase" which should therefore not re-ligate (in the case of the *Hin*dIII-*Eco*RI prepared vector due to incompatible ends and in the case of the *Hin*dIII digested vector should not due to the alkaline phosphatase treatment) and thus cells transformed by such vector should also appear blue (no

functional *lacZ*). The quality of the cells for transformation was checked by inclusion of cells transformed with "untreated vector". An "insert only" control was also included. No colonies will be seen with this treatment unless the insert is contaminated with (a possibly unsuitable) plasmid.

5.1.1.6 Preparation of competent cells

Competent cell preparation was performed with the calcium chloride method of Sambrook *et al.* (1989). XL1Blue, stored at -20°C plated from frozen on Luria agar containing 15mg ml^{-1} tetracycline were grown overnight at 37°C. Transformation of competent cells is more succesful if undertaken with a culture inoculated from a frozen stock (Sambrook *et al.*, 1989). A single colony was then isolated and placed in 5mls Luria Bertani (LB) broth and grown overnight at 37°C with shaking at 200r.p.m. XL1Blue is a weak strain and is sensitive to anaerobic conditions, needing at least 4/5 of the bottle size (in airtight bottles) empty in order to grow. Therefore cells were grown in 5mls LB broth in a 30ml universal tube. After this incubation period, 1ml was taken and added to 200mls LB broth in a conical flask plugged with sterile cotton wool. This culture was incubated for approximately 3 hours at 37°C with 200r.p.m. shaking until the culture assumed a milky turbidity. Cells were then pelleted at 4,000r.p.m. (in autoclaved 200ml tubes) for 10 minutes, following which the cell pellet from each tube was resuspended in 15mls of ice cold 0.1M $CaCl_2$ and stored on ice for 20-30 minutes. Bacteria were then once again pelleted at 4,000r.p.m. for 10 minutes after which the pellet was resuspended in 8mls of fresh ice cold 0.1M $CaCl_2$ and stored on ice for 2 hours (although overnight storage did not compromise transformation efficiencies). After a further 4,000r.p.m. spin for 10 minutes the pellet was resuspended in 8mls ice cold 0.1M $CaCl_2$ (2mls per 50ml of original culture). Transformation was performed using 200µl of this suspension in 1.5ml microfuge tubes on ice.

5.1.1.7 Transformation of competent cells

Ligated and control DNAs were added to separate, labelled 1.5ml tubes containing 200µl competent cell suspension and kept on ice for 30 minutes, after mixing by inversion. Then after a 90s 42°C heat shock cells were immediately placed on ice for 1-2 minutes after addition of 800µl LB broth and then incubated at 37°C for 45

minutes with shaking (this period allows the newly transformed bacteria time to express their ampicillin resistance genes that will be utilised in the next selection process).

5.1.1.8 Selection of recombinant colonies

Transformed bacteria were pelleted for 20s at 13,000r.p.m. and the liquid drained. The cell pellet was then resuspended in the residual broth and plated on Luria agar plates containing 100μg ml^{-1} ampicillin, 50μg ml^{-1} IPTG (Boehringer Mannheim 724815) and 50μg ml^{-1} X-GAL (BM 745740). Plates were incubated overnight at 37°C, then placed at 4°C for 2 hours to enhance the colour selection, following which the number of blue and white colonies were estimated (ratios of blue-white colonies in each treatment will provide an indication of success). White colonies (containing inserts) were picked from the plates with sterile toothpicks and replated by spreading on new plates containing ampicillin, IPTG and X-GAL at the same concentrations as before. These plates had been "divided" into approximately 1.5 cm^2 by drawing a lattice on the petri dish and colonies were spread within the areas demarcated by these squares. This procedure allowed growth of single colonies without the danger of coalescence of colonies containing different inserts. Plates were again incubated for 20 hours at 37°C and then 1-2 hours at 4°C. Colonies which remained white (this second plating step acted as a "double check" for α-complementation) were then analysed for the nature of their insert.

5.1.1.9 Mini-prep analysis of plasmid DNA

Restriction quality plasmid DNA was extracted from bacteria using a variant of Holmes and Quigley's (1981) STET mini-prep. The colony of interest was touched with a toothpick, which was then dropped into 3mls of LB broth in a universal tube and incubated overnight at 37°C with shaking. 1ml of this was used for plasmid preparation. The advantage of these variations to the technique was that the remaining bacterial suspension could be frozen, after addition of glycerol to 40% (v/v) and saved for future use if it proved to contain a target insert.

Bacteria from 1ml of suspension (in 1.5ml tubes) were pelleted (12,000g/20s) in a microfuge. The supernatant was then poured off, the remaining liquid blotted off with tissue and the pellet resuspended in 200μl STET buffer (0.1M NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA pH8.0, 5% Triton-X). After

addition of 20µl of 10mg ml^{-1} lysozyme (Sigma L6876) the tubes were left for 10min at room temperature and then placed in boiling water for 50s (to avoid the lids exploding off the tubes, the lids were replaced with pierced eppendorf tube lids which could be washed, autoclaved and reused. Unpierced lids were left attached to the tubes and could be replaced after the boiling water treatment). Tubes were placed on ice, then centrifuged for 10mins at 13,000r.p.m. Cell walls and chromosomal DNA (which in bacteria is attached to the cell wall) formed a white amorphous, floating pellet which could be picked out with a sterile toothpick and discarded. Recombinant DNA was then precipitated from this crude solution with 1vol of isopropanol (no salt was added as the buffer contained sufficient) for 10 minutes at -70°C following which tubes were centrifuged at 13,000r.p.m. for 15mins. The resultant DNA pellet was washed with 70% ethanol, spun for 10mins, dried and resuspended in 50µl TE buffer. In order to check for the presence of desired inserts, restriction digestion was performed on 10µl of this in a total volume of 20µl with 3U of the appropriate restriction enzyme. In the case of the *Hin*dIII/*Eco*RI clones, potential recombinants were analysed by digestion with *Hin*dIII, *Eco*RI and *Hin*dIII/*Eco*RI (to ensure double digestion had occurred) whilst the plasmids extracted from *Hin*dIII clones were examined only after digestion with *Hin*dIII. Restriction digests were examined under U.V. light after electrophoresis in 0.8% agarose gels and staining with ethidium bromide. In each case the approximately 2.96kb vector band will be seen along with the excised insert, the size of which can then be estimated in comparison to markers of known size.

5.1.2 Sequencing of insert ends

For this study the main purpose of cloning was not to amplify the quantity of DNA but to obtain a form of the DNA to be sequenced bounded by DNA of known sequence. This is necessary because the sequencing protocol, utilising Sanger's di-deoxy termination method (Sanger *et al.*, 1977), requires an element of strand-synthesis (replication) which as in the extension phase of the PCR process must begin from a double stranded region of DNA proceeding into the single stranded DNA. To obtain sequence information from cloned DNA the recombinant vector is made single stranded and a small piece of DNA (primer) used to prime strand-

synthesis, this DNA matching part of the sequence of the vector DNA. Such primers are available to match sequences in most commercially available vectors.

5.1.2.1 Preparation of sequencing quality DNA

Cloned vector DNA containing an insert of interest was further purified using Promega's Magic Miniprep™ system (Anon., 1993) prior to sequencing. This is a resin based DNA purification spin column. DNA passed through the column remains bound to the resin which is washed to remove contaminants such as proteins and salts, leaving the DNA able to be stripped off with hot water/buffer. DNA was first prepared using the STET prep procedure, but after boiling and removal of the chromosomal pellet the remaining solution containing the DNA (usually <100µl) was treated as per the manufacturer's instructions except that DNA was removed from the column using near boiling dH_2O (50µl), repeated once leaving the recombinant DNA in a final volume of 100µl. In order to use the correct quantity of DNA in the sequencing reaction the concentration of each template was measured using a Shimazu UV-1200 spectrophotometer, with quartz cuvettes, reading at A_{260} and A_{280}. The maximum absorbency of DNA occurs at 260nm and the reading at this absorbance level can be converted to concentration whilst the A_{260}/A_{280} ratio provides indication of any contamination (Sambrook *et al.*, 1989). A value of 1.8 indicates clean DNA, above 1.8 indicates contamination with RNA and below 1.8 suggests the presence of protein (or phenol if used) in the sample.

5.1.2.2 Sequencing

Sequencing was performed on the pure plasmid DNA using Pharmacia's T7 polymerase sequencing kit (Pharmacia 27-1682-01) and forward and reverse sequencing primers from Life Technologies (catalogue numbers 18257014 and 18424010). The Pharmacia protocol for sequencing double stranded templates was followed. For the labelling, ^{35}S labelled dATP (Amersham) was used. Because of the low activity of ^{35}S, no special precautions were required except use in a designated area and appropriate disposal of radioactive waste. Sequencing reactions, gel preparation and running and initiation of autoradiography were all performed in one day.

5.1.2.3 Acrylamide gel preparation and electrophoresis

The two plates of the sequencing rig (Anachem) are treated differently in order that the gel will stick to one plate (the rectangular one) but not the other (the notched plate). In order to facilitate this the notched plate was treated with ~10ml repel-silane (dimethyldichlorosilane, BDH 33164) by pouring on and wiping gently over, leaving for 2 minutes, blotting and then repeating the treatment. The plate was then polished with a small amount of ethanol. The rectangular plate was treated with bind-silane (20ml 100% ethanol, 200µl γ-methacryloxypropyltrimethoxysilane [Sigma M6514] and 600µl 10% acetic acid) by wiping 10mls over the plate using soft tissue paper then leaving to dry for 5 minutes, blotting and repeating. Finally the plate was polished with soft tissue and a small quantity of ethanol. The plates were then assembled in the sequencing rig, no taping of the glass plates was required. The gel mix was prepared using 31.5g of urea, 15ml 5x TBE buffer, 25.5ml dH_2O and 9.375ml 40% bisacrylamide solution (Integra Biosciences 077034) and dissolved by gentle heating under hot water. Once the rig was prepared, 450µl of 10% ammonium persulphate (APS) and 37.5µl TEMED (Sigma,T-9281) were added to the acrylamide mix to initiate polymerisation. This mix was then poured slowly between the plates with the sequencing rig laid flat and the glass plates tipped to facilitate movement of the mix and prevent formation of air bubbles. Once the gel mould was full the comb was inserted to just below the level of the indentation in the notched plate, so that all wells were 0.5-1cm below this level and the gel was then left for 1 hour to polymerise. After ensuring that the gel rig's gasket was clean, the module containing the polymerised gel was fitted into place and the appropriate quantity of 0.6xTBE poured into the top and bottom chambers (note that TBE from the top chamber can be re-used in the bottom chamber but not *vice versa* since the bottom chamber will contain residual radioactivity due to current flow from top to bottom). After removal of the comb the wells were flushed out using a Pasteur pipette to remove the urea which diffuses from the gel and also to expel any bubbles in the wells. The system was then pre-run to warm the gel and rig at 1000V for 20 minutes followed by 1350V for 10 minutes.

On completion of the pre-run, 4µl aliquots of the sequencing reactions were heated at 80°C for 2 minutes and then loaded onto the gel using duck-billed

tips (Anachem). The gel was run at 1450V for approximately 2.5 hours until the bromophenol blue marker migrated off the end of the gel.

Following gel running the plate module was removed and laid with the notched plate uppermost. By inserting a razor blade between the plates they were separated and the notched plate lifted from the gel which remained stuck to the other glass plate. This plate and gel were then immersed in 10% acetic acid (re-usable) for 10 minutes to fix the gel, following which the gel was washed under running tap water for 7.5 minutes. In order that the photographic film did not stick to the gel, the gel was dried by baking for 90 minutes at 80°C (until the gel surface was no longer tacky).

Following drying, a suitable length of Amersham hyperfilm was lain over the dried gel and a second glass plate placed over to keep this flat. The whole set-up was then clamped tight with bulldog clips, wrapped in black plastic and left in a darkroom for 16 hours to expose the film. Following this the film was developed, washed and dried before the base sequence was read.

5.1.2.4 Analysis of sequence

Base sequences were read from the bottom of the film upwards (5′ →3′) until the bands became compressed, at which point the sequence was ambiguous. Sequences were then edited using the AUTHORIN program (available to Seqnet users from the SERC Daresbury Laboratory). Suitable primer sequences were identified using the PRIMER program (Whitehead Institute for Biomedical Research, Lander Laboratory, Whitehead Institute, M.I.T., Cambridge, MA, U.S.A. Available by anonymous FTP from genome.wi.edu in the directory distribution/primer.0.4). In order to prepare the sequence data for the PRIMER program the sequences had to be represented as if they were contiguous sequences, that is, as if they were from one strand with the intervening unsequenced section missing. Thus although the sequence data from the autoradiogram is obtained from opposing strands of DNA and running in opposite directions one sequence was arbitrarily chosen and the other reversed and complemented (using the REVERSE program on Seqnet). Parameters chosen for the primers within the PRIMER program were the default options except for primer length which was chosen as 24 (minimum 20, maximum 25). Longer primers have higher melting temperatures and consequently higher

specificity (Dieffenbach *et al.*, 1993). The PRIMER program then takes every possible pair of primers and scans them for inter and intra primer complementarity. Suitable pairs are then offered for choice. After selection of suitable oligonucleotide sequences the oligos were prepared on a 0.2μm scale using purification from C.O.P. columns at the Cruachem oligonucleotide facility, Glasgow.

An attempt was also made to identify the particular sequences as sections of mtDNA encoded genes and to align them to published sequences. The Genetics Computer Group, University of Wisconsin (GCG) set of programs available through the Seqnet service of the SERC Daresbury Laboratory were utilised for this purpose including the BESTFIT program for alignment with the *Mytilus edulis* sequence (Hoffmann *et al.*, 1992) which was the closest relative of *P. maximus* with published sequence at the time except for the length variable region, 12S rRNA gene and 5 tRNA genes of *P. maximus* which are available in Genbank (see appendix H for details) and the BLASTX program for alignment of the sequence in all possible reading frames against all protein sequences in the Owl protein database. Virtually all protein coding genes are on the heavy (H) strand of the mtDNA molecule (Meyer, 1994) with only 5 tRNAs and the gene for ND6 on the light (L) strand, at least in vertebrates (Meyer, 1994) thus it is likely that only one sequenced strand will exhibit homology to any particular sequence. If the other is to show any complementarity it must first be reversed and complemented in order to show how the other strand would appear. Since it was not known whether the reverse or forward primer had initiated sequencing of the H strand, the sequences of both ends had to be reversed and complemented using the REVERSE program on Seqnet before comparisons could be made.

5.1.3 Positioning of the sequenced fragments on the mtDNA

Rigaa *et al.*(1993) provide a restriction map for *P. maximus* mtDNA on which the position of the *Hin*dIII 3.85kb fragment is shown. Because the 2kb fragment sequence contained a recognition sequence for *Nsi*I (see results) the position and orientation of this fragment could also be ascertained. *Nsi*I cut the mtDNA only once and therefore positioning of the fragment should be accurate providing that the mtDNA used by Rigaa *et al.*(1993) is not unrepresentative. Double digests of

*Nsi*I-*Eco*RI and *Nsi*I-*Hin*dIII were performed and the reaction products electrophoresed through 0.8% agarose.

5.2 Results

5.2.1 Cloning

The efficiency of ligation was generally low (as evidenced by comparison of the number of colonies from ligated plasmids *versus* uncut controls). This was subsequently found to be probably due to the mechanism of vector isolation from the gel. He *et al.* (1992) report that if vector and/or insert are purified from the gel by spinning through glasswool then the centrifugation should be no longer than 45-60 seconds performed at low speed (2,874g) otherwise ligation efficiencies are dramatically reduced, since contaminants from the agarose are centrifuged through and can be deleterious to the ligase. Since in this case the spin was for 10 minutes at 6,000g this was deemed the probable cause. Ligation efficiency was particularly poor for the *Hin*dIII cut insert-vector. This may have been a result of a harsh alkaline phosphatase treatment. Alkaline phosphatase can "chew back" into DNA making the ends incompatible if performed for too long or if the concentration is too high.

The numbers of colonies on plates from each treatment when *Hin*d III-*Eco* RI treated vector was used are given in Table 5.1.

Treatment	Number of Colonies
Cut vector only, ligase added	36 white
Insert only, ligase added	2 white
Cut vector only, no ligase	0
Uncut vector	> 3,000 blue
Vector and insert (2:3)	1276 white, 2 blue
Vector and insert (1:3)	792 white, 5 blue

Table 5.1: Number of colonies counted on agar plates following various transformation treatments with *Hin*dIII-*Eco*RI cut vector and insert.

The majority of the inserts were small, with the frequency of successful ligation and transformation decreasing with increasing insert size. Of all colonies screened only 5 (2.5%) contained the 2kb band, 3 (1.7%) contained a 1kb insert,

46% contained inserts smaller than 500bp whilst 50% either had no insert or the insert was so small that it was obscured by the RNA on the gel and so could not be sized. In spite of the low numbers of desired inserts, the numbers obtained were sufficient since only one of each kind was required for partial sequencing. A 1kb insert had not previously been seen in the limited number of *Eco*RI-*Hin*dIII digests performed.

For the *Hin*dIII only treated insert and vector the numbers of observed colonies is shown in Table 5.2.

Treatment	Number of Colonies
Uncut vector plus ligase	0
Unphosphorylated uncut vector plus ligase	46 blue
HindIII treated unphosphorylated vector + ligase	0
HindIII treated unphosphorylated vector. No ligase	0
HindIII insert and vector (phosphorylated) + ligase	49 white, 1 blue

Table 5.2: Number of colonies counted on agar plates following various transformation treatments with *Hin*d III cut vector and insert.

Of the 49 recombinant colonies 27 were screened for insert size. Of these, 20 appeared to contain no insert, 3 contained the 850bp *Hin*dIII fragment, 2 had an approximately 2.5kb band and 2 contained a 3.85kb band, which was the size of the target fragment.

Plasmids containing suitable sized inserts were then isolated for sequencing. The Magic-minipreps gave high yields (4.58µg ml^{-1} culture on average) of high purity DNA, with A_{260}/A_{280} readings that averaged 1.826.

5.2.2 Sequencing

Sequences exhibited sharp, clear and intense bands with 170-250 base pairs of unambiguously readable sequence, however up to 80bp of this was composed of vector sequence. Figure 5.1 shows a section of the sequencing gel. In each case the vector sequence (taken from the Stratagene catalogue) was demarcated from the insert sequence by the relevant restriction enzyme site sequence. Sequences were read by eye and orientated relative to each other before use for primer design.

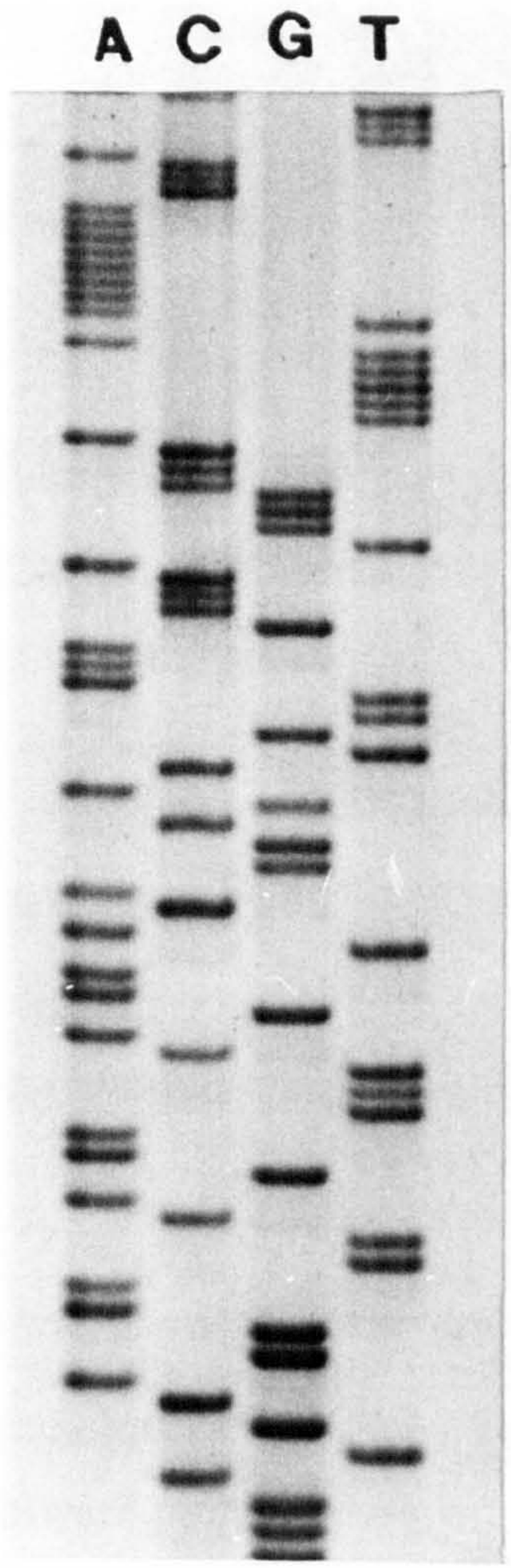


Figure 5.1. Section of autoradiogram displaying mtDNA sequence from *P. maximus*. Shown is a sequence of the 1kb *Eco*RI-*Hin*dIII fragment (initiated with reverse primer) sequence. The *Eco*RI sequence demarcating the vector from the insert sequence is marked. Sequence is read 5′-3′ up the gel.

The sequences as read from the autoradiograms were as follows (5′→3′) and the primers designed from these indicated by the boxed section. *Eco*RI sites are indicated by single underlines, *Hin*dIII sites by double underlines and the *Nsi*I site (see 5.1.3 and 5.2.3) by a dashed underline.

2kb fragment (primed with forward primer):

```
1           11          21          31          41
aagctttctt aaggggacgt cgtcctttgt tgtttggaaa tcggcctagg
51          61          71          81          91
tctttagagt gggtggttaa tcgtggtttt acttgtgtag ctcgccatac
101         111         121         131         141
ttttaaggag gtaatcgcta ttcgaatgat gcatgacgta actgagttcc
151         161         171
ataagaggtc tttagattct ttgg
```

2 kb fragment (primed with reverse primer):

```
1           11          21          31          41
gaattctagc aggggtctct atataatcac aaggaacaaa cctaccccca
51          61          71          81          91
tccagcaatc tgtatgggta gaaccgaacc ataaaaatca agatacaaca
101         111         121         131
acctccgcac acaccaacta gtcttttaca gt
```

3.85kb fragment (primed with forward primer):

```
1           11          21          31          41
aagctttatt ttggtctctg agttttttatg tggtggtatt gttgaggttt
51          61          71          81          91
gcttatttaa ctctttttga gcgtaaggtg ttggcagcat ctcaggtccg
101         111
taaagggcct gaga
```

3.85kb fragment (primed with reverse primer):

```
1           11          21          31          41
aagcttccca ttgcatatgg ataaacaaag aaacactgac cataactatt
51          61          71          81          91
gcacacccaa tagttctcca ctgattccaa taataataaa aaaataggat
101         111         121
agtctggaat acgacggcgca tacccct
```

1kb fragment (primed with forward primer):

```
1           11          21          31          41
aagcttacaa ttcatctaag taaaacaaat tcaattcagg tggaaatcaa
51          61          71          81          91
ggcattggaa atcaaaaagt ccatgttcaa aagtttataa tgtggatgaa
101         111         121
ttatgatgtc aaagattaaa ta
```

1kb fragment (primed with reverse primer):

```
1           11          21          31          41
gaattcagaa ttcagaata caggcgactg ttaaagccca tgggcccatt
51          61          71          81          91
tttataaaaa aaacccattt catttacgat accattgagt tacaattaca
101         111         121         131         141
atgtaaaaga gtaacgacag aatctccaat ttgtgtgtgt a
```

Primer pairs have been labelled as Pma1 (2kb region), Pma2 (3.85kb region) and Pma3 (1kb region). Due to the short size and unidentifed nature, sequences were not deposited in Genbank. Using the BLASTX program the 3.85kb (forward) sequence (translated to amino acid sequence) showed a high degree of similarity to the mitochondrial NADH1 gene sequence from many organisms for which this gene has been sequenced. A 46bp stretch of the sequence exhibited 73% homology to the NADH1 sequence from *Mytilus edulis* when compared using BESTFIT. The 3.85kb (reverse) sequence when reversed and complemented showed good homology to the mitochondrial cytochrome oxidase 1 gene from a number of organisms although the gene sequence ran in the opposite direction to that of the NADH1 sequence from the 3.85 (forward) sequence. The sequence of the 2kb (reverse) when reversed and complemented matched to some degree the cytochrome *b* gene of some invertebrates, whilst the other 3 sequences could not be matched to any known sequence.

5.2.3 Localisation of cloned fragments on the mtDNA

Comparison of *Hin*dIII-*Nsi*I and *Eco*RI-*Nsi*I double digests with *Hin*dIII and *Eco*RI single digests showed that patterns differed by only a single cut site. The 6.3kb *Eco*RI fragment was cut into approximately 4.3kb and 2kb fragments and the 2.55kb *Hin*dIII fragment was cleaved into 2.42kb and 0.13kb fragments. Therefore placement of the 2kb *Hin*dIII-*Eco*RI fragment is possible. From the sequence the *Nsi*I site is shown to be 129bp from the *Hin*dIII site of the *Eco*RI-*Hin*dIII 2kb fragment and so orientation can also be performed. Using these data the restriction map of Rigaa *et al.*(1993) was updated to indicate this and is shown in Figure 5.2. The position of the 3.85kb fragment is known but its orientation (to show the position of the primers) is not. Attempts to determine this using *Ava*II (a restriction site for *Ava*II is found in the sequence of the 3.85, forward primed fragment) were unsuccessful since the mtDNA contained far too many restriction sites to determine the restriction products of the 3.85kb fragment.

5.3 Discussion

In order to use restriction enzyme analysis of *Pecten* mtDNA to search for population variation it proved to be impractical to look at digests of the whole mitochondrial genome as a consequence of extensive length variation (Chapter 4).

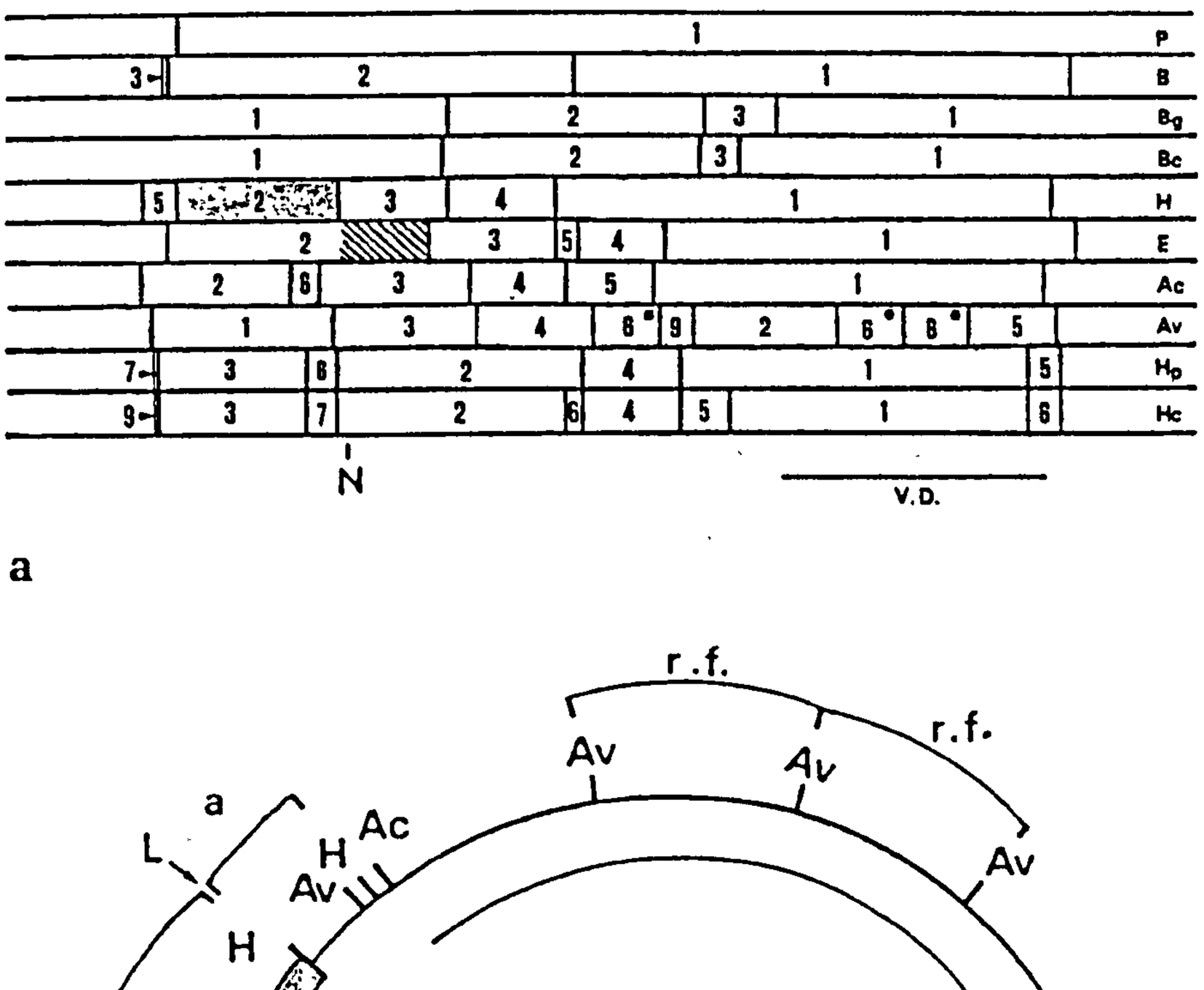


Figure 5.2. Restriction map for *P.maximus* mtDNA as published by Rigaa *et al.*(1993) but with additional information on the position of the 2kb and 3.85kb fragments isolated in this study. a) Cleavage map of mtDNA linearised at *Acc*I site close to 5′ end of small rRNA gene. P=*Pst*I, B=*Bam*HI, Bg=*Bgl*II, Bc=*Bcl*I, H=*Hin*dIII, E=*Eco*RI, Ac=*Acc*I, Av=*Ava*I, Hp=*Hpa*I, Hc=*Hin*cII. Numbers indicate relative size of fragments produced on digestion. VD=variable domain. ■=*Ava*I 1.6kb repeated fragment from the coding part of the genome with the same mobility as the 1.6kb repeated *Ava*I repeat (*) N=inferred position of *Nsi*I. Shaded region is the 3.85kb *Hin*dIII-*Hin*dIII fragment. Hatched section represents localisation of the 2kb *Eco*RI-*Hin*dIII fragment. b) Circular restriction map. The arc identifies a region that hybridises with the 1.6kb *Ava*I repeat fragment (r.f.). a=850bp *Hin*dIII fragment containing 12SrRNA gene. L=$tRNA^{lys}$ at 3′ end of the 12SrRNA gene. b=region hyridising with *Xenopus* 16SrRNA probe. Light shading shows 3.85kb fragment and heavy shading shows 2kb *Eco*RI-*Hin*dIII fragment. F=forward primer. R=reverse primer. E=position of one of the *Eco*RI sites (inferred from a) bounding the 2kb fragment.

To avoid this problem, a method involving restriction digestion of polymerase chain reaction (PCR) products distinct from the length variable region was developed. Regions of mtDNA fulfilling the appropriate criteria were identified by restriction enzyme digestion and cloned. This enabled sequencing for the design of PCR primers so that these sections could be subsequently amplified to create sufficient quantity of these areas of mtDNA for restriction enzyme digestion. Areas of mtDNA that were chosen due to their appropriate size and position were successfully cloned despite the inefficiency of the ligation. This inefficiency was probably caused by ligase inhibiting compounds extracted along with the DNA during DNA isolation from the gel. Any future attempts at cloning would take heed of the work of He *et al.* (1992) in order to boost ligation efficiencies and thus the number of suitable transformants. In this work He *et al.* (1992) state that if a 2,874g spin is used to elute the DNA from the gel then 83% recovery and 100% transformation efficiency can be expected if a 45s spin is used (although He *et al.* use the term transformation efficiency it is likely that what is being measured is ligation efficiency as inhibitors will affect the DNA ligase as the reaction is performed in a small volume whilst transformation is undertaken in a relatively high volume and inhibitors will consequently be diluted). Transformation efficiency drops to 86% whilst recovery improves to 92% if a one minute spin is used. When a 5 minute spin is utilised then recovery is at 96% but transformation efficiency drops markedly to 40% due supposedly to ligase inhibitors being spun from the agarose. As a 10 minute spin at 6,000g was used in this work then it can be appreciated that this is a likely cause of the low ligation efficiency. Plasmid DNA containing the appropriate inserts was isolated quickly and easily using simple mini-preps although commercial spin columns were employed to obtain sequencing quality DNA as contaminants such as RNA can cause problems to sequencing (Hillis and Moritz, 1990). This technique yielded high quality DNA (as evidenced by the A_{260}/A_{280} ratio) which proved to be ideal for sequencing and although sequences gained were short, they were clean, clear and unambiguous. Identification of the sequences was problematical due mainly to the short size of the insert sequences obtained and also to the lack of sequences of close relatives available in the DNA databases. The *Mytilus* mtDNA sequence (Hoffmann *et al.*,

1992) is the closest near complete sequence available. Some *P. maximus* sequences have been deposited in Genbank (see appendix H) but there was no match to these, including to the repeat sequence (Rigaa *et al.*, 1995). Despite these difficulties the ends of the 3.85kb clone showed similarities to the NADH 1 gene and cytochrome oxidase 1 gene of a number of organisms and the 2kb fragment showed affinity to the cytochrome *b* gene. The 1kb fragment did not match with any sequences. Caution does need to be taken when interpreting the results of such comparisons since a search can produce spurious but apparently complementary sequences since there is no fixed value at which 2 sequences become obviously complementary. In this case only matches between the *Pecten* sequence and sequences from multiple other organisms were considered significant. Further and less ambiguous identification of the sequences would require the complete sequences of the fragments for alignment to other mtDNA sequences. Accurate identification of constituent genes could suggest suitability of the fragments for measuring population level variation before any RFLP or sequence information is uncovered. Many genes evolve at similar rates in a variety of organisms, therefore if these fragments contained genes known to be rapidly evolving their suitability for answering the question at hand would be indicated. However genes that show variation in some organisms have proved unsuitable for other organisms e.g although D-loop sequences have been useful for answering population level questions in mammals, this has not been so for some fish species (Park and Moran, 1995).

Two of three PCR primer pairs have been shown to flank sequences distinct from the length variable domain (see Figure 5.2) and since no match was found between the sequence and the sequence of the repeat region (Rigaa *et al.*, 1995) there is almost no likelihood that the length variable domain will cause problems for data analysis when the PCR method is used, as it does for studies of the whole molecule (Chapter 4).

6.0 Mitochondrial DNA variability assessed by PCR-RFLP

6.1 Materials and methods

6.1.1 Sampling

Samples of *P. maximus* and *P. jacobaeus* were collected as in Chapter 2. Due to time considerations, a subset of samples were studied for mtDNA variation omitting the PS1, PS2 and ANG samples, since the Peel, Douglas and Chicken Rock samples were assumed to provide an adequate number of Irish Sea samples. Mitochondrial DNA isolated from *A. opercularis* (by the same method as for *P. maximus* as in Chapter 4) was also used. Two samples of isolated genomic DNA from the New Zealand scallop *P. novaezelandiae* were provided by Simon Bulman (Crop and Food Research, Private Bag 4704, Christchurch, New Zealand).

6.1.2 DNA extraction

Isolation of mtDNA followed the methods described in Chapter 4.

Total genomic DNA (gDNA) was extracted using a method based on Doyle and Doyle (1987)[§]. The extraction protocol is reportedly suitable for most tissue types. Because of its year-round availability in contrast to gonad tissue, adductor muscle was preferred.

Adductor muscle tissue (1-2mm^3, ≈50mg) was ground in a 1.5ml eppendorf tube containing 300µl CTAB buffer (100mM Tris-HCl pH8.0, 1.4M NaCl, 20mM EDTA, 2% CTAB, 0.2% β-mercaptoethanol) using a metal macerating rod and aided by a pinch of sterile sand. Following maceration and addition of proteinase K to 0.5 mg ml^{-1} tubes were incubated at 60°C for 30min. On completion, tubes were cooled to room temperature then the CTAB removed by extraction with 1vol chloroform-isoamyl alcohol (24:1) and centrifugation at 13,000r.p.m. for 3min. The organic phase and pellet of undigested tissue was then discarded and the aqueous phase containing the DNA, extracted with 1vol phenol-choloroform-isoamyl alcohol (24:24:1) to remove proteins, then centrifuged at 13,000r.p.m. Following removal of the aqueous phase to a clean tube the chloroform-isoamyl alcohol extraction was repeated and the aqueous phase transferred to another clean tube. DNA was subsequently precipitated from this at -20°C for 1hr following

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[§] method sent to the mollusc molecular news bulletin board by Andrew McArthur (University of Victoria, B.C., Canada).

addition of 2vol ice-cold ethanol and 0.1vol 3M sodium acetate and mixing by inversion. Following precipitation, DNA was pelleted for 10min at 13,000r.p.m. then the ethanol discarded and replaced with 0.5ml 70% ethanol to wash the pellet. The pellet was resuspended then centrifuged for 10min at 13,000r.p.m. After removal of the ethanol, any remaining liquid was blotted away with tissue and the pellet left to air dry for 10min. Once dry, the DNA pellet was left to dissolve in 50µl dH_2O overnight at 4°C (no attempt was made at resuspension until after dissolution had occurred, to avoid shearing the DNA).

6.1.3 Polymerase Chain Reaction

In all 10 primer pairs were tested on *P. maximus* DNA including the 3 pairs developed in Chapter 5 specifically for *P. maximus* and 7 other pairs (Table 6.1) either universal primers or primers developed for the Japanese scallop *Patinopecten yessoensis* (Boulding *et al.*, 1993; provided by E.Boulding, Simon Fraser University, B.C., Canada. Now University of Guelph, Guelph, Ontario, Canada). All primer pairs target mitochondrial DNA genes except the ITS primers directed at the nuclear rDNA repeats.

A standard PCR protocol was used for all reactions although modifications and optimisations (see 6.1.4) were required for each primer pair.

The standard reaction was prepared to 50µl containing 1x PCR buffer (provided with the *Taq* or *Tbr* DNA polymerase), 2.5mM $MgCl_2$, 200µM each dNTP, 25pmol each primer, 1U *Taq* polymerase and 1µl DNA solution. *Taq* polymerase (supplied by Promega) was used for most targets although *Tbr* DNA polymerase (supplied by NBL) worked better for the Pma2 primer pair. Addition of 1U *Taq* extender (Stratagene) increased the yield from amplifications using both Pma1 and Pma2 when added directly to the PCR cocktail. Amplification under the manufacturer's recommended conditions failed to amplify any product. Since yield from Pma1 amplification was sufficient for restriction analysis *Taq* extender was not used routinely in amplifications with these primers. Yield from amplification using the Pma2 primer pair was only adequate for further manipulation when *Taq* extender was added. DNA templates consisted of 1µl of a 1/10-1/100th dilution of mtDNA or 1µl of genomic DNA (100-500ng). A modification of the "hot start" method (Newton and Graham, 1994) was employed to reduce the chances of

Primer, Target and Source	Primer Sequences	Conditions
12S rRNA (Kocher *et al.*, 1989)	aaactgggattagataccccactat gagggtgacgggcggtgtgt	1′-1′-1′ 94°-55°-72°
717 & 641. Cyt *b* (Kocher *et al.*, 1989)†	gtacgtaccatccaacatctcagcatgatg aaactgaattccctcagatgatatttg	1′-1′-1′ 94°-47°-72°
717 & MVZ16. Cyt *b* (extended length ≈800bp)†	gtacgtaccatccaacatctcagcatgatg aaataggaa(a/g)tatca(t/c)tctggttt(a/g)at	1′-1′-2′ 94°-47°-72°
#53, #54. ND2;$tRNA^{thr}$ (Boulding *et al.*, 1993)	caggtggtttggggtgtggg cccggctcaacacctgacag	1′-30s-1′ 94°-48°-72°
#48, #49. ATPase 6/CoIII (Boulding *et al.*, 1993)	agggctctatccgctaggca cgcctccccgataatagcc	1′-30s-1′ 94°-48°-72°
#59, #60. end of cyt b (Boulding *et al.*, 1993)	cgtgtgcttgcccaggtagg aattctccgctctattccga	1′-30s-1′ 94°-48°-72°
Pma1. 2kb section of *P. maximus* mtDNA (Ch. 5)	ttttaaggaggtaatcgctattcg cagcaatctgtatgggtagaacc	45″-30″-2′ 94°-52°-72°
Pma2. 3.85kb section of *P. maximus* mtDNA (Ch. 5)	ttttatgtggtggtattgttgagg ataggatagtctggaatacgacgc	1′-1′-8′ 94°-56°-72°
Pma3. 1kb section of *P. maximus* mtDNA (Ch. 5)	aggtggaaatcaaggcattg tcagaatacaggcgactgttaaag	
ITS4 & psnDNA2p. ITS region of rDNA‡	ttcttcgcttattgatatgc gtccacacaccgcccgt	1′-1′-2′ 94°-55°-72°

Table 6.1: Primers tested on *P. maximus* DNA (given 5′→3′). Reaction conditions given as time periods at denaturation-annealing-extension temperatures which followed an initial 3′, 94°C denaturation step. † designed and provided by T.Griffiths, S.B.S., U. of Wales, Bangor. ‡ designed by G.Griffiths, S.B.S., U. of Wales, Bangor.

formation of primer-dimers (Mullis, 1991). For this, all reaction components except the *Taq* DNA polymerase mix (in 10µl of 1x *Taq* buffer and for the Pma2 primers containing 1U *Taq* extender) were added to individual 500µl micro test tubes then heated to 94°C in the PCR machine prior to addition of the *Taq*. 10µl

Taq mix was then pipetted into individual tubes whilst at 94°C, using a clean pipette tip for every addition and thoroughly mixed with the pipette tip to ensure complete homogeneity, essential for optimal PCR performance (Carbonari *et al.*, 1993). No mineral oil was added because a heated lid was fitted to the PCR machine, but this meant that addition of *Taq* had to be performed quickly to avoid evaporation of water from the reaction mix whilst the lids were open and condensation within the closed tubes whilst the heated lid was displaced.

Cycle parameters for Pma1 were optimised to an initial 3min at 94°C, followed by 35 cycles of 45secs at 94°C, 30 secs at 52°C and 2min at 72°C with a final extension time of 7min. The cycle parameters for Pma2 were 3min at 94°C, then 40 cycles of 1min at 94°C, 1min at 56°C and 8min at 72°C with a final extension time of 10min.

A control (no DNA) tube was used in every PCR run to increase the chances that any contamination would be detected.

6.1.4 Optimisation of PCR

Bands produced were starting points for optimisation trials to attempt to increase yield, specificity or reproducibility. If bands were faint or amplification was unsuccessful then repeat amplifications with varying Mg^{2+} concentrations, annealing temperatures, *Taq* manaufacturers, primers and DNA concentrations were performed. These are known to have effects on PCR reactions (Linz *et al.*,1990).

6.1.5 Restriction digestion of PCR products

5µl of PCR product was digested with 1-2U of the appropriate restriction enzyme in 15µl total (made up with dH_2O) containing 1.5µl 10x restriction enzyme buffer. Digests were performed in microtitre plates. In all, 9 4bp cutting enzymes (Promega) were screened against the amplified DNA sections: *Alu*I, *Cfo*I, *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3AI and *Taq*I. Following digestion and addition of 2µl loading dye, digestion products were electrophoresed alongside either 100bp or 123bp markers (Life Technologies), through 1.4% agarose in TBE buffer for 3-5hr at 100V, then stained with ethidium bromide, viewed under UV light and photographed. Restriction enzymes identifying polymorphic patterns in more than

1 of 20 individuals were screened against all animals. From the resultant fragment patterns homologous fragments were identified for use in data analysis and where possible the types of site changes that had occurred were identified, allowing either fragment or site data to be used in statistical interpretation of the data.

6.1.6 Statistical methods

Each polymorphic pattern generated by a restriction enzyme was labelled as A, B, C .....etc. For each individual animal a composite haplotype then described the patterns over all polymorphic enzymes, thus if 4 enzymes had been employed and an individual was described as AACB, restriction digestion with 4 different endonucleases would have produced patterns A, A, C and B.

From fragment patterns it was possible to produce a matrix of shared fragments between RFLP patterns (1 for presence, 0 for absence) and where sites were identifiable, a matrix of shared sites was prepared. The Restriction Enzyme Analysis Package (REAP, McElroy *et al.*, 1992) was used to analyse data obtained by PCR-RFLP. The input for REAP consisted of a binary matrix of presence/absence of sites or fragments for each restriction enzyme and the numbers of each composite haplotype in each population.

6.1.6.1 Variability at the haplotype level

From the haplotype frequencies in each population haplotype diversity ($\hat{h}$) was calculated. Haplotype diversity is the probability of encountering different haplotypes when 2 individuals are sampled from a population.

$$\hat{h} = \frac{n(1-\sum x_i^2)}{n-1}$$

Where x_i is the sample frequency of haplotype I in population X and n is the total number of composite haplotypes in that population. This measure is analogous to heterozygosity or gene diversity (Nei, 1987).

Frequencies of composite haplotypes were tested for heterogeneity via χ^2 using a Monte-Carlo procedure provided by the MONTE option of REAP with 10,000 randomisations. This follows the method of Roff and Bentzen (1989) to avoid the problems associated with empty cells and small sample sizes. χ^2 was performed both over the entire data set and in pairwise comparisons of populations.

6.1.6.2 Variation at the nucleotide level

The number of nucleotide substitutions per restriction site (d) can be estimated from either fragment or site data. When fragment sharing is used as input then first of all the expected proportion of shared fragments ($\hat{F}$) between 2 haplotypes is estimated by:

$$\hat{F} = \frac{2m_{XY}}{(m_X + m_Y)}$$

where m_X and m_Y are the numbers of restriction fragments in haplotypes X and Y and m_{XY} is the number of shared fragments. $\hat{F}$ is then used to calculate $\hat{G}$:

$$\hat{G} = \{\hat{F}(3 - 2\hat{G}_1\}^{\frac{1}{4}}$$

where $\hat{G}_1$ is a trial value of $\hat{G}$. An iterative computation is performed until $\hat{G}=\hat{G}_1$. From $\hat{G}$, d can then be estimated as:

$$\hat{d} = -\left(\frac{2}{r}\right)\log_e \hat{G}$$

where r indicates the length of the recognition sequence of the restriction enzyme, for example, 4, 5, 6 etc. or for those enzymes with multiple recognition sequences, 14/3 or 16/3 (see Nei, 1987).

Using site data d is estimated for each class of restriction enzyme (r value) using S, the fraction of shared sites (Nei and Tajima, 1981; Nei and Miller, 1990):

$$S_{ij} = \frac{2m_{ij}}{m_i m_j}$$

where m_i and m_j are the number of restriction sites in DNA segments i and j and m_{ij} is the number of shared restriction sites.

Then if there is only one class of restriction enzyme:

$$\hat{d}_{ij} = [-\log_e S_{ij}]/r$$

If there is more than 1 class of restriction enzyme used (enzymes of different r value) then:

$$\hat{d}_{ij} = \frac{\sum_k \overline{m}_k r_k \overline{d}_{ij}(k)}{\sum_k \overline{m}_k r_k}$$

for the k[th] class of restriction enzyme $\bar{d}_{ij}$(k) is obtained from the equation used to calculate for single enzymes (above) and $\overline{m}_k$ is $(m_{i(k)} + m_{j(k)})/2$

Once the number of nucleotide substitutions per site has been estimated either from fragment or site data, then using the frequencies of haplotypes within each population it is possible to estimate the average number of nucleotide substitutions for a randomly chosen pair of haplotypes(d_{ij}) in population X as:

$$\hat{d}_X = \frac{n_X}{n_X - 1} \sum_{ij} \hat{x}_i \hat{x}_j d_{ij}$$

where n_X is the number of sequences sampled and d_{ij} is the number of nucleotide substitutions per site between the i[th] and j[th] haplotypes.

Nucleotide divergences among haplotypes (calculated by D in REAP) were used to construct a bootstrapped majority rule concensus tree within PAUP (Swofford, 1989), to depict haplotype relatedness. Bootstrapping involves creating a new data set by sampling *N* characters randomly with replacement from the original data set so that some characters are duplicated and some ommited. The random variation of the results from analyzing bootstrapped data sets is typical of that which would be revealed from collecting new data sets.

Using data on haplotype frequency and inter-haplotype nucleotide substitution levels, divergence between populations was then estimated as the average number of nucleotide substitutions (uncorrected nucleotide divergence) between haplotypes from populations X and Y ($\hat{d}_{XY}$:).

$$\hat{d}_{XY} = \sum_{ij} \hat{x}_i \hat{y}_i d_{ij}$$

Where d_{ij} is the number of nucleotide substitutions per site between the i[th] haplotype from population X and the j[th] haplotype from population Y. However such a measure does not account for within population polymorphism. The net divergence) which does account for this is measured as:

number of restriction site differences between populations (corrected nucleotide

$$\hat{d}_A = \hat{d}_{XY} - \frac{(\hat{d}_X + \hat{d}_Y)}{2}$$

To depict population relatedness $\hat{d}_A$ values were used to construct a UPGMA dendrogram clustered using the SAHN option of NTSYS (Rohlf, 1990b) and plotted using the TREE option.

6.1.7 Gene flow

Although methods are available for calculating F_{ST} analogues from mtDNA data (Takahata and Palumbi, 1985; Birky Jr. *et al.*, 1989; Lynch and Crease, 1990; Weir, 1990) they are computationally laborious. Any F_{ST} (or equivalent) measure may also be inappropriate for mtDNA since the mtDNA molecule is effectively a single locus, transmitted as a single linkage unit and thus any F_{ST} estimate is not based on multiple, independently segregating loci and consequently may be inaccurate. Also, as the mtDNA mutation rate is elevated respective to that of allozyme loci (for which F_{ST} is suitable) then the assumption that the mutation rate is much lower than the migration rate is more likely to be violated (Neigel and Avise, 1993).

Since F_{ST} could not be assessed the only available way to estimate migration was Slatkin's (1985) private allele method where N_em is estimated using $\bar{p}(1)$ the average frequency of alleles found in only 1 site and $\bar{N}$, the average sample size. This theory is based on the supposition that alleles occur in only single populations if gene flow is restricted. The applicability of the equation for haploid data is not known.

$$N_em = \frac{e^{-[(\ln[\bar{p}(1)]+2.44)/0.505]}}{\bar{N}/25}$$

6.2 Results

6.2.1 PCR

For some primer pairs no product was amplifiable no matter what conditions were employed. These included the 3 pairs of mtDNA primers designed for *Patinopecten yessoensis* (#48 and #49, #53 and #54, #59 and #60; Boulding *et al.*, 1993), the extended cyt *b* pair designed for herpetological specimens (717 and MVZ16) and Pma3 designed from *P. maximus* mtDNA sequence. Why the Pma3 primer pair did not prove successful is unknown. The 1kb *Eco*RI-*Hin*dIII fragment which was cloned and sequenced to facilitate primer design was not seen in initial mtDNA digests. It was assumed that restriction site variation had resulted in the

presence of this fragment in the individual's mtDNA used for cloning however it is possible that it was a segment of foreign (bacterial) DNA that was cloned and sequenced. This would explain why the primers did not work on *P. maximus*, but the presence of multiple clones (5.2.1) containing this fragment argues against this. The 12S rRNA, Cyt *b* (short segment) and ITS region primers (Table 6.1) were all successful, amplifying products of 400bp, 375bp and 1kb respectively as were Pma1 and Pma2 primer pairs designed for *P. maximus* which amplified segments of the correct size. Of the primer pairs for which amplification was successful, since Pma1 and Pma2 were designed specifically for *P. maximus* (and therefore contamination by other organism's DNA would be less problematical) and amplified longer fragments thereby creating a greater chance of containing restriction sites these were chosen for continued screeening and restriction digestion. Limited screening of amplified ITS regions was also undertaken.

Neither the 2kb or 3.85kb amplified product exhibited any evidence of length variation when undigested PCR product was electrophoresed. On no occasion was contamination detected through either bands in the control lane or through differences in haplotypes between repeat amplifications from either the same DNA sample or separate extractions from the same individual. Amplification was successful from both mtDNA and gDNA. Products were "cleaner" from mtDNA preparations and when digested and electrophoresed did not exhibit streaks as did products amplified from gDNA, although these did not obscure any bands. Amplification and restriction digestion of PCR products from gDNA and mtDNA of the same individual always produced identical haplotypes.

Optimisation of reaction conditions produced dramatic changes in yield and realiability of some amplifications. Table 6.2 details those factors which produced responses in the PCR reaction.

6.2.2 Restriction analysis of PCR products

Table 6.3 details the number of patterns revealed by restriction enzyme digestion of mtDNA regions amplified using the PCR. No pattern suggested that there was heteroplasmy of mtDNA evident from these regions.

*Alu*I cut PCR fragments produced uninterpretable variation probably due to incomplete digestion. However there did seem to be an underlying polymorphism.

Factor	Response
Annealing Temperature	Decrease → reduced specificity but increased yield
Magnesium concentration	Increase of 1-2mM over optimum → increased yield but decreased specificity.
Quality of primers	>2 freeze thaws of primers caused failures of PCR reactions
Taq manufacturer	*Tbr* polymerase (NBL) fared better in some PCRs for longer targets
Taq extender	1U *Taq* extender (Stratagene) improved yield using primers Pma1 and Pma2 (not tested on others)
DNA concentration	Use of too much DNA (particularly gDNA) prevented PCR

Table 6.2: Effect of altering PCR reaction conditions on amplification success in standard PCR reaction.

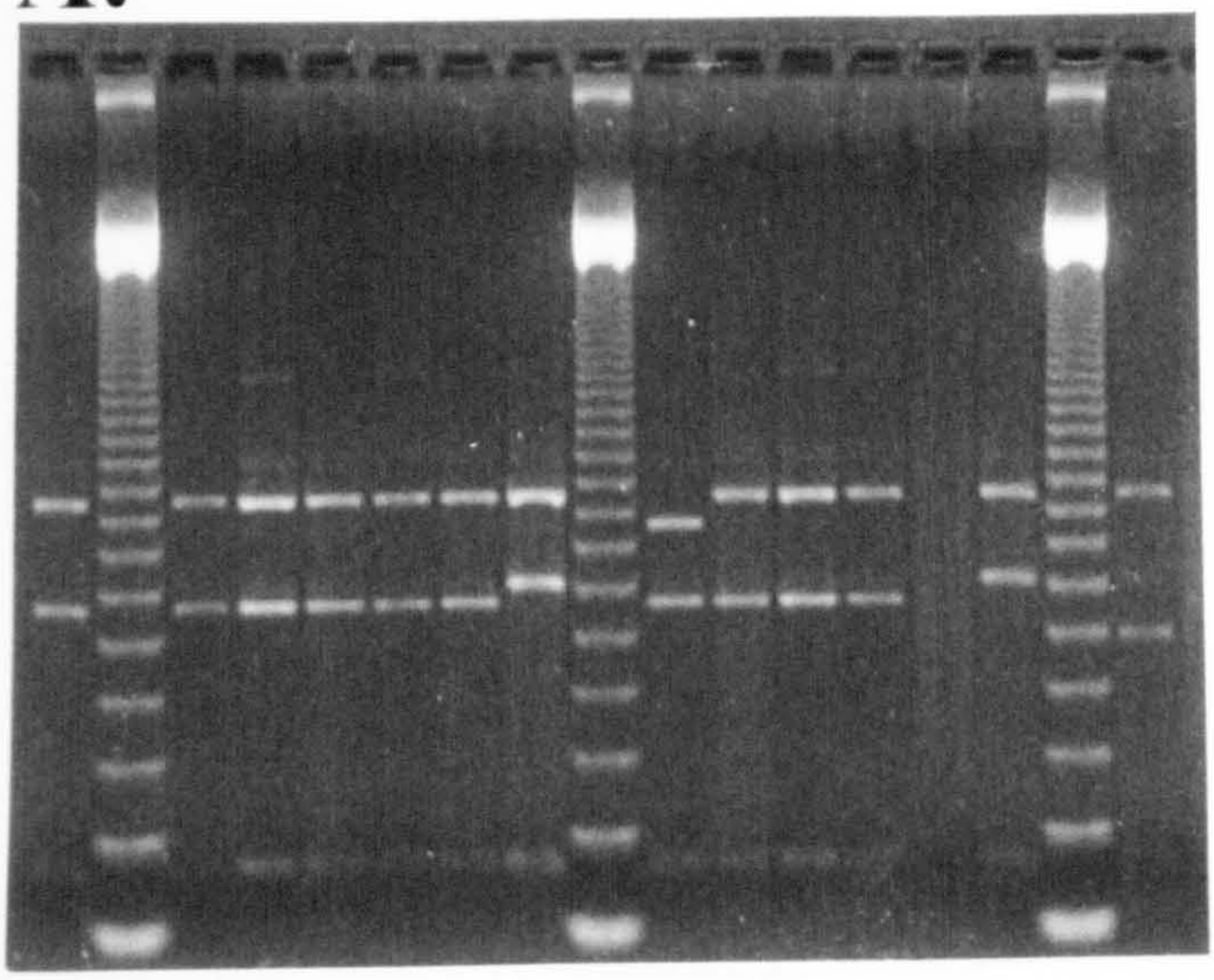
Restriction Enzyme	Pma1 fragment	Pma2 fragment
*Alu*I	Uninterpretable variation	
*Cfo*I	4	1
*Dra*I	1	6
*Hae*III	1	15
*Hin*fI	6	6
*Msp*I	6	9
*Rsa*I	9	8
*Sau*I	8	4
*Taq*I	4	4

Table 6.3: Number of haplotypes generated upon restriction digestion of Pma1 and Pma2 amplification products with a variety of restriction enzymes.

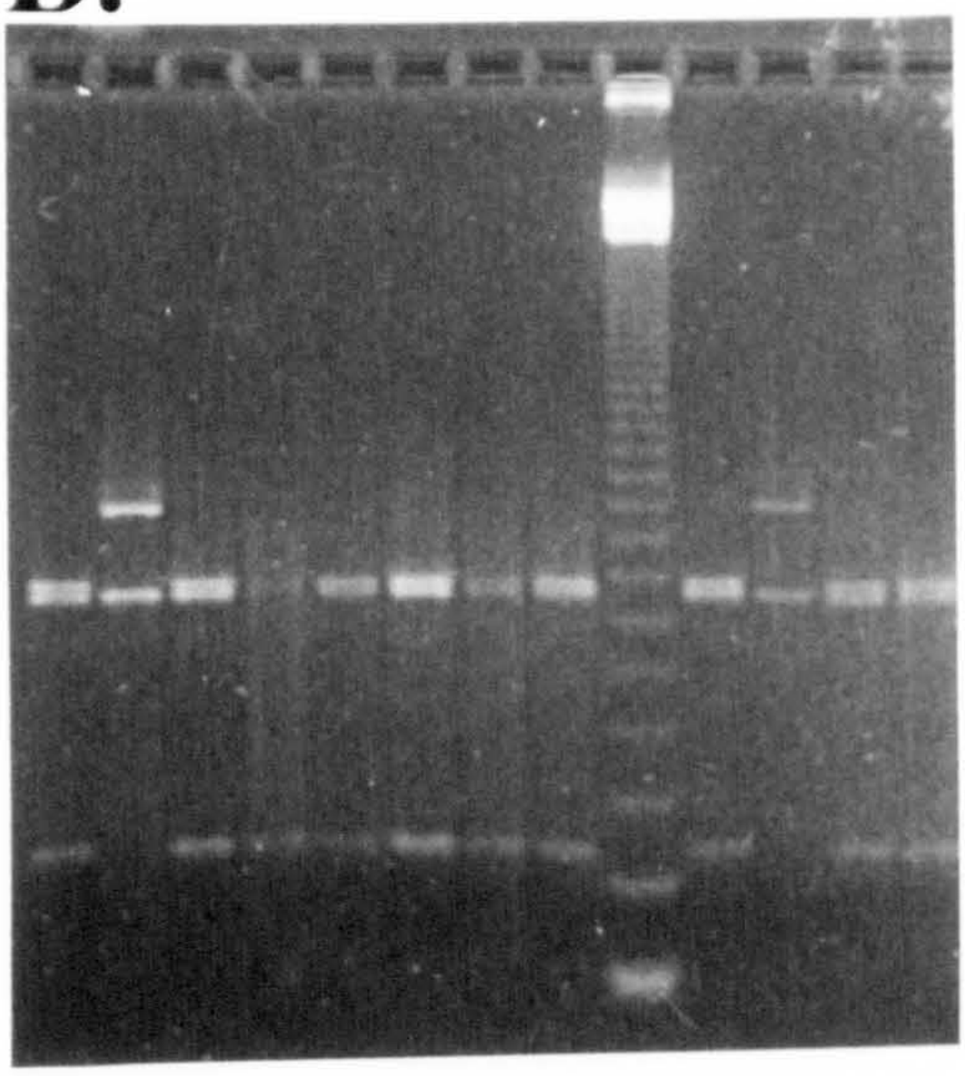
Examples of fragment patterns generated by each restriction enzyme for both the Pma1 and Pma2 amplified fragments are shown in Figures 6.1(a-h) and 6.2(a-h) respectively. Diagrammatic representations of RFLP patterns are shown in

Figure 6.1: Examples of RFLP profiles generated by restriction digestion of the Pma1 amplified fragment with A) *Msp*I B) *Taq*I C) *Cfo*I D) *Rsa*I E) *Sau*3AI F) *Hin*fI.

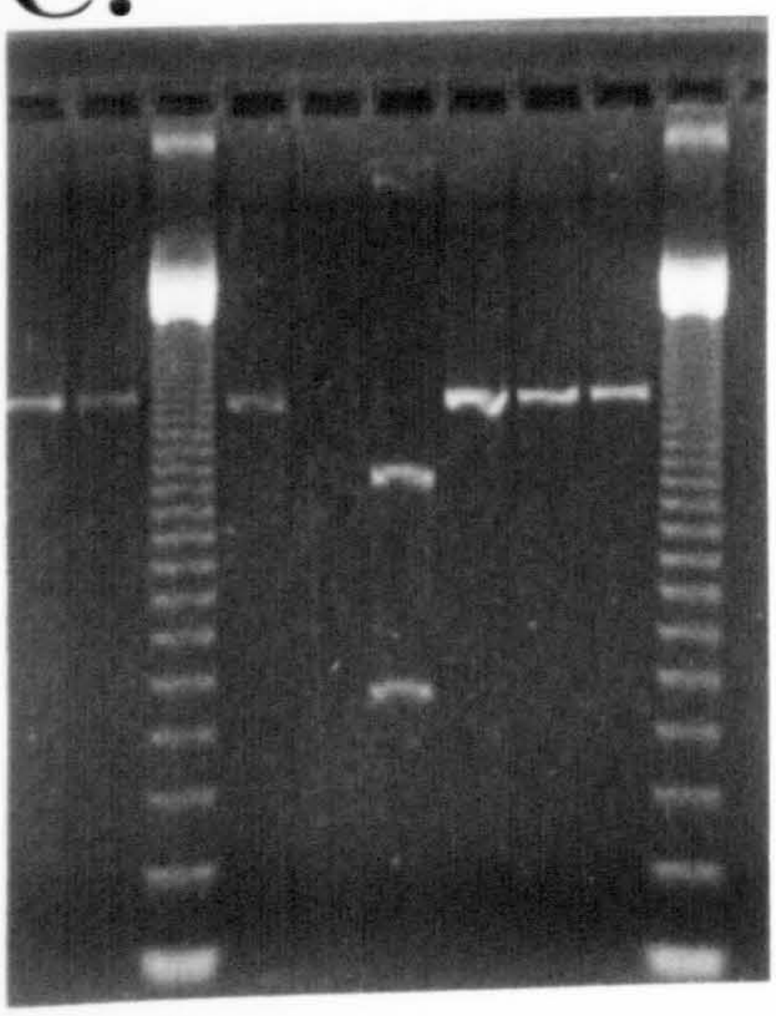
A.



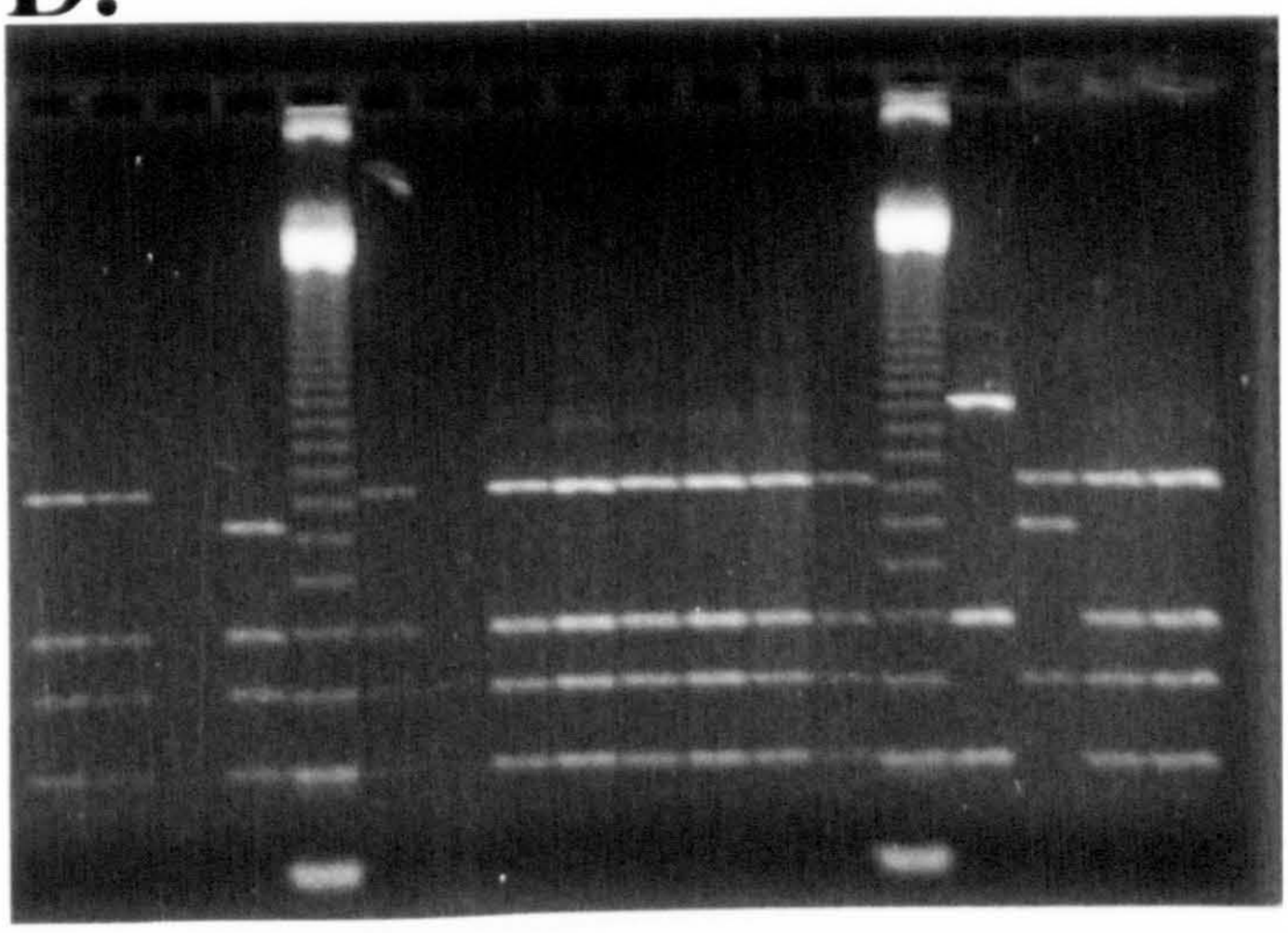
B.



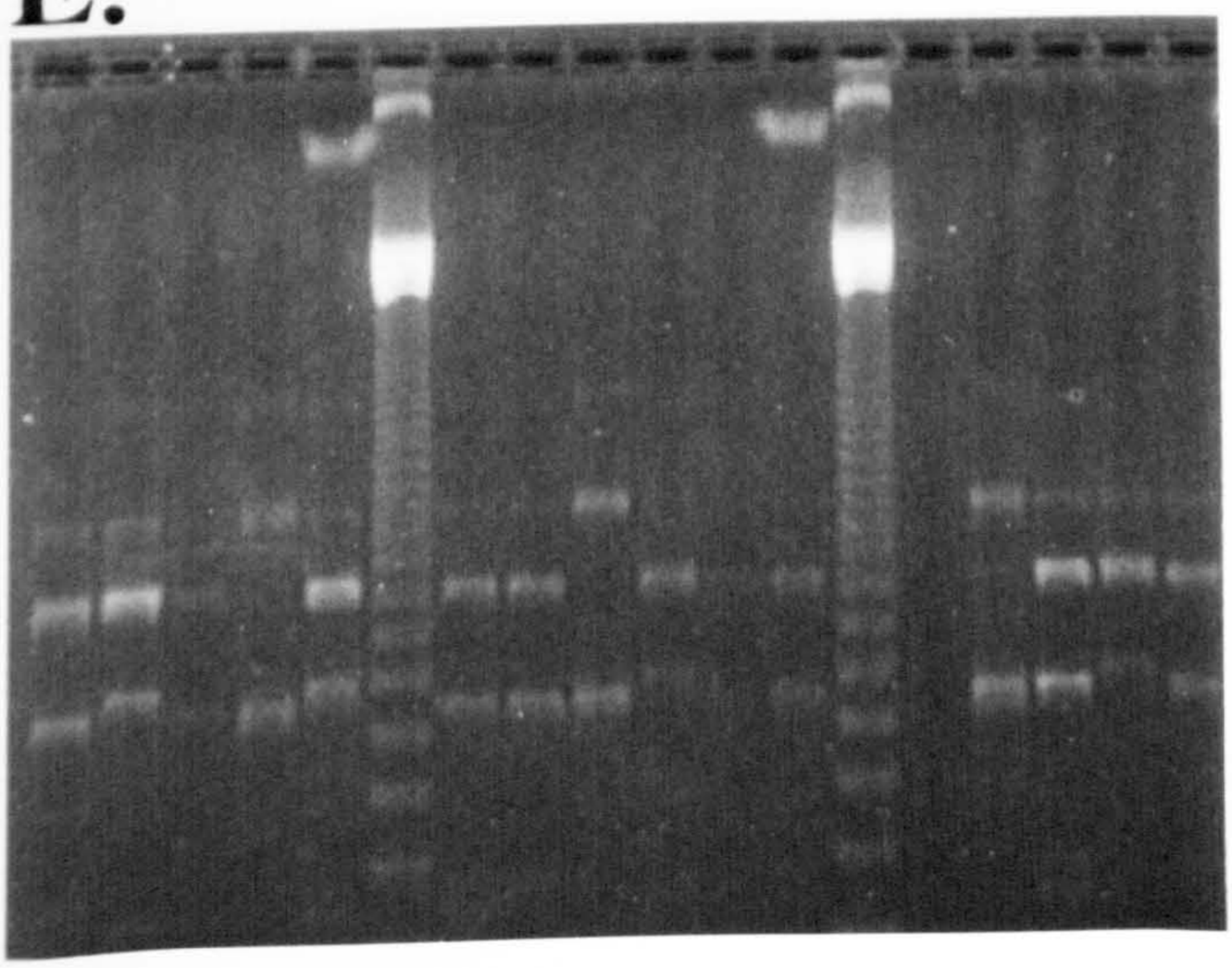
C.



D.



E.



F.

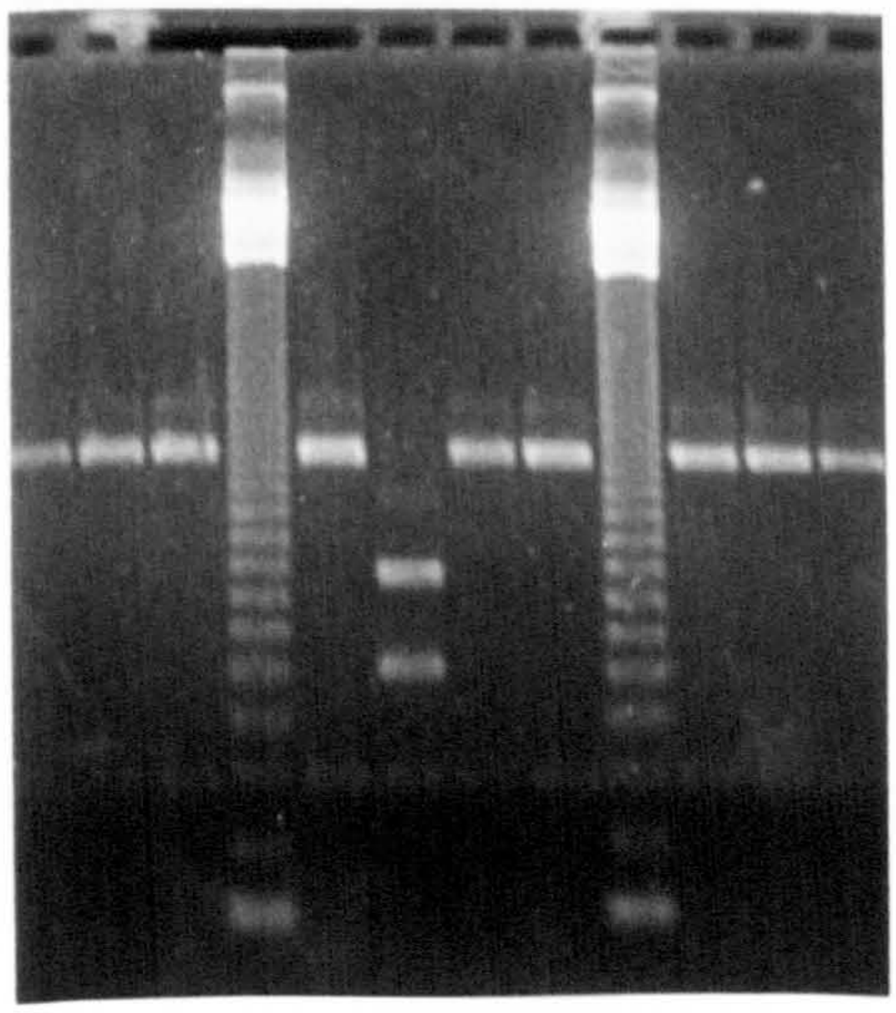
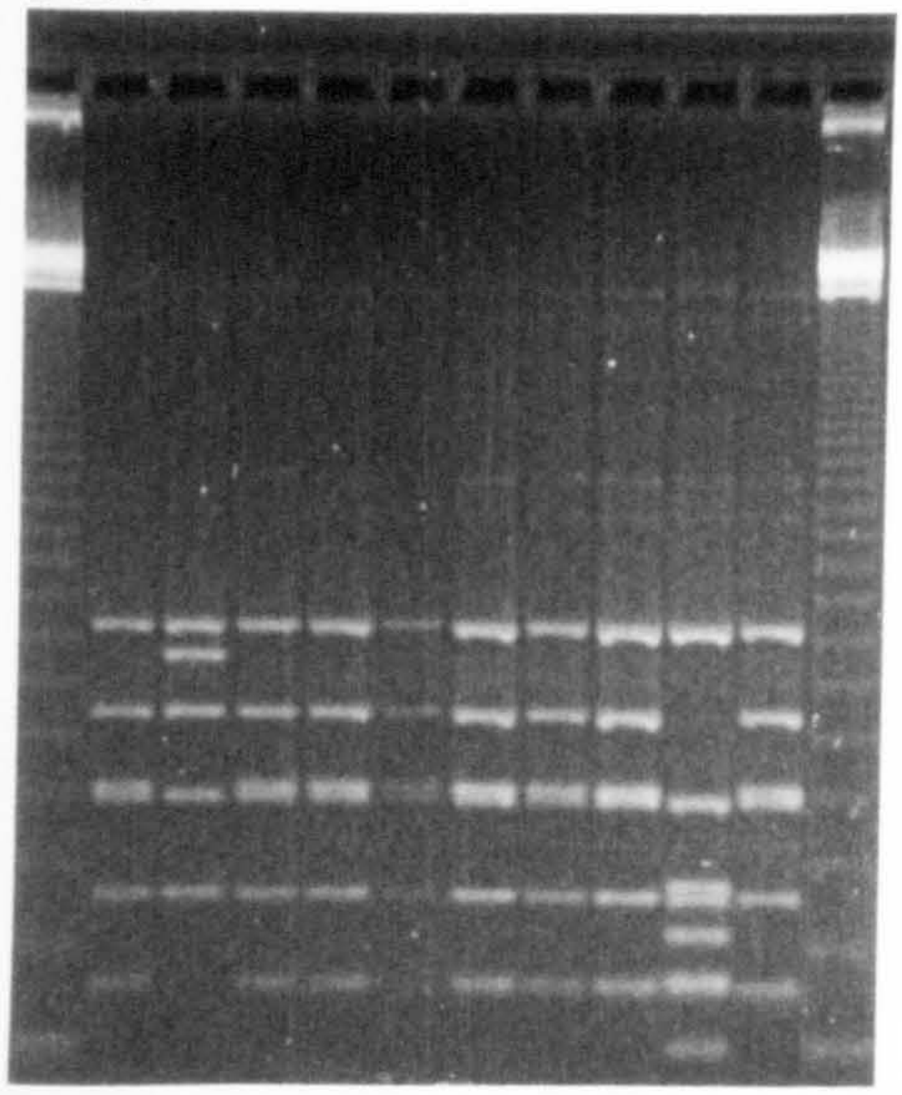
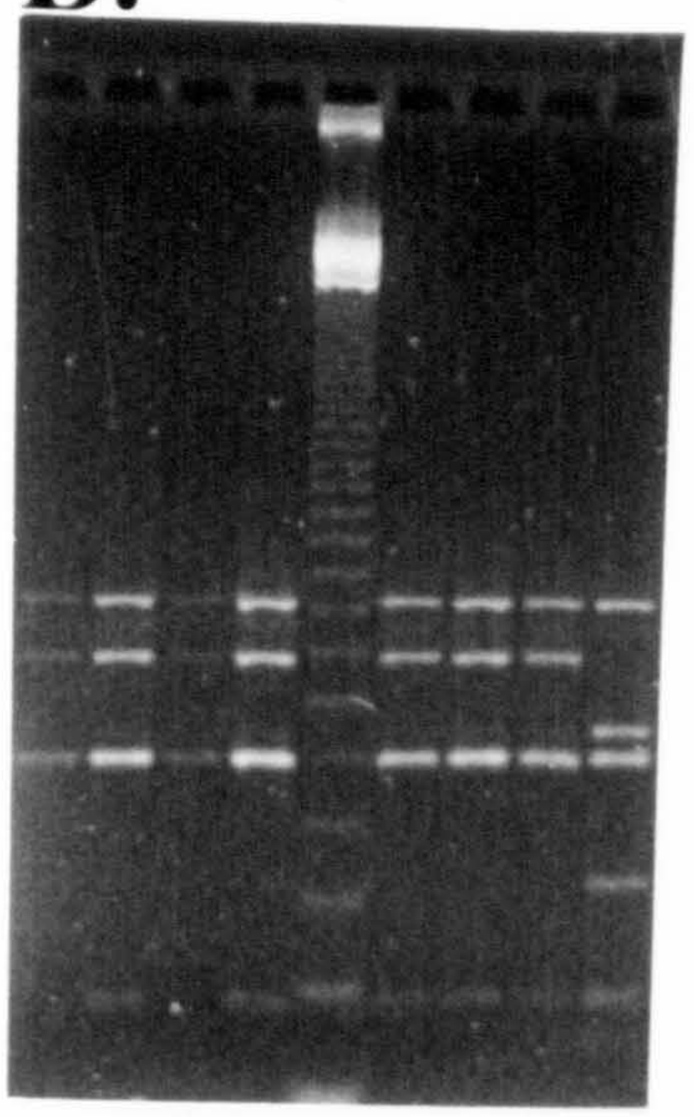


Figure 6.2: Examples of RFLP profiles generated by restriction digestion of the Pma2 amplified fragment with A) *Hin*fI B) *Taq*I C) *Sau*3AI D) *Msp*I E) *Dra*I F) *Rsa*I.

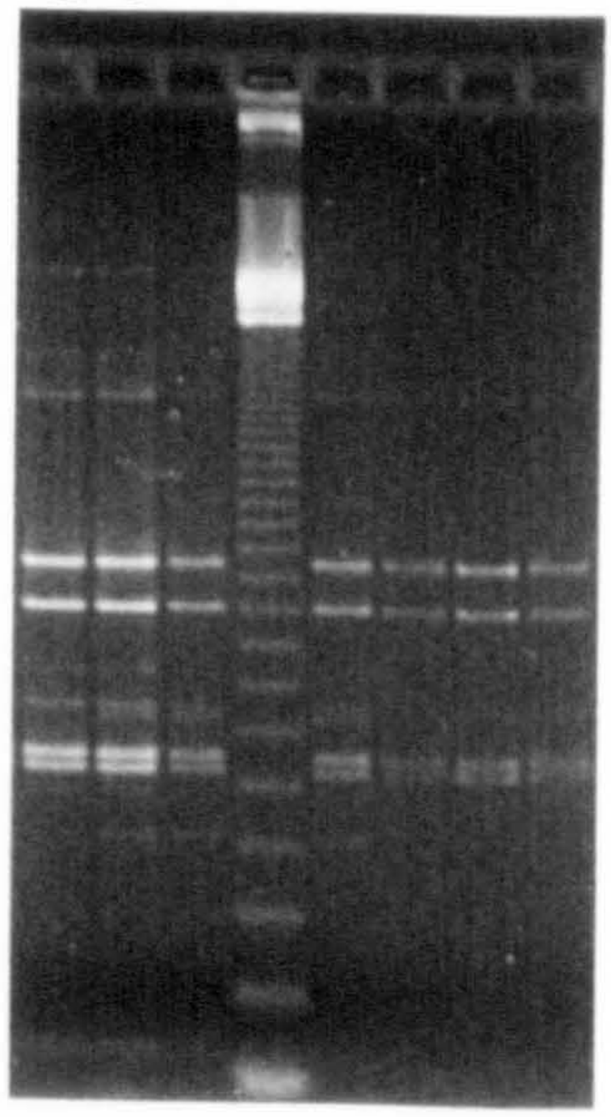
A.



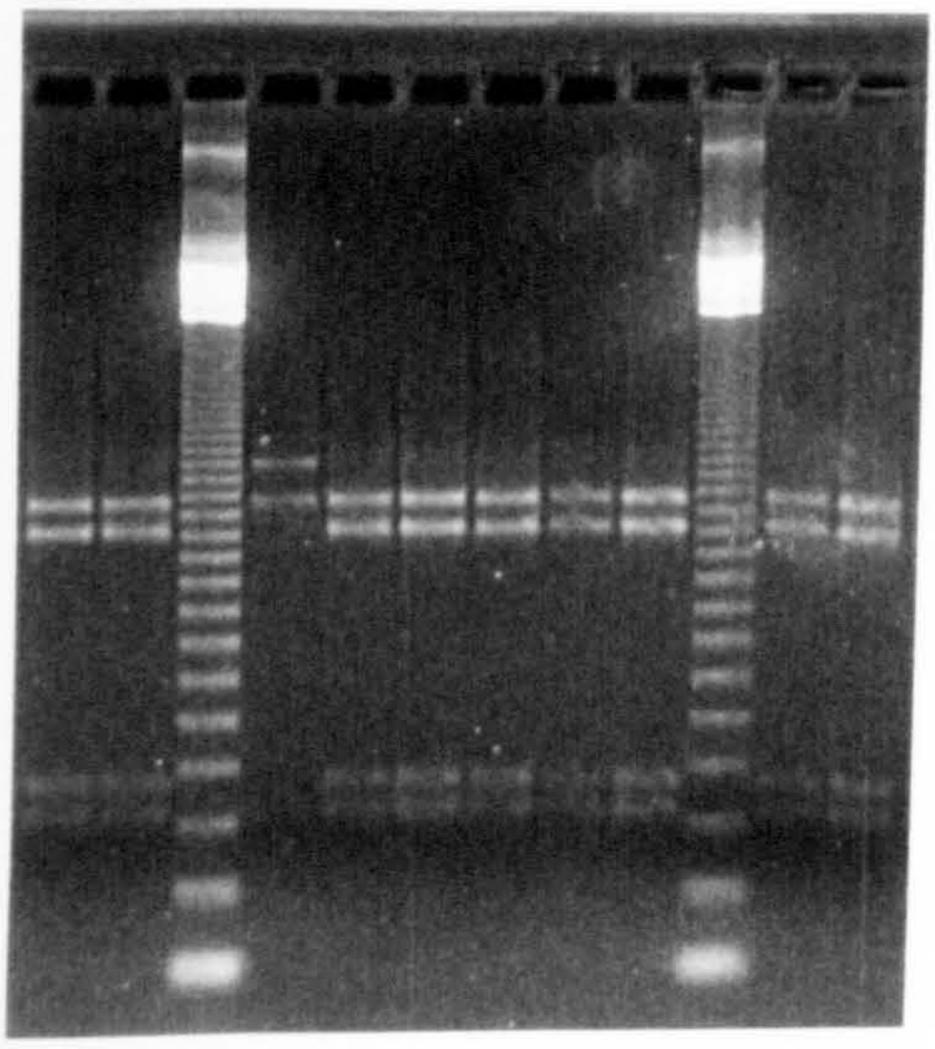
B.



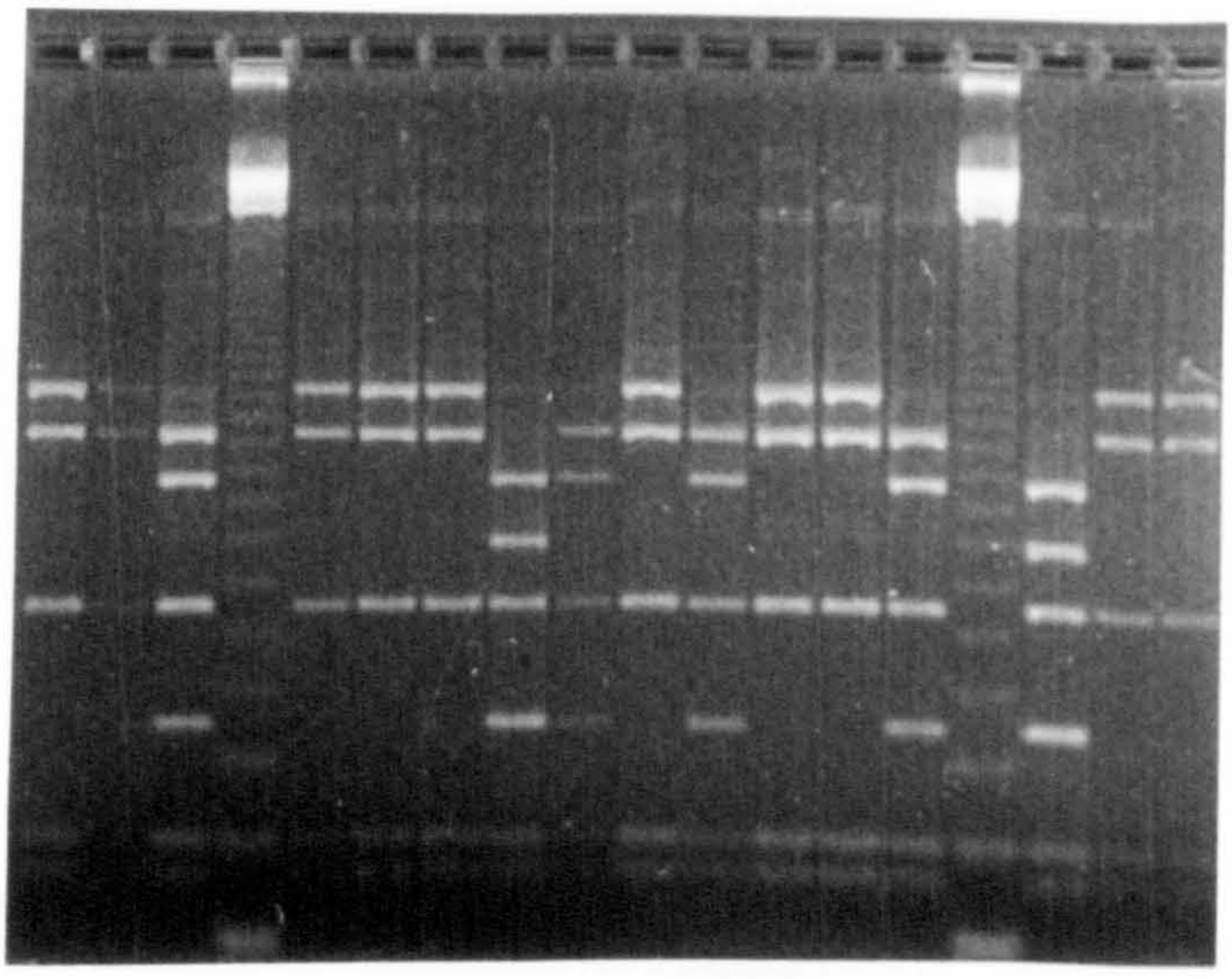
C.



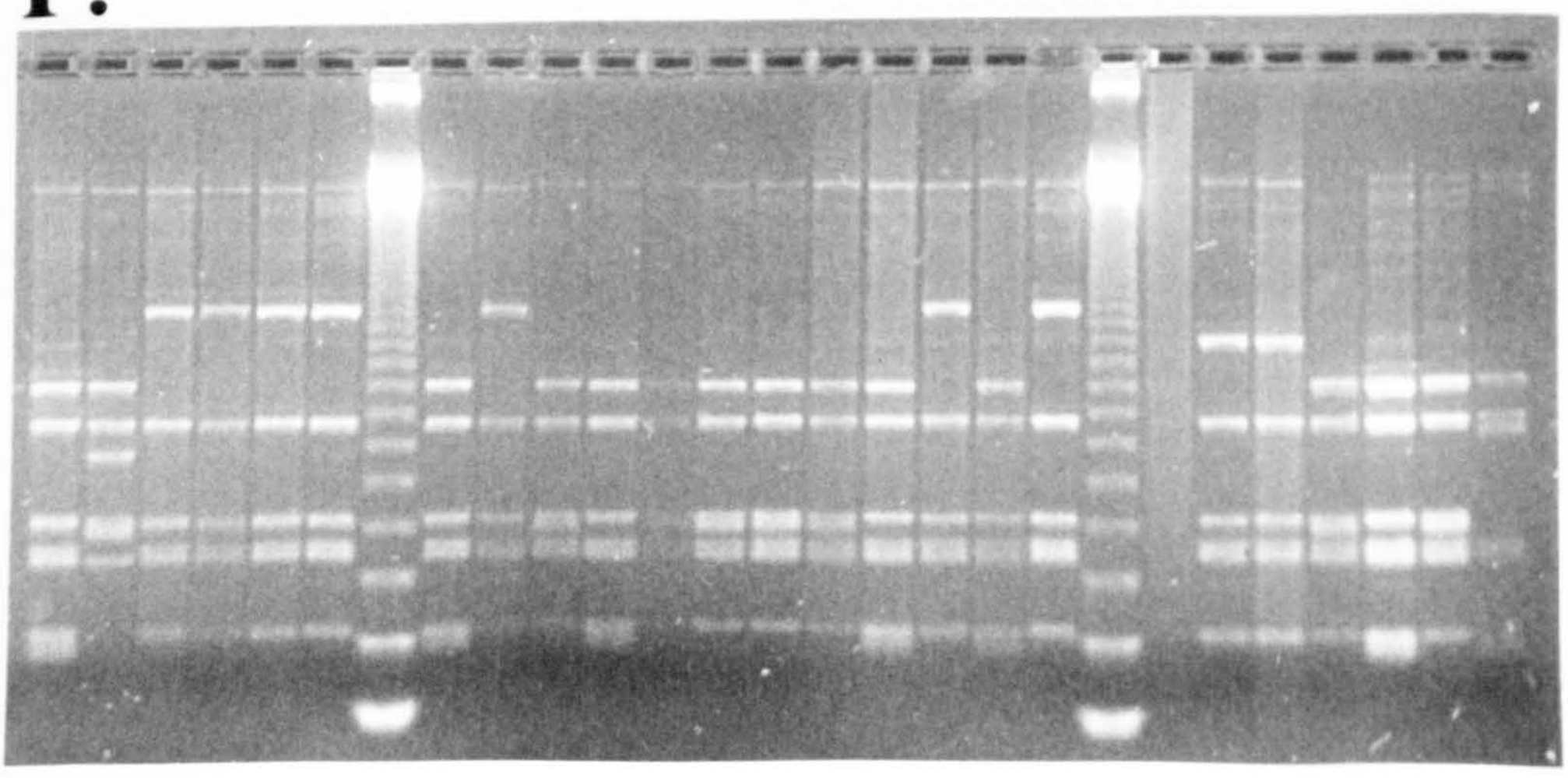
D.



E.



F.



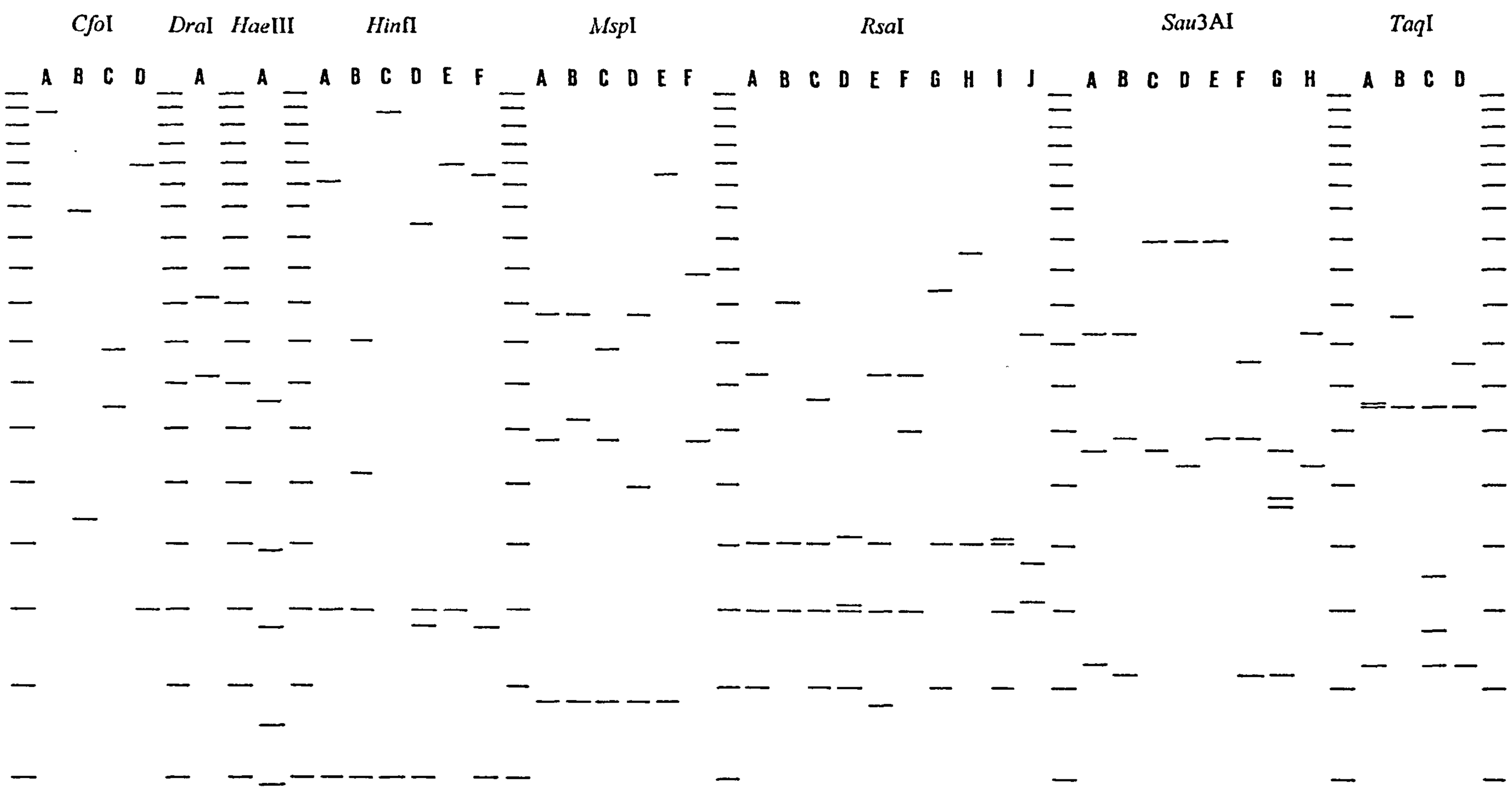


Figure 6.3. Patterns detected by restriction digestion of the Pma1 amplified product with various restriction endonucleases. Patterns for each enzyme are separated by 123bp ladder (Life Technologies).

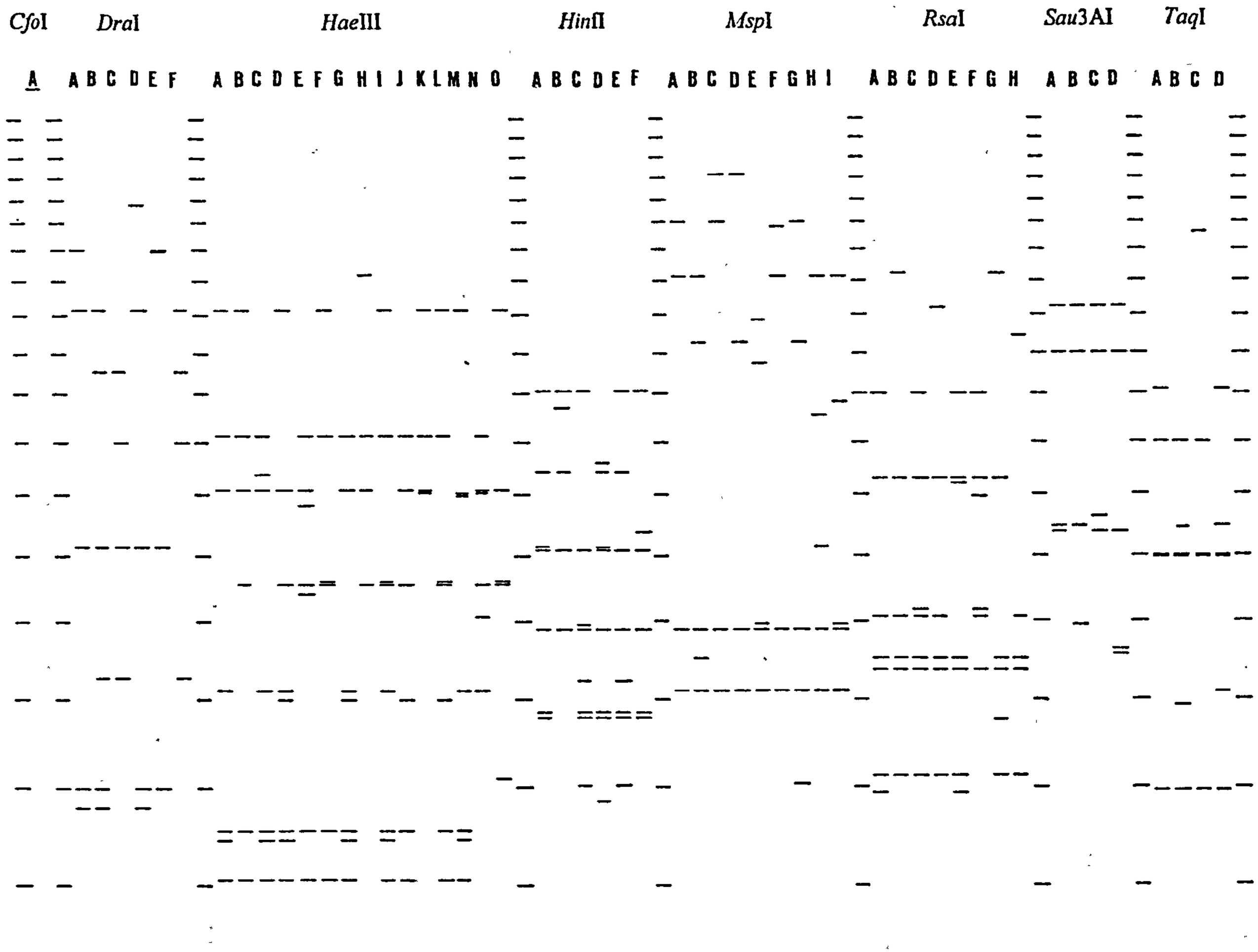


Figure 6.4. Patterns detected by restriction digestion of the Pma2 amplified product with various restriction endonucleases. Patterns for each enzyme are separated by 123bp ladder (Life Technologies).

Figures 6.3 and 6.4 for Pma1 and Pma2 amplified fragments respectively. For the Pma1 fragment, although each pattern did not sum to 2kb in all cases, it was possible to infer the number of site changes to account for these patterns (see appendix D for inferred restriction site patterns) in all except *Rsa*I patterns D and I. As can be seen from Figure 6.3 it appears that the fragments in these patterns do not add to the desired size (2kb). These patterns were reproducible and not due to length variation as digests involving other enzymes on DNA from the same amplification summed to 2kb, thus in these *Rsa*I profiles there must be fragments that have been unaccounted for either due to small sizes and therefore running off the end of the gel, or due to being an identical size to another fragment on the gel (doublets). Since fragments were easily visible down to 0.1kb in size, then, in order for these 2 patterns to be apparently small as a result of loss of fragments off the gel, it would require a large and inordinate number of closely spaced sites compared to other haplotypes. This is unlikely to have occurred and the most likely explanation is doublets on the gel being scored as a single fragment (see appendix D for interpretation).

Patterns revealed by restriction digestion of the 3.85kb fragment were much more complex (Figure 6.4) and due to migration of smaller fragments off the gel did not always sum to 3.85kb. In some instances it was possible to detect individual site differences by the loss of one fragment and the gain of 2 others but in most cases this could not be done with confidence, therefore restriction site data were not suitable for further analysis and it was only appropriate to analyse data from the 3.85kb fragment on the basis of fragment sharing.

6.2.3 Pma1 amplified fragment

Of 9 restriction enzymes tested against the Pma1 PCR amplified fragment 6 revealed polymorphic patterns (Table 6.3). Figure 6.5 details the distribution of single enzyme RFLP patterns within populations. The composite haplotype for this fragment is based on these 6 enzymes placed in alphabetic order (*Cfo*I, *Hin*fI, *Msp*I, *Rsa*I, *Sau* 3AI, *Taq*I). In all there were 63 composite haplotypes. Table 6.4 details these and the number of occurrences of each in every population. As can be seen many haplotypes were rare; forty-two occurred only once, 7 occurred twice, 3

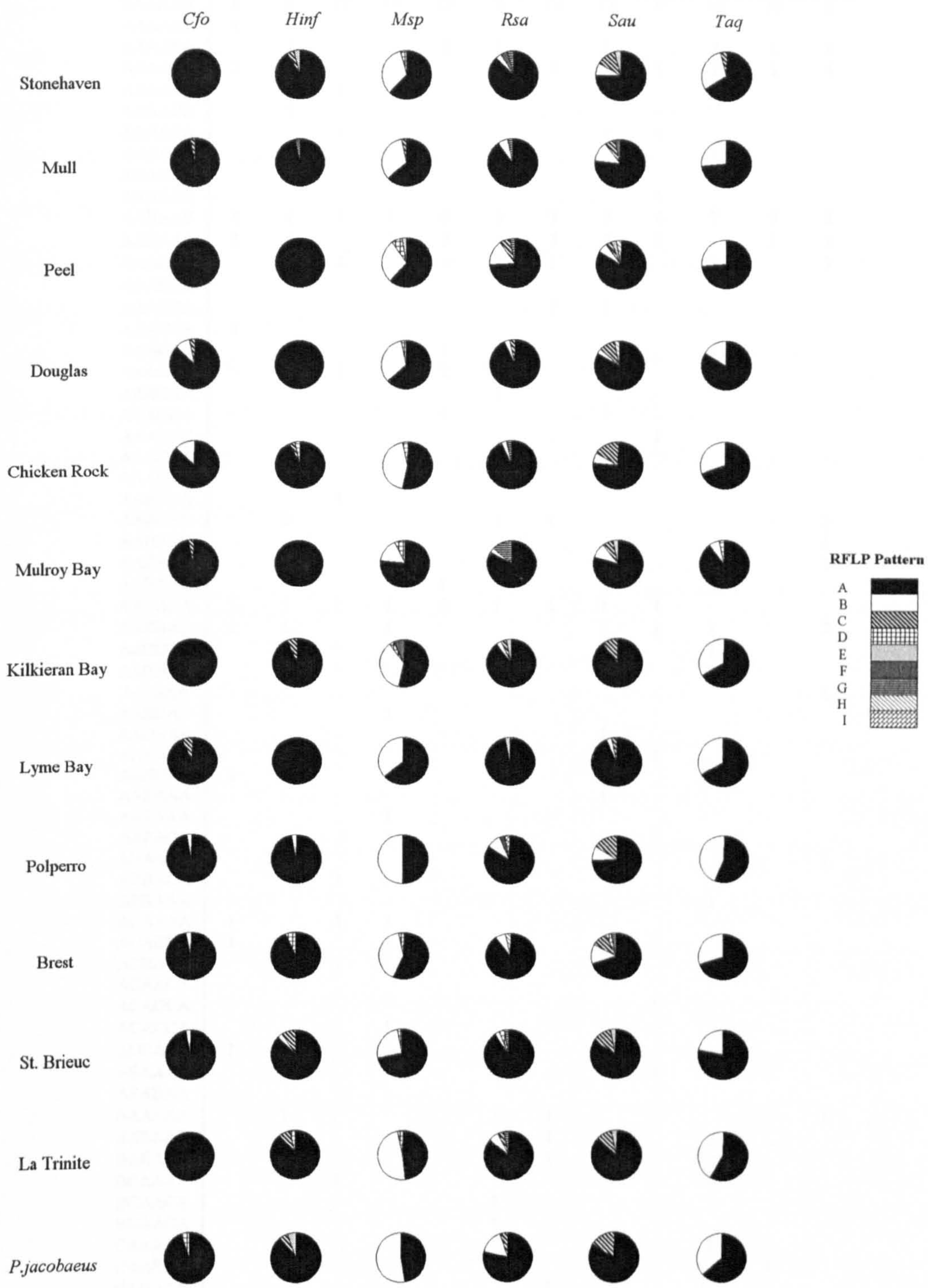


Figure 6.5. Pie diagrams representing frequencies of haplotypes from restriction digestion of Pma1 amplified mtDNA, in 12 populations of *P. maximus* and 1 sample of *P. jacobaeus*.

Haplotype	LAT	BRE	STB	KIL	MRY	CHI	DOU	PEE	STO	MUL	LYM	POL
AAAAAA	8	8	17	14	15	8	13	11	9	12	16	8
AAAAAB	1											
AAAABA		2			2	1		1		3	1	1
AAAACA	2	2				2	3	1	1	1	1	4
AAAACB			1									
AAAADA		1										
AAAAEA			1					1	1			
AAAAFA										1		
AAAAGA		1										
AAAABB									1			
AABAAB	8	4	5	7	2	6	3	5	4	7	9	8
AABAAA	3	4		1	2	4	3	2	2		1	1
AAABAA			1		1	1	1	2		1		1
AAABCB		1										
AAACHA							1	1				
AAACCA	1											
AAAGEA					1							
AAAGCA			1		2							
AAAGAA						1						
AAAGBA					1			1				
AAAFCC									1			
AAACAA								1				
AAACBA		1										
AABDCA			1									
AABABB		1				1	1					2
AABFAB												1
AABACB				1		1						1
AABAAD					1							
AADAAA	1	1	1	1	2	1	1	1	1			
AABBAB	1	1		1				2	1	1		2
AACGCA										1		
AABABA										1		
AABIAA												1
AABEAB				1								
AADBAB								1				
AABABB									1			
AABGAB	1											
AAEAAA								1				
AAFAAA				1								
AAFACA				1								
ABAAAA												1
ABBACA			1									
AFBAAA										1		
ACAAAA	1		1	1								
ACABEA	1											
ACBAAB			1									
ACAACA												
ACAGCA									1			
ACACCA				1								
ADBAAB	1	2										
AEAAAA									1			
AEABAA												
BAAAAA		1				1	1					1
BABAAA							1					
BABAAB						1	1					
BCAAAA			1									
BCAACA						1						
BDAACA						1						
CAAAAA					1					1		
CAAEAA											1	
CABAAA							1					
CABAAB											1	
DABAAB												
N	29	30	32	30	30	30	30	31	24	30	30	32

Table 6.4. Numbers of each composite haplotype from Pma1 amplified mtDNA, observed in 12 populations of *P. maximus* and 1 sample of *P. jacobaeus* (N=sample size). Order of restriction enzymes in composite haplotype: *Cfo*I, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3AI, *Taq*I.

were detected 3 times and 2 four times. Of the 63 haplotypes only 9 were seen 5 or more times and the four common haplotypes, AAAAAA, AABAAB, AABAAA and AAAACA accounted for 72% of all animals. However, each population had approximately the same number of observed haplotypes despite variation in sample size, for example, the *P. jacobaeus* (N=19) and Stonehaven (N=24) samples contained 10 and 12 different haplotypes respectively. The mean number of haplotypes observed in the other populations (N=29-32) was 11.91, with the only obvious discrepancy exhibited by the Lyme Bay population in which only 7 different haplotypes were seen. Haplotype diversity (the probability of encountering different haplotypes when 2 individuals are sampled from a population) was high (Table 6.5) ranging from 0.64-0.90 suggesting substantial variability of the mtDNA.

Sample site	Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity	
				Fragment	Site
La Trinité	29	12	0.8522	0.0148	0.0238
Brest	30	14	0.9011	0.0159	0.0256
St.Brieuc	32	12	0.7056	0.0132	0.0210
Kilkieran Bay	30	11	0.7425	0.0128	0.0200
Mulroy Bay	30	11	0.7471	0.0107	0.0184
Chicken Rock	30	14	0.8851	0.0153	0.0243
Douglas	30	12	0.8000	0.0109	0.0184
Peel	31	14	0.8538	0.0138	0.0230
Stonehaven	24	12	0.8442	0.0168	0.0265
Mull	30	11	0.7931	0.0146	0.0235
Lyme Bay	30	7	0.6414	0.0092	0.0145
Polperro	32	13	0.8710	0.0153	0.0249
P.jacobaeus	19	10	0.9006	0.0166	0.0255
Average		11.77(Σ=63)	0.8106	0.0138	0.0222

Table 6.5: Haplotype and nucleotide diversity in 13 populations of *P. maximus* estimated from Pma1 amplified product using fragment sharing and site approaches.

There were no haplotypes seen at high frequency in only one sampling site, although the frequency of the common haplotype (AAAAAA) ranged in frequency from 0.25-0.533 among populations. However there is no evidence from χ^2 estimates for any heterogeneity of haplotype numbers (Table 6.6). Monte-Carlo estimation of χ^2 over the whole matrix was insignificant (37.3% of the pseudo χ^2 were higher than the original χ^2) and all of the few apparently significant pairwise comparisons can be attributed to type I errors due to multiple testing if the method of Hochberg (1988) is used to adjust significance levels for multiple comparisons.

Both fragment and restriction site sharing data were used as input for further statistical analyses and from the fragment patterns produced by these 6 enzymes a total of 30 restriction sites were inferred. Nucleotide sequence divergence between haplotypes ($\hat{d}_{ij}$) estimated using either fragment patterns (Nei and Li, 1979; Nei, 1987) or restriction sites (Nei and Tajima, 1981; Nei and Miller, 1990) produced similar results, so site estimates were utilised. Site data are less ambiguous than restriction fragment data, for which homology can not be assured (although in this case due to the short fragments and ease of interpretation this would be unlikely to be problematic). Restriction site estimates of $\hat{d}_{ij}$ (average number of nucleotide substitutions between haplotypes) ranged from 0-0.0979. Since most haplotypes differed by only one or two sites most $\hat{d}_{ij}$ estimates were low. A consensus bootstrapped majority-rule tree using these as input (Figure 6.6) provides no evidence to suggest that those haplotypes confined to particular populations ("private haplotypes") cluster together, which would suggest population sub-division.

Although haplotype diversity was high, variable patterns differed by only 1-4 sites. Nevertheless within-population nucleotide diversity proved to be quite high and estimates from fragment patterns were similar, although slightly lower than those taken from inferred restriction site data (0.0092-0.0168 *vs.* 0.0145-0.0265). Nucleotide divergence estimates among populations (the proportion of nucleotide diversity in the pairwise comparison that cannot be explained by the within

	LAT	BRE	STB	KIL	MRY	JAC	CHI	DOU	PEE	STO	MUL	LYM	POL
LAT		14.8	22.84	14.69	24.25	14.16	16.42	17.65	16.98	13.57	20.19	15.05	17.9
BRE	0.856		29.32	22.25	21.8	15.98	13.73	15.68	18.24	16.6	21.15	19.06	16.75
STB	0.088	0.017		15.58	17.03	26.45	25.29	20.99	19.61	18.81	20.15	16.13	27.93
KIL	0.736	0.175	0.856		19.48	16.56	19.51	19.64	17.35	14.75	18.15	12.38	19.99
MRY	0.034	0.222	0.475	0.189		23.09	20.46	17.88	15.89	19.07	18.31	17.15	27.36
JAC	0.752	0.768	0.006	0.315	0.036		15.48	17.99	13.87	13.42	20.37	18.21	15.46
CHI	0.968	0.975	0.078	0.433	0.238	0.836		10.53	16.89	17	20.21	16.4	13.03
DOU	0.537	0.789	0.255	0.244	0.4	0.335	0.968		15.19	15.6	21.64	16.31	16.89
PEE	0.758	0.702	0.551	0.701	0.636	0.854	0.86	0.848		12.96	16.03	15.39	17.62
STO	0.932	0.796	0.563	0.876	0.342	0.88	0.925	0.864	0.954		18.81	14.73	19.11
MUL	0.207	0.262	0.271	0.469	0.212	0.092	0.321	0.105	0.748	0.438		11.82	16.95
LYM	0.339	0.093	0.338	0.771	0.077	0.021	0.311	0.194	0.464	0.357	0.693		16.48
POL	0.65	0.671	0.021	0.269	0.01	0.523	0.889	0.533	0.691	0.525	0.451	0.179	

Table 6.6. Pairwise χ^2 analysis of numbers of haplotypes (from restriction digestion of Pma1 amplified DNA) in 12 populations of *P. maximus* and 1 sample of *P. jacobaeus*. Estimated χ^2 values above diagonal and proportion of times this was exceeded after randomisation (=p) below diagonal.

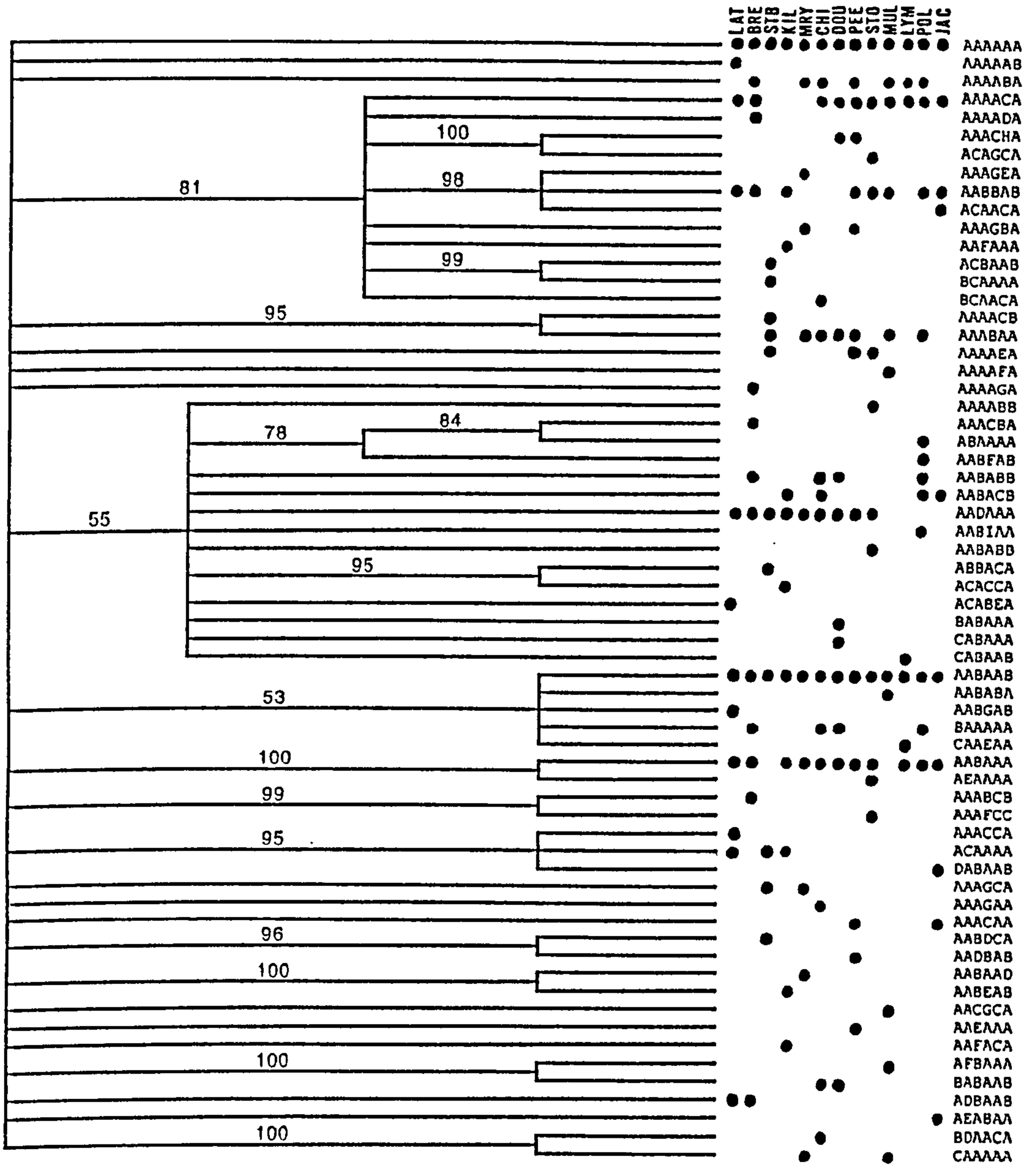


Figure 6.6. Bootstrapped majority-rule dendrogram of composite haplotype (from Pma1 amplified DNA) relatedness in *P.maximus* and *P.jacobaeus*. Bootstrap values indicate the number of times the group consisting of haplotypes to the right of the fork occurred among 100 bootstrapped repititions. Dots depict presence of hapotype in each population.

A)

	LAT	BRE	STB	KIL	MRY	JAC	CHI	DOU	PEE	STO	MUL	LYM	POL
LAT		0.015320	0.014221	0.013516	0.014272	0.015153	0.014912	0.013273	0.014429	0.015632	0.014817	0.011997	0.014734
BRE	-0.000049		0.014640	0.014384	0.013956	0.016154	0.015314	0.013417	0.014807	0.015849	0.014897	0.012648	0.015488
STB	0.000192	0.000060		0.012936	0.012237	0.015238	0.014213	0.011990	0.013611	0.014812	0.013799	0.011391	0.014874
KIL	-0.000280	0.000038	-0.000072		0.012800	0.014462	0.013937	0.011947	0.013364	0.014602	0.013674	0.010796	0.014094
MRY	0.001490	0.000624	0.000244	0.001040		0.015172	0.013831	0.011072	0.012621	0.014075	0.012838	0.010991	0.014856
JAC	-0.000556	-0.000107	0.000316	-0.000227	0.001498		0.015777	0.014005	0.015152	0.016542	0.015768	0.012824	0.015499
CHI	-0.000130	-0.000279	-0.000042	-0.000084	0.000825	-0.000158		0.012921	0.014738	0.015733	0.014802	0.012217	0.015255
DOU	0.000404	-0.000003	-0.000091	0.000100	0.000239	0.000243	-0.000174		0.012394	0.013750	0.012602	0.010100	0.013701
PEE	0.000117	-0.000056	0.000087	0.000074	0.000345	-0.000052	0.000201	0.000031		0.015081	0.014003	0.011658	0.014865
STO	-0.000184	-0.000518	-0.000216	-0.000192	0.000295	-0.000167	-0.000308	-0.000118	-0.000229		0.015248	0.012853	0.015875
MUL	0.000116	-0.000355	-0.000115	-0.000006	0.000172	0.000174	-0.000125	-0.000152	-0.000193	-0.000452		0.011855	0.015142
LYM	0.000009	0.000109	0.000191	-0.000170	0.001039	-0.000056	0.000004	0.000060	0.000176	-0.000133	-0.000017		0.012368
POL	-0.000339	-0.000136	0.000589	0.000043	0.001820	-0.000466	-0.000043	0.000577	0.000298	-0.000196	0.000185	0.000125	

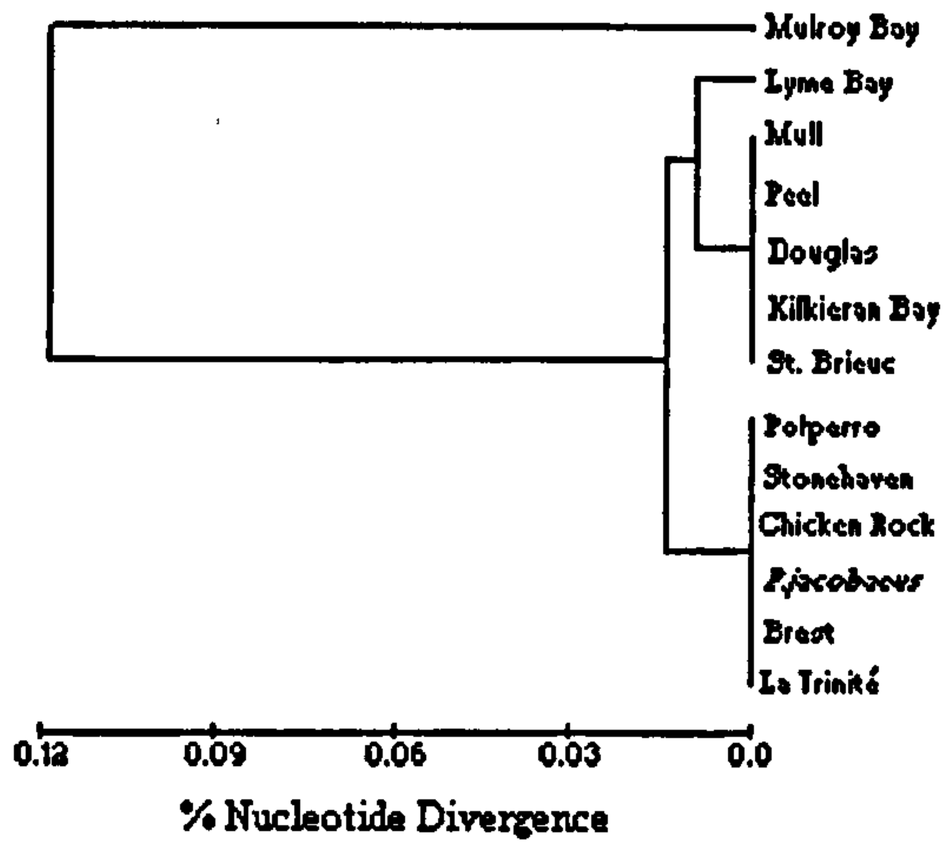
B)

	LAT	BRE	STB	KIL	MRY	JAC	CHI	DOU	PEE	STO	MUL	LYM	POL
LAT		0.024546	0.022681	0.021438	0.023221	0.023783	0.023701	0.021380	0.023395	0.024962	0.023691	0.018958	0.023848
BRE	-0.000125		0.023407	0.022913	0.022948	0.025406	0.024422	0.021877	0.024083	0.025351	0.024082	0.020398	0.024974
STB	0.000300	0.000106		0.020393	0.019886	0.023754	0.022606	0.019406	0.021929	0.023319	0.022095	0.018009	0.023846
KIL	-0.000452	0.000103	-0.000128		0.020630	0.022407	0.022052	0.019167	0.021410	0.023087	0.021765	0.016924	0.022641
MRY	0.002167	0.000974	0.000202	0.001437		0.024394	0.022517	0.018698	0.021236	0.022708	0.021320	0.017976	0.024371
JAC	-0.000834	-0.000130	0.000507	-0.000349	0.002475		0.024609	0.022194	0.024165	0.025940	0.024676	0.019966	0.024596
CHI	-0.000327	-0.000526	-0.000053	-0.000115	0.001186	-0.000284		0.021057	0.023687	0.024866	0.023686	0.019571	0.024358
DOU	0.000308	-0.000114	-0.000295	-0.000044	0.000324	0.000258	-0.000292		0.020463	0.022130	0.020736	0.016554	0.022358
PEE	0.000006	-0.000226	-0.000090	-0.000119	0.000544	-0.000089	0.000021	-0.000246		0.024407	0.022886	0.018813	0.024355
STO	-0.000182	-0.000713	-0.000455	-0.000196	0.000261	-0.000069	-0.000554	-0.000334	-0.000374		0.024324	0.020577	0.025588
MUL	0.000049	-0.000480	-0.000177	-0.000016	0.000375	0.000169	-0.000233	-0.000226	-0.000393	-0.000710		0.019065	0.024385
LYM	-0.000169	0.000351	0.000252	-0.000342	0.001546	-0.000026	0.000168	0.000107	0.000049	0.000058	0.000047		0.020058
POL	-0.000480	-0.000275	0.000887	0.000173	0.002740	-0.000598	-0.000247	0.000709	0.000389	-0.000133	0.000165	0.000354	

Table 6.7. Nucleotide divergence among *P. maximus* populations and 1 sample of *P. jacobaeus* calculated from composite haplotype frequencies generated from restriction digested Pma1 amplified DNA. A) Estimated from fragment data. B) Estimated from site data. Uncorrected above diagonal and corrected for within sample diversity below diagonal.

population diversity) were similarly high (Table 6.7A and B) and show that when within population diversity was accounted for most values are positive, although a number of negative values do occur. The negative divergences are obviously artefactual and are actually a result of large within-population diversity estimates. In order to make the results suitable for dendrogram construction these negative values must be replaced by zero (M.Nei, University of Pennsylvania pers. comm.) in a similar way that negative D values (from allozyme studies) are artefactual and are ignored (Nei, 1978). When the edited divergence matrix was used to construct a UPGMA phenogram (Figure 6.7A) due to the number of 0 values in the matrix there were a number of equally likely phenograms produced. Clustering proceeds by identifying the two most closely related points, merging these into a single cluster, updating the matrix to account for this merged pair and then repeating this process. Because of the multiple zero values there were a number of ties for the most similar points from which to initiate clustering and therefore a number of equally possible initial clusters and subsequent branch points, resulting in differing clustering patterns in alternative dendrograms. It is likely that some of these clusters defined by these branch points are not real, since they do not occur in every phenogram and it is important to identify the true cluster pattern. Each dendrogram revealed a number of clades, the most striking result of which was the clear separation of the Mulroy Bay population from all others. Three separate patterns were produced in the alternative dendrograms each with a basic pattern of 3 clades, Mulroy Bay being distinct alongside 2 other weakly supported clades. A consensus tree using all possible trees as input eradicated these weak clades but still supported the separation of Mulroy Bay (Figure 6.7B). However this does not necessarily mean there are no clades other than that containing Mulroy Bay. One method to establish confidence in the other branch point is through the use of alternative algorithms for the construction of the dendrogram (D.McElroy, Western Kentucky University, pers. comm.; Rohlf, 1990b). Because any clustering routine will by definition always produce branch points it is necessary to confirm which are real and which are "statistical noise". By using both single and complete-linkage methods to generate the dendrogram then it is possible to see which branch points are robust to deviations from the assumptions of clustering. As single and

A)



B)

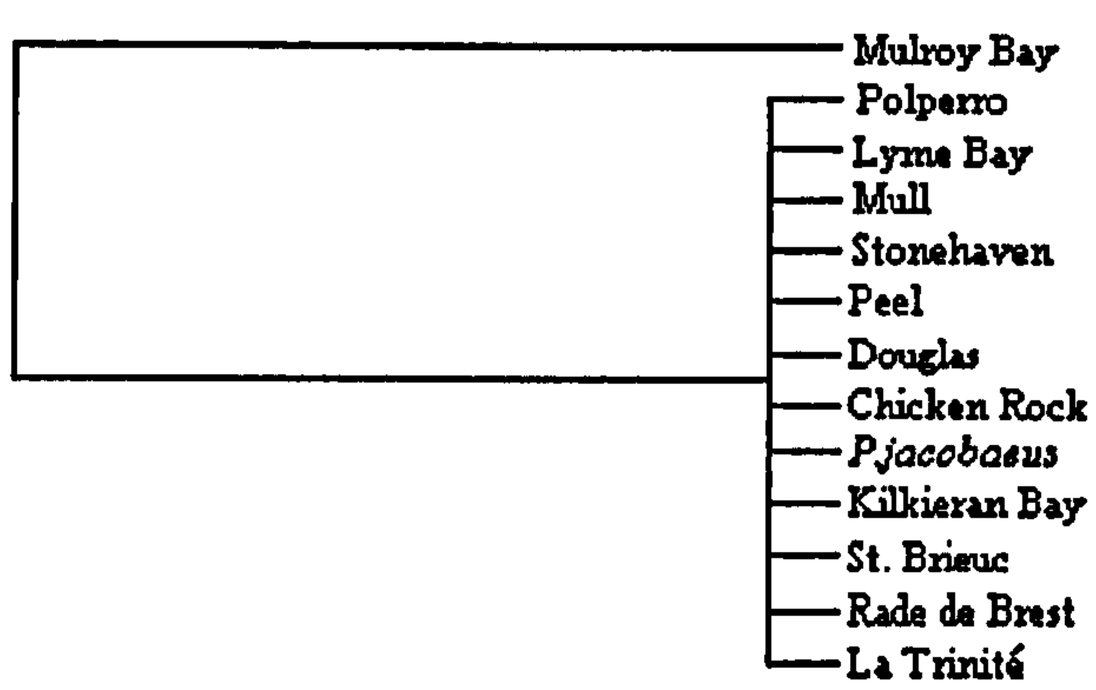


Figure 6.7. A) One of the equally likely UPGMA dendrograms from clustered nucleotide divergence values calculated from PCR-RFLP data on the Pma1 amplified fragment. B) Consensus dendrogram calculated from all possible dendrograms.

complete-linkage represent opposite extremes of clustering methods (with UPGMA methods in the middle) then if a branch point is seen using both the minimum distance between the members of 2 clusters (single linkage) and the maximum distance between members (complete linkage) the clusters can be assumed to be real. Clusters identical in both analyses are called "ball clusters" (Rohlf, 1990b) within which the most dissimilar populations are more similar to each other than any object within the cluster is to those outside. Using complete linkage methods the Mulroy Bay population split first and formed a clade with the St. Brieuc, Peel, Douglas, Mull and in some instances Kilkieran Bay populations whilst using single linkage the Mulroy Bay sample was the sole representative of this clade. Thus the branch point shown using the UPGMA method in which Mulroy Bay is represented as divergent from all other populations is a real branch, as expected from the nucleotide divergence ($\hat{d}_A$) values (Table 6.7) in which Mulroy Bay (MRY) was the only population to exhibit consistently positive values.

This differentiation of Mulroy Bay as suggested by nucleotide divergence would suggest reduced gene flow and indeed gene flow is suggested to be low from the N_em estimate via the private allele method (Slatkin, 1985) with $\overline{N}$=29 and $\overline{p}(1)$=0.114, at a level of 0.506 migrants per generation, a value lower than the level of 1 migrant needed to maintain similar alleles (Kimura and Ohta, 1971b). However as there is no certainty that the alleles implicated as private are actually restricted to single populations this gene flow estimate can not be accepted with confidence.

The Pma1 primer pair succesfully amplified a 2kb product from *P. novaezelandiae* DNA but was unsuccesful for *A. opercularis* mtDNA. Digestion of Pma1 PCR product amplified from *P. novaezelandiae* DNA produced patterns similar to those of *P. maximus* and *P. jacobaeus*. However the 2kb *Rsa*I haplotype J (Figure 6.3) was only seen in one of the 2 *P. novaezelandiae* samples. With only a sample size of 2 there is no way to quantify the inter-specific differences.

6.2.4 Pma2 amplified fragment

The 3.85kb fragment amplified using the Pma2 primer pair was also highly variable, with extensive polymorphism for most restriction enzymes. The patterns produced by RFLP analysis of this larger fragment are shown in Figures 6.2 and

Haplotype	CHI	POL	PEE	KIL	STB	MUL
AAAAAA	3	6		4	6	3
AAAACA	2		1	1	6	4
AABAAA	1		1		1	
BFAABA	1			1		
ACABAA			1			
BBAABA	3	4	1	2	3	4
AAAEAC			1			
BEAABA		1			1	
AAAAFA		1		1		
AAAGCA		1				
AAABAA		1				1
DAAACA					1	
AEAHAA					1	
AACAAA					1	
AABGAA					1	
AAAIAA					1	
AAAAEA						1
AAABCA						1
CBAABA				1		1
BBBADA						1
AACACA						1
AGAAAA						1
AAACBA						1
AAAFEA						1
BEAABC				1		
AAAACB				1		
AABACA	2			1	1	
AAADHA				1		
BBAAAA				1		
ABAACA	1			1		
BMBAAA	1					
ANEACA	1					
ADAACA	1					
BHAABA	1					
ADAAAA	1					
BBAADA			1			
N	18	14	6	16	23	20

Table 6.8. Numbers of composite haplotypes observed in 6 populations of *P. maximus* (N=sample size) after digestion of the Pma2 amplified fragment with 6 restriction enzymes: *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I and *Sau*3AI.

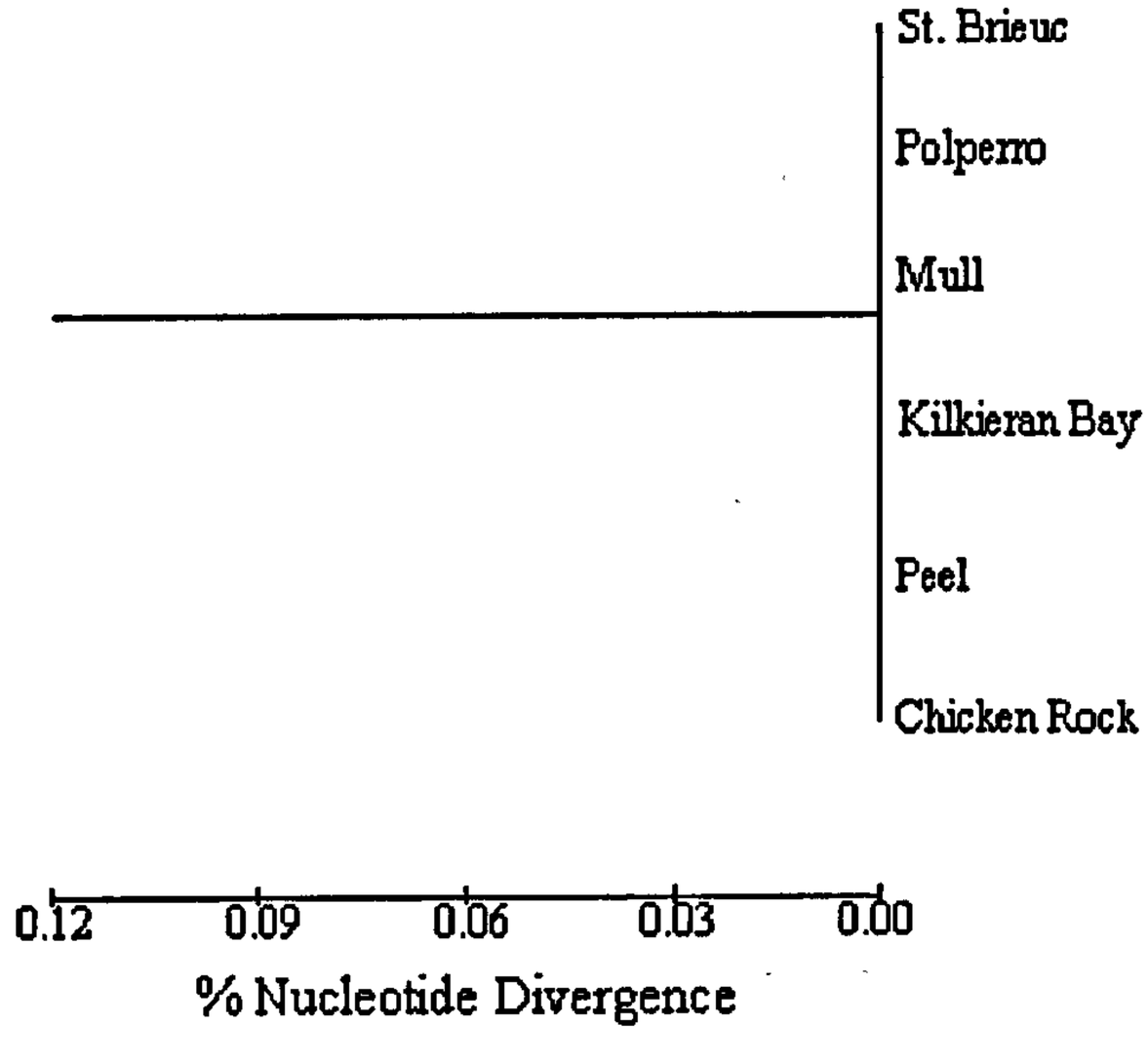
6.4. Seven of the 9 enzymes produced variable patterns (*Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3AI and *Taq*I) although there were not enough data from *Taq*I for inclusion in the analysis (*Taq*I was not screened initially). The distribution of 6 letter composite haplotypes is shown in Table 6.8. Due to the larger size of this fragment and hence greater number of fragments produced upon digestion, interpretation of RFLP patterns was more complex and as a result of fragments being lost off the end of gels it was impossible to accurately infer site differences and analyses had consequently to be confined to fragment sharing methods. It proved impossible to type many specimens for variability of this fragment as amplification of samples became unsuccessful mid-way through screening. Attempts to alleviate this problem through new primers, new *Taq*, alternative *Taq* manufacturers, new dNTPs, different PCR machine and re-optimisation proved completely unsuccessful and analysis of this fragment had unfortunately to be abandoned. However some results were obtained for a few populations (Table 6.8).

Haplotype diversity for the Pma2 fragment (Table 6.9) was of a similar order to that seen with the Pma1 fragment but nucleotide diversity was less. Due to the smaller sample sizes, inability to score all fragments and consequently possibly biased analysis this cannot be regarded as necessarily accurate.

Sample site	Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity
St.Brieuc	23	11	0.8696	0.00802
Kilkieran Bay	16	12	0.9417	0.01118
Chicken Rock	18	12	0.9477	0.01089
Peel	6	6	1.000	0.01348
Mull	20	12	0.9211	0.00992
Polperro	14	6	0.7692	0.01348
Average	16.17(Σ=97)	9.83(Σ=36)	0.9082	0.01024

Table 6.9: Haplotype and nucleotide diversity in 6 populations of *P. maximus* estimated from Pma2 amplified product using fragment sharing methods.

A)



B)

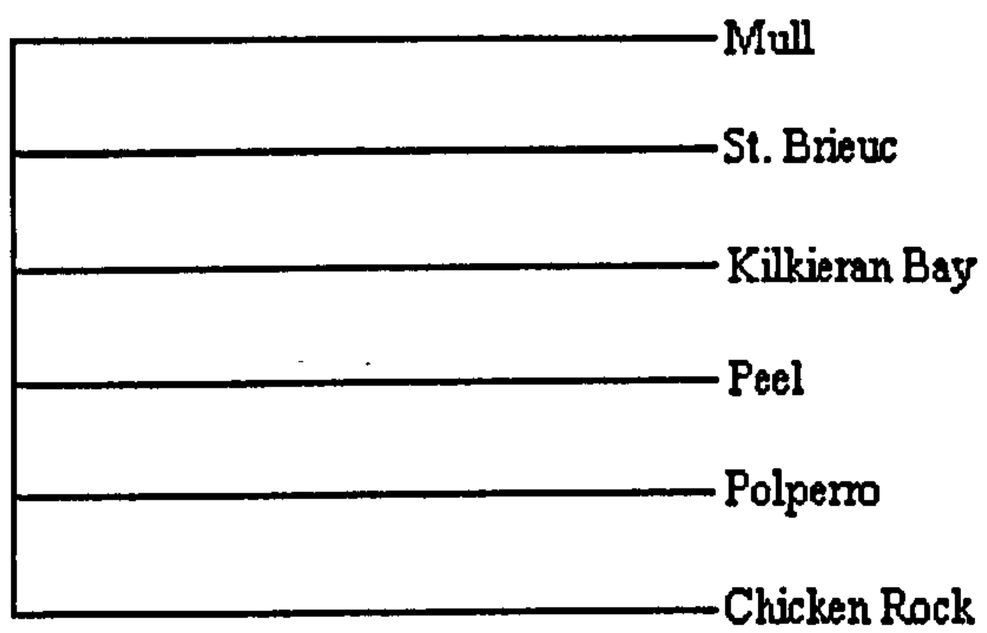


Figure 6.8. A) One of the equally likely UPGMA dendrogram from clustered nucleotide divergence values calculated from PCR-RFLP data on the 3.85kb fragment (drawn to identical scale as Figure 6.7a). B) Consensus dendrogram.

Dendrogram construction on the same scale as the dendrogram produced from the Pma1 PCR fragment data confirmed the findings from that data set, that is, that there were no apparent differences among these 6 populations (Figure 6.8A and B). Since technical difficulties prevented Pma2 being applied to samples from Mulroy Bay there are no data to verify the differentiation of this population suggested by the 2kb fragment.

6.2.5 ITS fragment

The ITS primer pair amplified a 1kb product from gDNA of *P. maximus* and *P. jacobaeus* in which no length variation was detected. This amplified product was subsequently found to contain restriction sites for the enzymes *Alu*I, *Hae*III, *Hin*fI, *Msp*I and *Rsa*I although only *Msp*I exhibited variability. Through screening of amplified ITS regions with *Msp*I, 8 patterns were detected (Figure 6.9) and digestions of repeat amplifications were consistently identical. Variation in the numbers of these patterns observed within 6 populations is shown in Table 6.10.

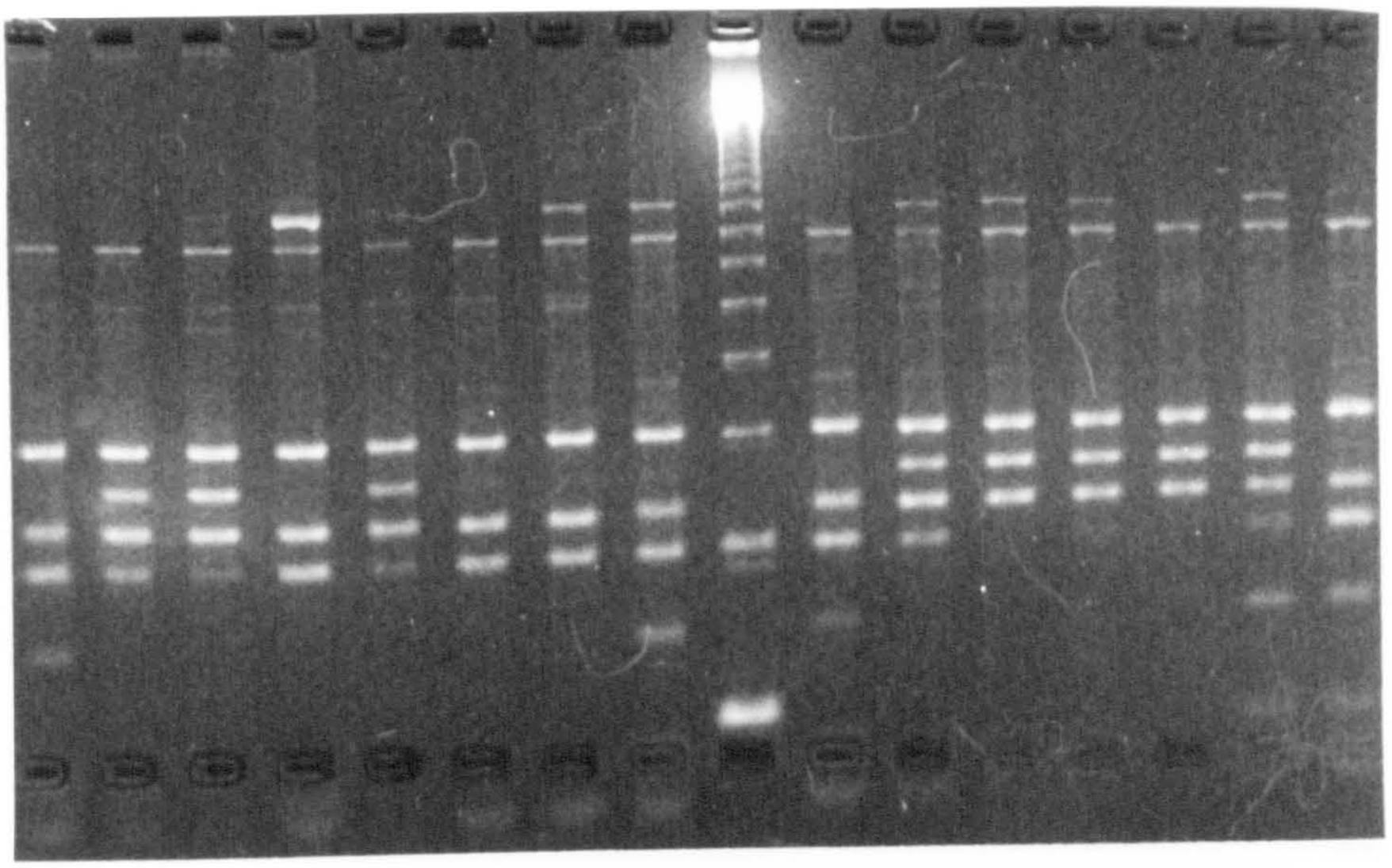
Population	A	B	C	D	E	F	G	H	Σ
St. Brieuc	6	4	8	5	4	1			28
Stonehaven	3	3	8	5	4				23
Lyme Bay	2		13	7	1				23
Mulroy Bay	1		15	10	1		1		28
Brest	5		11	4	1		1		22
La Trinité		3	12	5	2			1	23
P.jacobaeus			3	1					4

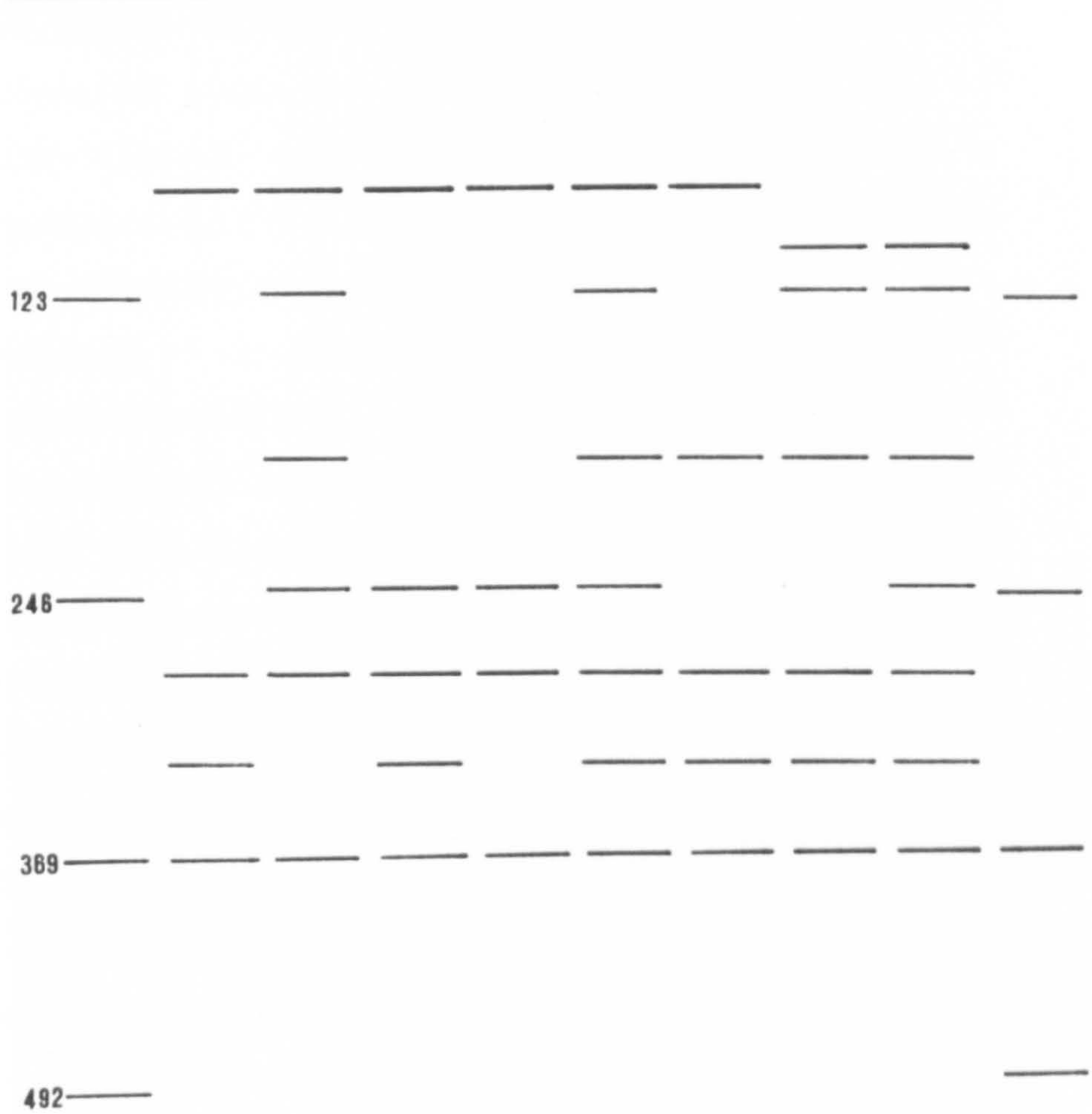
Table 6.10: *Msp*I generated patterns of rDNA variation in 6 populations of *P. maximus* and *P. jacobaeus*. Letters A-H refer to RFLP patterns (see Figure 6.9).

There was no evidence of heterogeneity in haplotype frequencies from χ^2 analysis. Using the MONTE option of REAP the calculated χ^2 value (43.81) was exceeded in 1074 randomisations, producing p=0.1074.

Because the rDNA is a diploid (multi-copy) gene, heterozygotes and homozygotes should be differentiable but the genetic basis of the polymorphism could not be inferred.

Figure 6.9. Variability of *Msp*I digested ITS fragment in *P. maximus*. Above: Example of patterns. Below: Haplotypes observed (A-H), m = part of 123bp ladder, sizes in bp.





m A B C D E F G H m

6.3 Discussion

6.3.1 Intraspecific variability

Examination of mtDNA variability has proved to be a useful tool in examining population structure of marine organisms and for fisheries research (e.g McLean *et al.*, 1983; Skibinski and Edwards, 1986; Edwards and Skibinski, 1987; Blot *et al.*, 1990; Reeb and Avise, 1990; Billington and Hebert, 1991; Brown and Paynter, 1991; Carr and Marshall, 1991; Karakousis and Skibinski, 1992; Boulding *et al.*, 1993; Geller *et al.*, 1993; Boom *et al.*, 1994; Silberman *et al.*, 1994; Blake and Graves, 1995; Hurst and Skibinski, 1995; Quesada, Beynon and Skibinski, 1995; Shields and Gust, 1995). Most studies have utilised restriction site analysis across the whole mtDNA molecule, however in this study restriction site variability was examined in PCR amplified segments of mtDNA. Such a method has previously been used in systematic studies and species identification (Glenn Hall and Muralidharan, 1989; Whitmore *et al.*, 1992; Chow and Inoue, 1993; Garnery *et al.*, 1993) and in population analysis (Cronin *et al.*, 1993, 1994; Bartlett and Davidson, 1994; Cronin *et al.*, 1994; Hall and Nawrocki, 1995).

The application of specifically designed primers provided valuable information on population structure of *P. maximus.* PCR-RFLP performed on the 2kb PCR fragment produced a large body of results in contrast to the application of the method to the 3.85kb amplified fragment for which initial amplification was successful but later, unexplainedly, completely failed. However the small body of data taken from the 3.85kb fragment produced results in accord with those of the 2kb fragment for the 6 populations.

As for other studies of mitochondrial DNA variability within species (Reeb and Avise, 1990; Boom *et al.*, 1994; Shields and Gust, 1995) two haplotypes predominated with other rarer haplotypes confined to certain sampling sites ("private haplotypes"). Since these were encountered only once in any population it is impossible to state with certainty that they are truly "private". If more than one individual in any one population had a haplotype found in that population alone it would be more convincing evidence that they were indeed private haplotypes. This pattern of a single or few common haplotypes dominating the frequency distribution has been attributed to either recent colonisation, high reproductive

outputs of animals possessing the common haplotypes which therefore contribute disproportionately to the surviving haplotypes, selection for the common haplotype or to some form of hitch-hiking effect with either another mitochondrial gene or with a nuclear gene (Shields and Gust, 1995). Disproportionate reproductive input from certain individuals is possible. Hedgecock *et al.* (1992) argue that highly fecund species (such as scallops) have high variability in fertility among individuals and should these possess a common haplotype then private haplotypes would remain comparitively rare. If certain individuals are overrepresented then the effective population size should be less than the total population size and indeed gross disparities between these have been shown for oysters (Hedgecock and Sly, 1990).

If any population were effectively isolated then mutations within haplotype (maternal) lineages will give rise to new, related haplotypes. These will then likely remain "private" in the absence of gene flow. Through continued mutation new haplotypes will result from both the widespread haplotypes pre-dating the split and the newly evolved mtDNA. Thus private haplotypes in isolated populations may be expected to be more similar to each other and to the widespread haplotypes than they are to haplotypes from other populations. Therefore private haplotypes may cluster by region if there is population genetic heterogeneity. There is no evidence to suggest this in this study. Such a phenomenon is often seen in terrestrial mammals, for example, in a study of pocket gophers Avise *et al.* (1979) examined 87 animals for mtDNA restriction site variability and 23 composite haplotypes were identified. In this study two distinct regions were identified in which although some haplotypes were widespread others were localised and the local clones were usually differentiated from the widespread clone of that region by only one or two base substitutions. However haplotypes from the two regions differed by at least 9 substitutions. This is evidence of substantial population differentiation.

P. maximus haplotype frequency distributions also provide little evidence to suggest geographic heterogeneity. For Pma1 amplified mtDNA, those haplotypes that occur in only single populations can not be regarded as evidence for population structure as the sample sizes used (19-32) would not be sufficient to reveal very rare haplotypes in every population. Although the frequencies of the

two commonest Pma1 haplotypes AAAAAA and AABAAB appear to suggest heterogeneity, χ^2 analysis does not support this whether performed over all populations or in pairwise comparisons (Table 6.7). The frequency of the common haplotype (AAAAAA) is interesting in that the geographic variation changes markedly in a manner not dissimilar to the geographic variation in allele frequencies seen in *A. opercularis* (Beaumont, 1982a). Thus St. Brieuc appears more similar to Irish Sea populations than the neighbouring Rade de Brest population which is itself similar to the sample from La Trinité (for queen scallops a population from nearby Belle Isle was studied). All Irish Sea samples are similar (although Chicken Rock appears to have a lower frequency of this haplotype). There are however no great differences between Irish Sea populations and those from the West Coasts of Ireland and Scotland. This weak pattern is by no means convincing but it does suggest some similarities in genetic structure of the *P. maximus* and *A. opercularis.*

Variation at the nucleotide level, assessed through nucleotide diversity within and among sampling locales showed that the estimated values of nucleotide diversity are over an order of magnitude lower than examples from the literature, for example, *Strongylocentrotus* urchins (Palumbi and Wilson, 1990) and *Argopecten* scallops (Blake and Graves, 1995). However such figures are not directly comparable since these values were estimated from restriction sites across the whole mtDNA molecule whilst the estimates in this study were based on data from a just a 2kb section of the molecule. The values are an order of magnitude higher than that estimated from PCR-RFLP in *Patinopecten yessoensis* (Boulding *et al*, 1993).

From these nucleotide divergence values there is a striking differentiation of the Mulroy Bay population as evidenced from nucleotide substitution values ($\hat{d}_A$). In every comparison involving Mulroy Bay the $\hat{d}_A$ value is positive. Such a result is indicative of a significant difference (Ovenden, 1990). Although there are positive values for pairwise comparisons involving other populations they are not consistently positive and, indeed in some cases are negative. Although negative values are artefactual (therefore assumed to be 0), no differentiation is suggested from the remaining positive values and as a result of some $\hat{d}_A$ values being positive

and others 0, dendrograms involving such populations do suggest divergent clades but there are a number of equally likely dendrograms suggestive of different branching patterns and alternative clades. These weakly supported clades in equally likely alternative UPGMA generated dendrograms are lost upon generation of consensus trees and are implicated as "statistical noise" from comparison of single- and complete-linkage dendrograms. However the branching-off of the Mulroy Bay population from all others is supported as true.

The results from this analysis of population structure initially suggest something of an enigma since although χ^2 values generated through Monte-Carlo simulations suggest no heterogeneity of haplotypes, nucleotide divergences implicate a divergent Mulroy Bay population. After separation of populations from a single ancestral stock, the 2 populations should first show evidence of differentiation in haplotype frequencies before any suggestion of differentiation on the basis of nucleotide substitution (Cronin, 1993). Thus it can be more appropriate to use haplotype frequencies rather than divergence levels to study population structure in species having undergone recent gene flow. However the Mulroy Bay population has shown the opposite of this with evidence of separation on the basis of nucleotide substitution but not haplotype frequencies. The nucleotide divergence levels among haplotypes do not suggest that any haplotype in Mulroy Bay is disproportionatly diverged from other *P. maximus* haplotypes in such a way that it would bias the nucleotide divergences between Mulroy Bay and other populations. Since there are no substantial shifts in haplotype frequency and no haplotypes capable of elevating the nucleotide divergences in pairwise comparisons of populations involving Mulroy Bay, this differentiation is likely due to low, statistically insignificant variation in frequencies of a number of haplotypes that together affect the nucleotide divergence. Such subtle changes in haplotype frequency could come about due to genetic drift in an isolated population causing shifts in haplotype frequencies, or another possibility is that the Mulroy Bay sample does not adequately represent the population. Since the animals represent only a single year class the sample may be unrepresentative of the population as a whole. Without further sampling and study this is impossible to determine.

Most studies of population variability in mtDNA study the complete molecule by restriction enzyme digestion. Here the main body of results came from just a 2kb portion. Even though diversity was examined at only a relatively few nucleotide positions (30 restriction sites across 2kb of a 20-25kb molecule) the method has proved effective. Thus there seems to have been no disadvantage to using such a small stretch of mtDNA. Martin *et al.* (1990) have shown that estimates of diversity do not vary greatly with the number of bases examined (their study was based on sequencing studies) although the range and standard deviation of replicate estimates declines with sample size. More of the molecule could be studied if amplification of the 3.85kb section was consistently successful but there is no evidence to suggest this would provide further resolving power.

6.3.2 Inter-specific comparisons

A similar pattern of haplotype frequencies to that in *P. maximus* was exhibited in *P. jacobaeus* with the common haplotypes in comparable frequencies and a number of rare "private" haplotypes. χ^2 analysis provided no evidence to suggest that the 2 species differed on the basis of haplotype frequencies (Table 6.7). Nucleotide substitution levels similarly provided no evidence for significant differences between the 2 species (Table 6.6; Figure 6.7). Indeed the Mulroy Bay population of *P. maximus* was substantially more differentiated from conspecific populations than *P. jacobaeus* was from its congener.

If *P. jacobaeus* and *P. maximus* are true species then differentiation would be expected. The lack of detectable divergence could come about as a result of recent speciation resulting in the mtDNA lineages having had little time to diverge. The likely time of divergence of these species is 5m.y.a. at the time of the Messinian salinity crisis at the very end of the Miocene (T.Waller, National Museum of Natural History, Smithsonian Institution, Washington D.C. pers. comm.). It is difficult to compare the divergence level with estimates from other congeneric species that diverged at a similar time as unless effective population sizes and pre-divergence diversities are similar, then the resultant genetic differentiation will certainly be different. For example speciation in *Drosophila* is widely believed to be a result of founder effects whilst this is unlikely to be the case for *Pecten*. However, for the sea urchin *Strongylocentrotus purpuratus* and

S.droebachiensis, also considered to have diverged approximately 5m.y.a. and having a similar life history, estimated interspecific differentiation is two orders of magnitude greater at 6% nucleotide divergence across the complete mitochondrial genome (Palumbi and Wilson, 1990).

Since differentiation of Mulroy Bay *P. maximus* from other populations was detected with this method then differences would be expected between *P. maximus* and *P. jacobaeus*. Although the differences observed for Mulroy Bay *P. maximus* may be a result of inadequate sampling, a far higher differentiation would still be expected between the two species in comparison to intraspecific comparisons.

Similar haplotype frequencies could be maintained after separation of the two species if both species had large effective population sizes, although some changes in haplotype frequency would be expected by random lineage extinction (Avise, 1994). However mutations within the two gene pools should nevertheless result in detectable nucleotide level variation over the 5 million years since separation given that animal mtDNA is believed to evolve rapidly at 2% per million years (Brown *et al.*, 1979). It may be that the region of the DNA molecule studied is unrepresentative of this rate of evolution for the mtDNA although the average variability (compared to other species) observed through PCR-RFLP argues against a slow rate of evolution for this region of mtDNA. A decelerated rate of evolution for poikolotherm mtDNA has been suggested (Rand, 1994) but the rate would need to be particularly low to explain why no detectable differentiation has occurred over 5 million years of separation. Further analysis at other loci is needed to examine the reasons behind this.

From a very small sample size (N=2) it also seems that the mtDNA of the New Zealand scallop *P. novaezelandiae* is not dissimilar to the mtDNA of European *Pecten*.

6.3.3 ITS fragment data

Using PCR-RFLP the ITS region of *Pecten* proved to be polymorphic with 8 patterns revealed by *Msp*I digestion, although the genetic basis of this polymorphism could not be resolved. Concerted evolution should eradicate any within organism variation, however during homogenisation there will be a transient

phase during which repeat units exhibit polymorphism. Results from this amplification are difficult to interpret since it is not known whether variation exists within a single unit, within a number of units or on a single chromosome. If the latter, then homozygotes and heterozygotes should be differentiable but this could not be undertaken. The variation in intensity of certain bands raises the possibility of variants existing in low copy number (one or a few units) thus amplification has not occurred to the same extent as that of the high copy number units. Until the genetic basis of this polymorphism is discerned it is not particularly useful for population comparisons of *P. maximus* since there was no heterogeneity in pattern frequency.

7.0 General Discussion

7.1 Intraspecific differentiation

Despite a relatively long lived larval phase and few distinct barriers to larval dispersal suggesting a potential for extensive gene flow among beds, stock structure with a genetic basis may exist among *P. maximus* populations. In particular previous studies have identified the St. Brieuc Bay population as potentially genetically distinct from neighbouring populations. Evidence suggests that differences in reproductive cycle between St. Brieuc *P. maximus* and those from Rade de Brest and the Bay of Seine (Paulet *et al.*, 1986, 1988; Lubet *et al.*, 1995) do not solely result from environmental induction, due to maintenance of the differences following transplantation (Cochard and Devauchelle, 1993; Mackie and Ansell, 1993). Two other lines of evidence also indicate that this population may be genetically differentiated and perhaps self recruiting. Firstly inferential evidence from hydrographic studies (the presence of a gyre) implies containment of larvae which may suggest self recruitment (Dare *et al.*, 1994). Gyres and other hydrographic features centred around other scallop grounds also invoke the possibility of self recruiting populations elsewhere (Sinclair *et al.*, 1985; Dare *et al.*, 1994). Secondly, because *A. opercularis*, a species with a similar distribution and life history to *P. maximus* exhibits considerable population differentiation including separation of the St. Brieuc population on the basis of allozyme frequencies (Beaumont, 1982a), divergence of the St. Brieuc *P. maximus* population is perhaps expected. Indeed *A. opercularis* displays pronounced stock structure throughout its range (Beaumont, 1982a) which has not been observed for *P. maximus* despite the obvious similarities in distribution, length of larval life and spawning periods between the two species. Similar patterns of genetic differentiation in the two species are expected and indeed given that *A. opercularis* has an increased capacity for larval dispersal relative to *P. maximus* due to its enhanced byssal drifting capability (Beaumont and Barnes, 1992) gene flow in *P. maximus* is expected to be lower and thus genetic differentiation greater. Nevertheless, in spite of expectations, past efforts to discern *P. maximus* populations from throughout the species' range including comparisons of St. Brieuc and the neighbouring Rade de Brest population (one of the populations for

which reproductive cycle comparisons were made) using allozymes, proved unfruitful (Beaumont *et al.*, 1993). The overall conclusion was that populations throughout the species' range were genetically homogeneous on the basis of data from the allozyme loci examined. Such a finding raises questions over how reproductive differences can be maintained on transplantation if there is no genetic divergence and why *P. maximus* does not display the stock structure seen for *A. opercularis*.

In order to undertake a detailed study of genetic differentiation of *P. maximus* populations to further examine this discrepancy, *P. maximus* was sampled throughout most of its range and the specimens used for comparative morphometric, allozyme and mtDNA studies. The expectation is that the most representative picture of genetic relatedness among geographically isolated populations would be provided by study of the genes themselves (the DNA) or by allozyme methods (the primary products of genes). Genetic inferences from the study of the phenotype through morphological differences may be prone to the influence of non-genetic factors, particularly developmental or phenotypic plasticity which can be a major problem for morphometric analyses of population level variation or systematic studies, if misinterpreted as having a genetic basis.

The same samples were used for all methodologies permitting removal of the possible influences of differential spatial sampling of specimens for the respective analyses or temporal effects on populations between sampling times that may affect any conclusions afforded the combined results.

The results from the different studies provided contrasting pictures. *P. maximus* from the populations around U.K. and Eire were undifferentiable from each other based on morphological comparisons but did differ from two of the French samples, with the Brest and La Trinité scallops having shorter hinges and, these populations and the Lyme Bay scallops differed on the basis of the third principal component (which could not be attributed to any recognisable aspect of shell shape). Whether these differences reflect actual genetic differences is debatable, however it is notable that the two French populations fall into the Celtic Sea (Brittany coast) province of *A. opercularis* genetic differentiation (Beaumont, 1982a) and thus if genetic differentiation of *P. maximus* populations does follow

the same pattern as that of *A. opercularis* these populations should be differentiable from others. Although this would suggest that the hinge length differences may have a genetic basis there is other evidence that morphology does not reflect genetic differentiation. As the Mulroy Bay population proved genetically divergent (on the basis of mtDNA results) from other populations including *P. maximus* from Kilkieran Bay and, because these two Irish samples were both ongrown in Kilkieran Bay, then if the morphological characters measured in this study accurately reflect genetic divergence, morphological differences should be seen. However if morphology as measured here is primarily influenced by the environment then such differences will be negligible since both sets of animals would have been subject to identical conditions during growth. The Mulroy Bay scallops could not be discriminated from Kilkieran Bay scallops on the basis of morphology and if as presumed, the measures of shell dimensions used in the analysis adequately describe shell shape then this is evidence to suggest that shell shape does not accurately reflect genetic differences but is plastic and affected considerably by the environment as occurs in *Mytilus edulis* (Seed, 1968) and *Crassostrea virginica* (Galtsoff, 1964). This issue could be further studied through examination of shells of animals raised in Mulroy Bay itself. If these prove differentiable from other shells, including the transplant Mulroy Bay animals this would provide evidence for environmental influence on the morphological characters used in these comparisons. Whether these potential environmental influences devalue the apparent separation of the French animals from other scallop populations is impossible to say and this is the prime failing of morphological criteria, that the influence of the environment relative to the effects of heredity is unknown. A thorough examination of this would require a parallel study of environmental factors to determine if any can be traced to morphology and a coincident breeding study using controlled conditions to evaluate their effects on growth. Because of the effort required and paucity of information to be gained relative to DNA data, morphometric studies are not recommended for such a population analysis, however if morphological comparisons are to be made then an increase in the number of measurements taken is needed, using not only the gross dimensions, in order to more adequately describe shape. Mulroy Bay scallops are

often skewed in shape with asymmetrical anterior and dorsal "halves", a phenomenon not seen in other populations (D.Minchin, Fisheries Research Centre, Abbotstown, Dublin, pers. comm.) and the measurements taken here would not capture this and, therefore would not reveal what may be a population specific phenomenon.

The conclusion from the allozyme results is in agreement with that from a previous study (Beaumont *et al.*, 1993) that populations of *P. maximus* are inseparable on the basis of allozyme data despite high variability and, this therefore suggests negligible genetic divergence among *Pecten* populations. Because population subdivision was expected (at least of the St. Brieuc population) and, due to the inherent problems with allozyme methods, the allozyme results were contrasted with results from DNA loci. For some species, the most notable example being the American oyster *Crassostrea virginica* (Karl and Avise, 1992; Karl and Avise, 1993), DNA methods which target the genetic material directly and therefore remove the problems of hidden variation and, often have increased resolution, have proved useful in revealing population structure in species where allozyme methods had suggested genetic uniformity. Examples of discordant patterns of mtDNA and allozyme differentiation include American oysters, *Crassostrea virginica* (Reeb and Avise, 1990), the horseshoe crab *Limulus polyphemus* (Saunders *et al.*, 1986) *Drosophila mercatorum* (DeSalle *et al.*, 1987) and the river blackfish *Gadopsis marmoratus* (Ovenden *et al.*, 1988). DNA data can also enhance the conclusions of allozyme studies and provide additional detail to the picture of population structure depicted by allozyme frequencies as is the case for *Tigriopus californicus* copepods (Burton, 1994). Hurst and Skibinski (1995) have also contrasted the effects of allozyme data and mtDNA RFLPs for estimating gene flow in the limpet *Patella vulgata* and concluded that gene flow estimates from mtDNA were far lower than estimates from allozyme data. In general it seems that DNA markers have the capability to reveal differentiation of populations not apparent from allozymes (Mitton, 1994) and indeed "so long as variation exists, differences among populations will be more readily detected with mtDNA than with nuclear genes" (Moritz, 1994) reflecting the increased

evolutionary rate of mtDNA (resulting in increased variation) and increased susceptibility to drift.

Within the genome there is a huge array of potential DNA loci for study (Kreitman, 1991) but the mitochondrial DNA has considerable advantages over nuclear DNA for population genetic studies. Because of the lower effective population size (due to haploidy and in most species maternal inheritance), mtDNA can show genetic differentiation at levels of gene flow and population size that would obscure differentiation at nDNA loci. Also because of an enhanced evolutionary rate, there is an increase in mtDNA variation which coupled with the increased sensitivity to drift should make the mtDNA a particularly useful population marker. As in virtually all scallops studied so far (Gjetvaj *et al.*, 1992; Repin and Brykov, 1993), the mtDNA of *P. maximus* is peculiar in the great amount of length variation that is present (Rigaa *et al.*, 1993). In some instances (Wirgin *et al.*,1989; Chapman, 1990; Wilkinson and Chapman, 1991; Brown *et al.*, 1992) situations have been described in which there is no geographic variation of restriction site variability but heterogeneity of length variants, the inference being that the distribution of length variants could be used to delineate populations. However the mechanics of the origin, maintenance and heredity of length variants is not fully understood, making their usefulness as population characteristics debatable and without substantial effort the correct measurement of mtDNA length in *Pecten* proved difficult and prone to error. Length variation also causes difficulties for RFLP studies of mtDNA since the separation of the length variation from restriction site variation is problematical and it would require considerable effort to alleviate these problems if the mtDNA as a complete molecule is to be studied. This problem was able to be circumvented through use of the PCR which permitted amplification of regions of mtDNA distinct from the length variable region allowing RFLP studies of mtDNA to be undertaken without the associated problems of length variation. Although the mtDNA is only a single locus, the differential evolutionary rate of its constituent genes means that separate regions may evolve more quickly than others and thus be of more use in population studies. Whether the sections studied here through PCR-RFLP are evolving at a rate suitable for population studies was not known beforehand since the primers

were newly designed and the complete sequences of the amplified fragments were not determined, thus the constituent genes were unidentified.

The variation revealed by PCR-RFLP was, however high, identifying numerous closely related haplotypes. Distinct mtDNA haplotypes were not revealed at high frequency in any area as would be expected if there was major population subdivision, although "private haplotypes" (haplotypes seen only in individual populations) were observed in individual animals. However in contrast to the genetic homogeneity suggested by the allozyme results, frequency differences of haplotypes and reduced within-population sequence diversity for the Mulroy Bay population resulted in a clear separation of this sample on the basis of average between population sequence diversity. There was no clear evidence for any population structure elsewhere although the frequency of the Pma1 amplified common haplotype (AAAAAA) appeared to change markedly among populations, albeit without causing differences detectable with χ^2 tests. Where such differences have been shown to be non-significant they would normally be dismissed, however because the pattern partially matched that expected (that of allele frequency in *A. operculatis*) they cannot be totally disregarded. The pattern of frequency of this haplotype does seem to reflect to some extent the population subdivision apparent for *A. operculatis* (Beaumont, 1982a) with Brest and La Trinité having similar haplotype frequencies (Brest and Belle Isle had similar genotype frequencies for queen scallops) but with St. Brieuc resembling more closely the Irish Sea populations (although Chicken Rock displayed a much lower frequency of the common haplotype). However the frequency of this haplotype in the Mull population did not differ significantly from Irish Sea populations which would be expected if the pattern of haplotype frequency matched the pattern of genotype frequency in queen scallops. Further evidence for similarity between queen scallop and *P. maximus* population differentiation comes from a separate study of allozymes in *P. maximus*, in which Igland and Nævdal (1995) detected significant differences in allele frequencies between Norwegian samples and published allele frequencies of U.K. scallop populations which is concordant with the substantial differences in *PT-A* allele frequencies among queen scallops from these areas.

MtDNA haplotype frequencies also suggest that the Lyme Bay population may be genetically differentiated from the other south coast population, Polperro, due not only to distinct differences in the frequency of the common haplotype but because of the low haplotype diversity of the Lyme Bay population. Low numbers of haplotypes and consequently depressed haplotype diversity may occur in a self-recruiting population due to the smaller effective population size. Beaumont (1982a) only examined one population from the south coast of England (Plymouth), thus there is no way of knowing whether the difference in frequency of Pma1 haplotype AAAAAA in *P. maximus* between Lyme Bay and Polperro samples is akin to stock structure in *A. opercularis.* The observed differentiation could be due to reduction of larval movement in and out of the Lyme Bay population as suggested by current movements in this vicinity, which often display a strong gyre potentially capable of maintaining scallop larvae within the bay (Salomon and Breton, 1993) and projected larval drift patterns (Dare *et al.*, 1994). Lyme Bay scallops have previously been suggested as self-recruiting (Clover, 1994) but the evidence for this claim was not obtainable (C.Clover, Daily Telegraph pers. comm, J.Edwards, Devon Wildlife Trust, pers. comm.).

Despite the suggested population structure, aside from the separation of Mulroy Bay there were no significant differences detectable in mtDNA among other populations. Carvalho and Hauser (1995) state that "a lack of significant genetic heterogeneity should be interpreted with caution and, where feasible, additional sources of information should be sought or conservative measures deployed". In a case such as this where there is some suggestion of stock structure (in the pattern of Pma1 haplotype AAAAAA) it is important to recognise that although self-recruitment of populations has not been shown, evidence of an underlying population structure (especially in this case when it is akin to that seen in a related species) implies that gene flow may be low. Given that only a small number of migrants is necessary to homogenise gene frequencies then the variation in haplotype frequency could be due to low gene flow which implies low numbers of migrants (i.e. reduced larval movement). Should the putative stock be overfished, then although migration (of larvae) probably is occurring, since larvae do not for some reason move freely among populations then the influx of larvae

would almost certainly not be enough to replenish the stock in the short term. This would have ramifications for management of the *P. maximus* fishery. It is thus imperative to accurately determine the stock structure in this case. A lack of significant population structure could result from a number of possibilities (Carvalho and Hauser, 1995). Firstly there may be sufficient gene flow to maintain near panmixia. The available evidence cannot disprove this but argues that it is not the case. Secondly occasional "sweepstake" events such as sporadic recruitment from distant areas may occasionally occur to homogenise allele frequencies. Once again although the larval transfer may partially homogenise gene frequencies it would be insufficient to repopulate an overfished stock and thus if such a process is responsible for affecting haplotype frequencies it requires detection. However such a process would likely obscure any differences in haplotype frequency thus the pattern of haplotype AAAAAA observed would not be expected. Thirdly there may be balancing selection on the studied locus. Selection may act on mtDNA (although mtDNA is often considered as effectively neutral it does contain predominantly coding sequence which could be affected by selection) and, since it is just a single locus this may go undetected, unlike allozymes where multiple loci are studied and thus selection, which often acts on only single loci may be indicated. Further examination of more DNA loci is required if this is to be ruled out. A fourth possibility is recent divergence. This is possible if current patterns have recently changed to effectively isolate certain grounds without leaving sufficient time to alter allele frequencies to a detectable level at the studied loci. Finally use of unsuitable loci or insufficient sample sizes may not adequately reveal the true picture of genetic differentiation. Use of further loci may answer the question. The latter two possibilities are believed to be the most likely.

If the two scallop species do share a common population structure then it is strange that an allozyme locus (in *A. opercularis*) revealed a more striking picture than that of presumably rapidly evolving mtDNA in *P. maximus*. The differentiation of *A. opercularis* populations was primarily due to frequency differences at a protein coding locus (*PT-A*) of unknown function whilst *PGM*, *ODH* and *LAP* allele frequencies did not exhibit the same pattern. Since these are enzyme coding loci they may have been under the influence of balancing selection

(Altukhov, 1991). The *PT-A* locus (Beaumont, 1982a) may be of low functional constraint and therefore rapidly evolving, possibly at a rate higher than the section of mtDNA studied for *P. maximus*. Indeed whether the Pma1 amplified section from which the main body of results was generated is the most useful region of the mtDNA is not known although the limited body of data from Pma2 amplified mtDNA did not contradict or enhance the conclusions. It is possible that examination of different regions of mtDNA or alternative loci in *P. maximus* may prove useful in discerning a more distinct pattern of population differentiation, perhaps as clearly defined as that of queen scallops. Although mtDNA is often hailed as the most suitable locus for population level questions it has generally been of most value in studies of mammals. The evolutionary rate of mtDNA in endotherms is apparently less than that of ectotherms (Rand, 1993, 1994) and because *P. maximus* is hermaphrodite the effective population size is not reduced to the same extent as in dioecious species with its consequential effect on haplotype sorting and susceptibility to drift. Thus there may be other DNA loci of more use for this kind of study in *P. maximus*. Alternative rapidly evolving (neutral) DNA markers may provide an enhanced picture of population subdivision. Potentially useful options include design of PCR primers for amplification of anonymous scnDNA loci (Karl and Avise, 1993), analysis of introns such as calmodulin gene introns (Côrte-Real *et al.*, 1994) or DNA fingerprinting methods. These include a range of possibilities including minisatellites and microsatellites or amplification of VNTRs with the PCR. Fingerprinting methods are often applied to organisms for which alternative methods have failed to reveal sufficient variability. Such lack of variability has certainly not been the failing of either the allozyme or mtDNA data for *P. maximus*, rather the problem has been that although variation is evident and appears to suggest some population differentiation, the results are inconclusive. Other loci may however provide data to resolve the issue. PCR based VNTR methods have been useful in suggesting population differentiation in *Placopecten magellanicus* (E.Kenchington, Marine Gene Probes Laboratory, Halifax, Nova Scotia, pers. comm.) for which allozyme frequencies were homogeneous.

Despite the unclear nature of the pattern of differentiation over most of the species' range, the mtDNA method was successful in revealing differentiation of the Mulroy Bay population which appeared, on the basis of mtDNA PCR-RFLP results divergent from other populations. This is not the only line of evidence for differentiation of Mulroy Bay scallops which have differences in colour from other Irish populations (Minchin, 1991). Mulroy Bay is also known to harbour animals rarely seen elsewhere (D.Minchin pers. comm.), for example, Couch's Goby (Minchin, 1988) which may indicate the existence of an enclosed body of water and therefore the population may be essentially self recruiting.

There are a number of possible explanations for why the Mulroy Bay population appeared different when other populations appeared (statistically) genetically homogeneous. Firstly the Mulroy Bay sample may not have been representative of the population. The animals used from this region were reared from a single collection of wild spat and thus represent a single year class. If there is temporal variation in mtDNA haplotype frequencies then a single year class may not be adequate to depict the genetic structure of the population as a whole. Temporal variation in allele frequecies of recruits is known to occur (Hedgecock, 1994), possibly due to selection acting on larvae or variance in individual reproductive success. Only a study of temporally separated samples would resolve this issue.

The second possibility is that the clear separation of this population is typical of populations within similar types of sea lough. Mulroy Bay represents a type C lough in the classification of Milne (1972), that is, a loch with an entrance sill and a further sill separating the "upper" and "lower" lochs. The structure of such sea lochs results in reduced water transferring in and out of the lough and therefore presumably reduced larval transfer. In extreme cases this could cause confinement of larvae within the lough and therefore result in self recruitment. However if the differentiation is typical of type C lochs then differences may be expected between upper and lower loch basins. No such differences were noticed in allele frequencies at the *PT-A* locus of *A. opercularis* between upper and lower Loch Creran (a type C loch) samples of *A. opercularis* (Beaumont, 1982a).

A study of scallops from similar sea loch systems is necessary to differentiate between this and the possibility that Mulroy Bay may represent a unique case. Due to an unusual geographic or hydrographic barrier, there may be negligible or curtailed transport of larvae into the lough, producing a self recruiting population. The North Water of Mulroy Bay is separated from the Atlantic by at least two sills and, due to an unusual tidal regime there is very little water movement (Minchin, 1981, 1983). This population may therefore be a unique (or limited) example of a self-recruiting population.

The final possibility is that the *P. maximus* in this population have been through a bottleneck, altering gene frequencies through stochastic processes to such an extent that they have become different from populations elsewhere and genetic diversity has thus been reduced. mtDNA is particularly prone to loss of variability during bottlenecks (Wilson *et al.*,1985). Settlement of scallops in Mulroy Bay is known to have been drastically reduced in the mid 1980s and this has been linked to the use of tributyltin (TBT) on salmon nets in the area (Minchin *et al.*, 1987). However since the banning of the use of TBT, settlement levels have increased (Minchin *et al.*, 1987; Minchin, 1995) indicating that the population may have bottlenecked with consequent effects on genetic variability. Changes in mtDNA allele frequency can occur within "ecological time" if the effective number of females is small (Moritz, 1994) as would occur after a bottleneck which could possibly cause divergence. However without a coincident reduction in gene flow from outside the lough the gene frequencies would be brought back into line as soon as TBT levels decreased to levels allowing settlement. Since settlement has increased after TBT levels fell, whilst divergence is still apparent, if bottlenecking was the cause of the observed mtDNA pattern there must be some coincident restriction to gene flow. Certainly variability in the Mulroy Bay population as represented by nucleotide diversity is quite low (only the Lyme Bay population has a lower level) but it is not vastly reduced and there is still a high haplotype diversity. If bottlenecking was not the cause, this reduction in nucleotide diversity could be explained by the lower effective population size that would be a consequence of a self recruiting population.

Whatever the cause, there is evidence that Mulroy Bay scallops are self-recruiting which has consequences for the management of the population. Although Mulroy Bay scallops have been dredged in the past the only fishing now allowed is by pole-net (Minchin, 1983) thus recruitment overfishing is not a threat to this population. However decisions on restocking need to be taken in context of these results. Mulroy Bay has been proposed as a suitable site for collection of scallop spat for reseeding efforts (Slater, 1994) and work has been undertaken to establish the suitability of the site for reseeding to bring the population to a level at which it could withstand removal of spat for transfer to other sites (Minchin and Ni Donnachada, 1995). This study provides no evidence for unique genotypes in Mulroy Bay that should be conserved to avoid loss of genetic variability, but it has indicated a potentially self recruiting population within which the animals may, through selection, have become adapted to their environment and thus may not perform optimally in alternative conditions. Indeed differences in gonad indices throughout the year have been recognised between native and transplant Mulroy Bay animals in Kilkieran Bay (Mark Norman, Muirin Teoranta, Cill Chiarain, Conamara, County Galway, Ireland, pers. comm.).

If the differentiation exhibited by Mulroy Bay is real (i.e. not due to sampling effects), whether actually restricted to Mulroy Bay or characteristic of this type of sea lough then mtDNA analysis has revealed population subdivision not apparent from allozyme data. Potential explanations for such discrepancy between methodologies have been suggested (Karl and Avise, 1992; Reeb and Avise, 1990).

1. A higher rate of interpopulation gene flow mediated by sperm rather than eggs resulting in higher gene flow for nuclear loci (allozymes) in comparison to the maternally inherited mtDNA. However in species such as *P. maximus* with planktotrophic larvae, this would be an unlikely explanation since even if sperm were able to move further than eggs the time period of gamete survival is negligible compared to that of the larvae. Thus differential gamete movement would be swamped by larval transfer. However if scallops reproduced disproportionately as males (*P. maximus* is hermaphrodite) then gene flow for mtDNA may be reduced and this may cause a discrepancy between allozymes and mtDNA in patterns of genetic variation. If scallops were predominantly

young then such a phenomenon is possible, since there is a general tendency towards protandry in scallops (Beninger and le Pennec, 1991) thus young animals may spawn only as males although there is no known evidence that any population is composed of predominantly young animals. In *Argopecten irradians* low food and temperature can also cause suppression of oogenesis and mainly male gametogenesis (Sastry, 1968; Beninger and le Pennec, 1991) but again there is no known evidence for such an effect in *P. maximus*. This hypothesis is unlikley since the widest discrepancy between nDNA and mtDNA was shown by Mulroy Bay and there is no obvious reason why suppressed mtDNA gene flow would affect only this population.

2. Directional selection favouring haplotypes in the diverged areas. mtDNA is assumed to be a neutral marker in most studies but in some instances has been shown to have an effect upon physiology or fitness (reviewed in Mitton, 1994). If selection promotes certain haplotypes in Mulroy Bay then divergence would be apparent. However there were no substantial shifts in haplotype frequency for the Mulroy Bay population thus there is no obvious evidence for selection. Since the mtDNA is a single locus such an effect would be difficult to detect in this kind of study. Analysis of alternative nDNA loci would aid in disproving this hypothesis since selection should not act similarly on alternative loci (unless it is balancing selection on enzyme loci) thus differentiation shown at other loci would sugest that selection has not affected the mtDNA. Use of neutral or nearly neutral loci would be advisable.
3. A smaller effective population size for mtDNA causing a faster rate of lineage sorting from the ancestral gene pool than that for nDNA (Wilson *et al.*, 1985; Nei, 1987). Due to haploidy and maternal inheritance for mtDNA the effective population size ($N_{f(e)}$) and time to shared ancestry is ¼ that of nuclear genes although in hermaphrodites such as *Pecten* maternal inheritance does not reduce the effective population size thus $N_{f(e)}$ is ½ of that of nuclear genes. The time to reciprocal monophyly for nuclear genes is therefore twice that of mtDNA. There is no distinct evidence of lineage sorting between the two clades, with no haplotypes at high frequency in the Mulroy Bay animals but not in scallops outside the lough which would implicate this. However the population may be

recently diverged and thus not yet be at equilibrium. This possibility would become more evident as equilibrium is approached.

4. "Hidden" variation for allozymes which would distinguish the lineages in a manner equivalent to the results seen for mtDNA. This is certainly a possibility. If cryptic variation exists it may accurately depict the divergence apparent in the mtDNA results but not the allozymes although such variation would be exceptionally difficult to uncover.
5. A slower evolutionary rate of change in allozyme frequencies in comparison to mtDNA. Differentiation should then be apparent at more rapidly evolving nDNA loci or at more rapidly evolving allozyme loci. The *PT-A* locus of *A. opercularis* (Beaumont, 1982a) may be an example of a rapidly evolving allozyme locus perhaps with a low functional constraint.
6. Balancing selection at the allozyme loci producing apparent genetic homogeneity in spite of curtailed gene flow. Balancing selection has been implicated for the geographic uniformity of allozyme frequencies but pronounced population subdivision evident from mtDNA haplotype frequencies in the oyster *Crassostrea virginica* (Karl and Avise, 1992), in which scnDNA RFLPs revealed a genetic break concordant with that evidenced by mtDNA. Balancing selection is believed to be a widespread phenomenon (Altukhov, 1991). If scnDNA markers revealed the Mulroy Bay population to be genetically divergent from other populations as for mtDNA (but not for allozymes) then balancing selection on allozyme loci would be implicated.

Although in this study the mtDNA data has proved successful in suggesting there was some population structure not apparent from allozymes, no differences were seen involving the St. Brieuc population which was *a priori* expected to exhibit differences. No differences were seen in morphological comparisons although St. Brieuc scallops are more highly coloured with purple right valves commonplace and frequent occurrences of upper valves with dark and white flecks. Coloured lower valves are found only rarely in other populations. Pigmentation of the lower valve is commonplace in Brittany populations (Minchin, 1991) which has been postulated as being due to an increased need for crypsis when overturned by

the strong currents in this area (Minchin, 1991), however the other Brittany populations (Brest and La Trinité) did not share this pigmentation. Whether such colour differences reflect genetic differentiation as in *Argopecten* (Adamkewicz and Castagna, 1988) and thus provide an indication of genetic differentiation of St. Brieuc scallops is unknown but these limited phenotypic differences are not corroborated by the genetic comparisons. As in previous studies (Huelvan, 1985; Beaumont *et al.*, 1993) there was no evidence for differences in allele frequency at any allozyme loci and, neither were significant differences detected in mtDNA haplotype frequencies or sequence divergence levels. The lack of discernible genetic differentiation raises important questions over the maintenance of the dissimilarity in reproductive cycles evidenced by the St. Brieuc and neighbouring populations (Lubet, 1986; Paulet *et al.*, 1986,1988; Cochard and Devauchelle, 1993; Mackie and Ansell, 1993; Lubet *et al.*, 1995). Perhaps the hydrographic isolation of St. Brieuc Bay is relatively recent and although gene frequencies have not had time to diverge to a detectable level, mutations at certain loci have resulted in the reproductive differentiation. If this is the case then DNA methods with a greater resolution would be required for identifying genetic differences.

Reproductive differences which are maintained on transplantation have been seen for *C.virginica* transplanted from Long Island to Delaware Bay and maintained for six generations, which as in *P. maximus* have not as yet, been separable with any genetic marker (Barber *et al.*, 1991). As stated by Barber *et al.* (1991), "the fact that discrepancies exist between allozyme frequencies, mtDNA patterns and physiological processes such as reproduction, point out that there is no simple means of delineating genetic boundaries among oyster populations". Differences in reproductive cycles have also been observed among populations of *Placopecten magellanicus* but by contrast in this species they do not appear to have a strong genetic component since differences are not maintained on transplantation (Dadswell and Parsons, 1994).

Despite the lack of separation noted for the St. Brieuc population where separation was expected, overall PCR-RFLP has proved to be a useful and cost-effective (following primer development) method. If the apparent separation of Mulroy Bay is real and not a product of poor sampling then PCR-RFLP on the

Pma1 amplified section of mtDNA has provided a method for detecting population subdivision not previously available and, there is the suggestion that further detail of population structure may become apparent with continued effort.

7.2 Interspecific comparisons

P. maximus and *P. jacobaeus* can be easily recognised by simple visual comparison based on the cross-sectional shape of the ribs, the size of the striae and the concavity of the upper valve. The number of ribs proved to be significantly greater in *P. jacobaeus* than in *P. maximus* (as suggested by Wagner, 1991). Rib number has previously been used as a taxonomic aide in studies of *Argopecten* subspecies (Blake, 1994) but whether it has a genetic basis in *Pecten* is unknown. In contrast, the dimensions used in the PCA were poor discriminators between the species. This is surprising for two reasons. Firstly because the two species are so obviously different on the basis of morphology, raising doubts over the utility of the measurements used for taxonomic (and population level) studies and, secondly, because *P. jacobaeus* were taken from an obviously different environment (deep, Mediterranean water) and if morphology is affected by the environment the shells of the two species would be expected to show differences. Discrimination between these species was also poor on the basis of allozymes and mtDNA. Comparisons involving *P. jacobaeus* in the allozyme study had the highest Nei's D values but there was no blatant divergence of the two species apparent from the dendrogram and the D value is consistent with the average value expected in comparisons of conspecific populations, not congeneric species (Avise, 1976) as has previously been suggested (Beaumont, 1991b). MtDNA haplotypes suggested *P. jacobaeus* was not differentiated from its congener with sequence divergence levels much lower than those in comparisons between the Mulroy Bay *P. maximus* population and its conspecifics. Limited divergence of the two species on the basis of allozymes has been seen previously by Huelvan (1985), but the lack of differentiation for mtDNA is surprising if this mtDNA region is rapidly evolving.

There is evidence that *P. maximus* and *P. jacobaeus* can hybridise with normal fertilisation rates and development to D-larvae (Huelvan, 1985). Hybrids were ungrown and shown to have an intermediate shell structure (J-C. Cochard, IFREMER pers. comm.). This evidence suggests a reevaluation of the taxonomy is

required but because there are no data on the viability of hybrids (animals died before a second spawning was undertaken) and because of the gross morphological differences, differences in ranges and limited loci examined in this study there is insufficient evidence for changes to the taxonomic standing of *P. jacobaeus* at present. Indeed despite the low genetic separation of the species they do fit the definition of non-sibling species of Avise (1976), that is, exhibiting morphological differentiation and reproductive isolation (presumed on the basis of distribution, but not backed up by genetic data). *P. maximus* does extend along the south coast of Spain into the Mediterranean and is then succeeded by *P. jacobaeus*. The change in species corresponds with the genetic break in *Mytilus galloprovincialis* populations (Quesada,Beynon and Skibinski, 1995; Quesada, Zapata and Alvares, 1995) where there is a sharp front which would be a strong barrier to larval movement (Quesada, Zapata and Alvares, 1995) thus it seems that the species are probably reproductively isolated. However the lack of observable differentiation suggests further investigation is warrented and indeed Waller (1991) has stated that the taxonomy of the Pectinidae is in need of drastic revision. Further evidence from a variety of DNA loci will help in this cause.

In the extremely limited (two individuals) study of *P.novaezelandiae* mtDNA using the Pma1 primer pair (identical sequence to that used in *P. maximus* and *P. jacobaeus*) the only obvious difference was the *Rsa*I haplotype of 1 individual. Obviously no measures of haplotype frequencies between this and the other two species can be taken but either differentiation of mtDNA within the genus *Pecten* seems overall to be unexpectedly low, or evolution of this region of mtDNA is slow, since extreme differences would be expected between species separated by thousands of kilometres of ocean. This primer pair did not work on *A. opercularis* thus no comparison can be made with a different scallop genus.

7.3 Future prospects

As there seems to be a pattern emerging that suggests there may be population structure in *P. maximus* akin to that seen in queen scallops, it is necessary to examine further loci to investigate this. The conclusions for *P. maximus* were based on data from mtDNA data (a single locus) and although data from a single locus can provide strong evidence for population subdivision

(providing the locus is effectively neutral) not only will similar conclusions from other loci be more convincing evidence, but loci that evolve more rapidly may provide a more striking picture (as for *PT-A* in *A. opercularis*). Gathering further data from the mtDNA by designing primers for alternative regions is unlikely to provide additional information. The study of nDNA loci is recommended.

Further analysis of the Mulroy Bay population is certainly required to resolve the question of whether the observed differences firstly are indeed representative of the Mulroy Bay population, by examining adult animals collected from within the lough and, secondly (and dependent on the results of the above) to investigate the extent of these differences to decide if they are limited to the North Water of the lough or whether they extend into the Broad Water and thirdly to determine if the scallops within the lough are differentiable from animals immediately outside the lough. The nearest exploited population occurs within the gyre off Glengad Head (D.Minchin pers. comm.) but limited numbers probably exist outside the lough entrance. Finally, do these differences extend to other sea lough populations particularly in type C loughs (Milne, 1972), or is Mulroy Bay a unique case. Milne (1972) provides examples of other type C lochs in western Scotland; Lochs Creran, Teacuis, Etive, Feochan, Leven, Linnhe and Tarbert, some of which may contain *P. maximus* deserving of continued study.

If the differences are limited to Mulroy Bay then they would likely be seen in other organisms and genetic differentiation of populations of different species (including a Mulroy Bay sample) should be studied.

Why the St. Brieuc population does not seem genetically divergent from other populations remains a mystery, but continued study using different loci may yet provide an answer. It may be beneficial to concentrate on why reproductive differences exist rather than why there is little detectable genetic differentiation.

Valuable information may also be gained by looking at mtDNA variation in *A. opercularis*. Intraspecific differentiation is expected to be similar for both species but conclusive proof of this is difficult without examing the same genetic marker in both species. *P. maximus* does not exhibit a directly equivalent and variable locus to the *PT-A* locus of *A. opercularis* (Beaumont and Gruffydd, 1975) and the mtDNA primers for P.maximus were designed specifically and do

not work on *A. opercularis*. Examination of samples of both species at a range of DNA loci may prove useful.

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Appendix A. Shell measurements analysed in Chapter 2.

Population	Length	Depth	Height	Weight	Hinge-l	Ridges
PS2	122.2	29.9	118.4	102.38	58.7	13
PS2	120.7	29.3	107.1	96.4	54.2	13
PS2	131.9	35.6	117.5	125.7	53.9	13
PS2	117.3	31.1	106	106.32	47.6	13
PS2	125	32.2	113	116.16	62.9	13
PS2	124.5	32.1	110.9	110.1	58.2	12
PS2	102.5	29.7	92.4	75.03	50.1	12
PS2	129.5	35.6	113.5	130.31	60	12
PS2	130	31.6	116.5	117.35	62.1	12
PS2	105.1	27.2	96.5	78.01	52.5	13
PS2	142	35.8	123.7	142.42	65.4	13
PS2	115.6	29.8	102.2	98.91	53.4	13
PS2	120.9	29.4	106.6	100.82	55.1	13
PS2	136	37.6	122.7	136.31	60.7	12
PS2	114.1	28.9	97.6	77.98	51.9	13
PS2	128.5	33.7	115.5	127.88	57.4	12
PS2	137.6	37.3	122.4	159.08	65.1	13
PS2	110.6	29.3	100	84.3	49.3	13
PS2	128.3	31.6	115.1	119.6	56.4	12
PS2	108.7	28	98.5	77.06	51.1	13
PS2	133.38	36.3	117.2	135.67	59.2	12
PS2	128.9	35.8	112.5	124.2	58.6	12
PS2	112.4	30.7	101.8	91.4	50.7	13
PS2	116.3	27.2	103.7	92.08	55.3	13
PS2	111.6	27	98.9	82	43.5	13
PS2	108.2	28.5	97.6	86.08	51	12
PS2	131	33.7	118.3	118.18	-	12
PS2	122.9	36.1	109.7	121.81	58.3	13
PS2	106.2	26.2	94.5	71.02	49.9	12
PS2	135	35.7	119.5	152.3	67.5	13
PS2	127.7	34	111.8	128.18	-	13
PS2	106.1	29.2	93.5	87.44	49.7	13
PS2	126.6	32.3	111.6	130.94	62.5	13
PS2	126.8	33	113.9	134.37	56.5	14
PS2	104.3	27	93.4	66.89	46	12
PS2	105.6	28.6	94.5	79.22	46.6	13
PS2	107.9	29.5	96.6	77.08	45.7	13
PS2	109.2	28.1	97.4	85	55.1	13
PS2	116	30.7	101.1	98.3	58.5	14
PS2	132.2	31.4	113.4	124.2	60.9	16
PS2	119.6	31.2	111.3	109.3	59.3	13
PS2	135.4	36.4	117.6	136.2	65.2	12
PS2	118.1	30.8	108.3	108.52	54.5	13
PS2	131.7	34.1	116.4	131	64.8	13
PS2	136.6	36.6	117.8	138	69.6	14
PS2	125.5	32.9	106.4	108.2	59.4	12
PS2	115.4	31.2	101.8	96.85	54.1	12
PS2	121.7	30.3	110	99.35	57.9	12
PS2	103.7	28.5	94	80.48	53	13
PS2	120.2	34.7	106.5	127.21	59.9	12
PS2	102.9	26.3	93.6	69.58	47.4	13
PS2	127.5	34.6	111.9	126.87	65.4	13
PS2	135.7	34.7	120.8	126.5	62.4	13
PS2	109.6	28.3	97.2	85.9	53	12

PS2	122.7	34.2	109	110.6	58.9	12
DOU	118.9	31.3	101.7	94.58	58.1	14
DOU	140.7	37.1	121.6	152.96	64.4	13
DOU	127.8	32.9	114.6	120.6	58.1	12
DOU	103.7	25.2	91.5	68.71	51.8	13
DOU	126.1	33.2	108.9	119.49	60.6	13
DOU	113.7	29.6	102.4	95.26	55.3	13
DOU	132.1	37.6	119.8	136.99	64.7	13
DOU	100.9	26.7	91.5	74.08	55.2	12
DOU	99.3	26.9	88.6	66.39	46.3	13
DOU	95	25.1	86.5	59.11	48.4	14
DOU	139.6	37.3	123.6	148.72	64.7	13
DOU	116	30	104.3	101.01	60.7	12
DOU	110.2	29.9	99.4	88.59	-	13
DOU	116.6	26.2	96.7	74.48	56.9	13
DOU	119.8	30.8	113	110.8	61.2	11
DOU	118.2	30.5	105.4	91.68	59.2	13
DOU	99.1	28.2	89.9	73.54	50.5	13
DOU	118.6	28	98	85.88	58.7	12
DOU	122.7	33.3	110.1	106.96	57.4	13
DOU	91.1	22.7	83.1	52.08	50.9	13
DOU	117.8	32.2	107	101.59	58.6	13
DOU	92.3	24.3	81.8	54.83	44.6	12
DOU	99.4	25.6	91.9	68.02	50.7	12
DOU	105.8	28	92.9	79.96	49.5	12
DOU	112.2	32.6	95.3	87.8	57.3	12
DOU	95.8	25.5	87.4	66.72	48.5	13
DOU	95.7	23.4	81.7	52.36	46.2	13
DOU	117.5	30.8	106.3	107.2	57.3	14
DOU	113.4	29.3	99.6	84.39	54.5	13
DOU	95.8	24.2	86.4	58.94	47	14
DOU	135.5	34.2	121.3	138.61	68.8	13
DOU	97.2	25.5	89	63.8	48.1	12
DOU	103.1	25.7	92.1	70.98	53.7	13
DOU	101.7	25.9	89.8	66.3	49.1	13
DOU	95.2	25	85.6	62.97	51	12
DOU	102.4	27.7	92.1	67.88	50.7	13
DOU	117	30.5	104.7	95.43	58.1	13
DOU	113.6	29	104.1	94.22	56.7	12
DOU	122.1	32.8	107.1	117.44	60	13
DOU	117.6	28.2	101.6	83.7	61.2	12
DOU	127.3	33.2	110.5	110.11	67.7	13
DOU	120.2	32	109.3	99.72	59.1	13
DOU	95.1	23.8	87.5	61.18	49.7	13
DOU	97.3	26.3	88.9	68.03	50.7	13
DOU	114.2	28.6	99.3	82.43	58.8	13
DOU	111.5	29.9	100.3	91.9	55.5	13
DOU	106	29.4	95.1	83.29	-	12
DOU	115.4	29.6	101.9	103.5	57.8	13
DOU	102.3	29.9	111.2	85.7	53.8	14
PEE	117.1	31.1	103.8	99.51	57.1	15
PEE	123.3	31.9	108.3	109.11	60.4	13
PEE	126.4	33.6	112.9	115.86	56.7	14
PEE	113.9	30.7	102.3	93.23	55.5	13
PEE	120.4	32.3	109.6	121.7	56.9	13
PEE	117.3	29.7	109.2	105.71	-	12
PEE	97.2	24.6	91.1	65.14	48.3	13
PEE	117.7	32.5	104.1	109.22	-	13
PEE	103.5	31	95.8	126.68	52.3	14

PEE	120.4	31.1	105.6	86.41	59.2	12
PEE	123.9	35.2	111	102.1	-	13
PEE	109	29	99.1	92.21	51.6	12
PEE	113	29.3	100.7	96.4	51	13
PEE	103.4	27.8	94.5	75.58	51	13
PEE	99.7	27.2	91.5	69.46	-	12
PEE	116.8	34.4	103.9	107.97	56.2	13
PEE	123.7	31.8	110.2	108.98	60.6	13
PEE	118	33.6	103.7	123.79	61.5	13
PEE	118.6	34.6	108.3	111.54	57.5	14
PEE	90.6	25.4	80.5	60.22	46.7	13
PEE	118	28.2	103.6	90.36	55.7	13
PEE	114.3	29.6	101.5	97.61	54.2	14
PEE	100.8	27.8	91.7	77.56	46.6	13
PEE	119.7	28.4	103.7	93.55	59	14
PEE	120.1	32.8	110.6	128.38	-	13
PEE	117.5	30.5	106.4	89.31	-	13
PEE	123.1	33.4	111.2	118.1	59.7	13
PEE	117.8	32	105.5	106.9	57.7	13
PEE	109.2	29.2	99.7	94.78	-	14
PEE	123.2	30.6	106.1	101.72	-	14
PEE	105.9	27.8	92.1	74.78	49	13
PEE	116.2	31.6	103.2	105.6	59.8	14
PEE	77	18.5	70.7	32.62	39.1	14
PEE	113.5	32.2	102.8	102.42	58	12
PEE	122.8	34.6	114.5	119.72	58	13
PEE	114.8	28.7	102	85.43	54	12
PEE	93.5	23.1	82.2	55.2	44.8	13
PEE	115.9	30.3	103	99.25	52.4	13
PEE	110.5	28.5	97.7	86.51	57.7	13
PEE	124.7	31.2	108.7	106.19	62	13
PEE	121.9	31.5	105.7	107.9	58	12
PEE	116.1	31	105.4	102.88	58.3	14
PEE	100.5	27.1	90.7	76.34	47.4	13
PEE	112.5	30.5	100.2	95.83	53	13
PEE	114.2	30.9	105.6	104.78	51.4	14
PEE	92.4	24.8	83.7	61.99	43.4	13
PEE	125.2	30.7	110.3	111.35	59.1	14
PEE	101.4	28	91.9	82.28	51.4	14
PEE	122.3	33.6	108.2	128.38	60	13
PEE	121.8	29.9	108.1	106.7	60.1	13
CHI	116.3	32.2	102.9	99.6	59.2	12
CHI	121.9	33.4	110.6	122.87	64	13
CHI	108.2	29.1	97.4	88.46	48.6	14
CHI	119.9	30.9	107	108.4	-	13
CHI	125.7	32.7	115	126.58	61.7	13
CHI	122	33.1	108.3	112.61	60.6	13
CHI	93.2	24.7	85.1	63.53	43.1	13
CHI	126.3	29.4	112.7	110.4	61.6	14
CHI	141.6	35.3	126.3	144.85	68.4	13
CHI	120.9	31.3	111.1	113.96	57.5	14
CHI	120.2	30.3	106.7	110.4	57.1	12
CHI	117.5	28.6	104.8	98.16	58.4	13
CHI	110.9	28.7	97	77.6	55	13
CHI	75.4	20.8	70.5	38.98	40.1	13
CHI	106.8	28.9	100.7	89.13	53.3	13
CHI	73.3	19.3	66	31	38.2	13
CHI	122.8	33.7	110.9	124.18	64.3	14
CHI	116.6	32.3	103.6	105.28	58.3	13

CHI	125.8	33.1	112.4	122.37	66.4	13
CHI	102.7	26.9	89	70.18	50.2	12
CHI	107.2	28.5	96.5	85.56	49.2	12
CHI	114.6	29.6	103.6	94.09	57	13
CHI	97.5	27.6	90.5	68.5	51.1	13
CHI	112.2	30.1	100.3	86.28	52	13
CHI	116.1	30	106.3	103.4	57.7	13
CHI	111.1	27.8	97.3	86.9	55.8	13
CHI	99.9	26.2	90	69.38	50.2	13
CHI	109	30.8	97.2	89.7	55.5	12
CHI	121.2	27.5	112.3	100.71	67.5	12
CHI	106.1	27.4	95.3	81.5	54.2	13
CHI	124	33.1	108.7	122.36	61.7	13
CHI	128.3	32.7	113.1	126.48	66.1	12
CHI	101.3	25.6	88.3	65.06	48.4	13
CHI	112.7	29.1	101.3	95.81	53.2	14
CHI	121.5	28.7	109	104.41	58.2	13
CHI	102.7	26.3	94.9	73.67	47.6	13
CHI	97.4	25.2	89.9	69.99	48.7	12
CHI	74.7	19.1	68.4	34.2	37	13
CHI	117.5	30.4	103.9	106.78	57.6	13
CHI	105.7	27.3	93.9	73.69	53.1	13
CHI	111.5	27.9	110.1	80.88	49.9	13
CHI	111.2	27.2	99.3	77.37	52.8	13
CHI	101.4	27	93	78.82	52.3	14
CHI	93	25.4	84.4	59.73	46.6	13
CHI	121.5	31.9	109.2	104.79	57.1	13
CHI	127	32.8	112.8	116.38	53.3	13
CHI	111.4	26.7	99	82.08	49.7	13
CHI	120.1	32.3	107.5	117.91	60.7	13
CHI	110.9	31.7	99.4	85.28	54.5	13
CHI	75.8	20	70.3	34.88	-	13
CHI	93.3	24.7	103.5	75.07	48.1	13
CHI	101.2	25.4	95.5	71.66	54.9	13
CHI	114.8	28.2	102.5	88.81	49.3	13
CHI	124.1	33.8	113.3	126.92	58.5	12
CHI	100.9	28.9	89.8	79.68	52.5	13
CHI	115.6	30.4	104.4	94.86	57.9	12
CHI	116.8	30.5	104.6	103.38	51.8	13
CHI	121.6	33.2	111.6	120.41	55.1	14
CHI	124.6	32.7	110.9	108.22	61	14
CHI	126	30.6	112.5	104.49	63.6	13
CHI	115.4	29.9	105.3	94.7	55.3	12
CHI	122.4	31.2	105.2	109.29	55.3	13
CHI	114.4	33	106	105.5	55.3	12
CHI	122.6	31.1	107.2	101.9	57.1	13
CHI	108.7	28.9	99.9	87.06	51.3	13
CHI	107.7	26.8	97.3	81.52	46.6	13
CHI	118.4	31.4	108.4	111.53	51.9	12
CHI	121.7	34.6	113	124.87	61.1	13
CHI	120	30	107.5	98.1	59.2	13
CHI	117.5	32.5	106.2	115.35	49.1	13
CHI	110.4	33.4	99	102.3	49.4	12
CHI	110	29.2	102.3	84.38	56	13
CHI	106.5	26.2	93.7	74.3	49.1	15
CHI	120	29	111.4	98.14	57.8	14
CHI	124.4	32.3	111.6	111.33	58.1	13
CHI	120	32.6	105.4	113.51	62.5	12
CHI	125.1	33	115.6	127.4	58.7	13

PS1	125.8	33.8	111.3	115.89	66.2	13
PS1	133.3	34.2	122	141.11	57.9	12
PS1	130.4	36.5	117.4	128.32	63.9	13
PS1	130	38.9	118.4	149.1	60.2	13
PS1	114.4	28.6	103.2	88.42	55.4	13
PS1	134.6	35.6	120	147.3	64.1	13
PS1	110.1	30.5	102.1	103.38	55.9	12
PS1	133.2	31.1	115.6	117.56	68.2	12
PS1	128.1	32.1	111.6	117.47	58.1	13
PS1	136.1	35	123.7	136.9	-	13
PS1	121.8	29.8	105.9	98.22	55.5	13
PS1	129	31	113.4	119.88	58.7	12
PS1	113	30.3	100.6	93.3	54	13
PS1	118.3	29.9	103.9	95.31	53.1	13
PS1	129.8	34.4	104	117.92	63.5	12
PS1	134.9	35.1	121.7	149.6	63.9	13
PS1	119.9	29.5	105.5	98.85	-	13
PS1	111	26.6	102.1	85.5	55.5	12
PS1	123.2	28.5	106.2	96.9	57.7	15
PS1	122.6	32.4	105.4	106.82	58.4	12
PS1	129.2	34.8	118	131.54	59.3	13
PS1	122	29.7	106.6	103.16	57.7	13
PS1	139.7	36.9	121	149.9	58.8	13
PS1	147.8	37.4	130.9	178.6	67	12
PS1	122.2	30.5	110.4	100.55	59.9	13
PS1	116.5	30.2	101.5	89.81	49.7	13
PS1	114.5	30.3	101.8	96.93	57.9	12
PS1	128.2	32.1	112	120.23	65.4	13
PS1	85.6	21.2	76.3	35.89	36	13
PS1	121.3	32.7	108.2	118.2	67.7	13
PS1	122	31.8	108.1	105.68	51.9	14
PS1	120.2	30.7	108.4	103.22	58.3	13
PS1	119.2	33.4	106.2	105.72	55.4	12
PS1	126	33.3	113.7	127.08	54.7	13
PS1	116.7	30.3	103.6	96.77	57.1	13
PS1	116.3	31.9	106.9	113.22	53.5	14
PS1	133.9	38.1	117.9	148.6	60	14
PS1	116.9	28.3	102.1	91.5	53.3	13
PS1	117.4	31.3	107.3	103.88	59.4	13
PS1	133.4	32.8	114.9	123.36	63.8	12
PS1	127.9	32.9	114.2	122.43	58.4	13
PS1	125.5	31.6	110.3	111	63.3	13
PS1	133	37.7	119.8	131.49	60.1	12
PS1	112.1	30.5	101.6	86.63	54	13
PS1	136	33.8	120.1	134.12	65.5	13
PS1	120	31.2	106.1	100.8	53.7	12
PS1	129.1	34	118.8	128.08	65.6	13
PS1	117.2	32.9	105.8	105.49	54.5	13
PS1	144.7	38.1	128.2	166.97	67.3	13
PS1	146.4	40.2	130.9	176.13	67.9	12
PS1	114.8	29.9	103.5	94.9	55.1	13
PS1	126	31.3	111.8	116.7	55.5	13
PS1	87.2	21.6	80	41.61	44.3	13
PS1	120	30.7	103.7	100.52	59.3	13
PS1	111.2	29.4	99.7	84.88	52.1	14
PS1	117.8	31.6	105.9	103.81	58.7	13
PS1	85.2	20.5	75.7	39.54	44.3	14
PS1	99.3	26.8	87	62.32	48.9	14
PS1	75.9	20	69.1	29.79	37.4	13

PS1	76.3	21.1	69.6	35.59	-	14
PS1	120.3	31.3	107.4	110.43	59.5	13
PS1	102.9	27.6	94.7	79.5	47.8	13
PS1	148.6	41.6	130.5	186.51	68.1	12
PS1	98.9	24.7	90.9	64.24	50	14
PS1	119.2	32.2	105.7	108.41	54.2	12
PS1	112.1	29	100.4	95.13	50.6	13
ANG	116.4	29.1	101.7	95.87	51.7	13
ANG	135.2	34.9	117.5	139.19	64.3	13
ANG	106	28.1	100.1	80.76	54.8	12
ANG	132.2	36.3	117.9	142.58	56.7	12
ANG	120	32.8	109.8	113.49	55.2	13
ANG	117.3	31.2	107.6	111.22	50.7	12
ANG	129.9	32.6	119.6	134.85	66.7	12
ANG	118.1	30.9	107.3	109.11	56.9	13
ANG	108	26.7	94.7	73.9	54.4	14
ANG	135.5	33.7	118	135.5	68.9	14
ANG	131.3	33	116.5	125.88	64.2	12
ANG	116.6	28.6	101.5	81.1	53.5	13
ANG	120.9	32	108.4	104.41	61.1	12
ANG	123	33	109.5	109.49	53	13
ANG	134.8	36.1	118.3	147.04	60.1	14
ANG	128.4	31.7	114.7	117.51	61	13
ANG	140.1	35.4	122.8	144.12	-	13
ANG	121.8	33.9	108.6	114.43	54.2	13
ANG	127	33.2	113.8	125.7	-	13
ANG	130.09	32.3	116.1	126.4	62.1	12
ANG	129.9	36.1	119.8	170.11	64.7	13
ANG	113.3	31	100.1	99.89	56.7	13
ANG	119.1	29.4	103.4	92.6	58.5	13
ANG	131.9	32	117.5	128.48	-	13
ANG	104	26.3	91.2	65.98	45.6	13
ANG	116.7	29.3	102.2	89.47	60.7	13
ANG	107.9	31.6	96.9	88.58	-	13
ANG	135.2	36.2	121.4	164.69	74.8	14
ANG	125	30.4	111.9	107.48	-	13
ANG	124.1	32.5	110	118.37	61.1	12
ANG	119.3	32	110.9	115.42	66.2	13
ANG	123	32.4	108.7	116.11	64.3	13
ANG	118.9	31.7	103.8	97.16	56.3	13
ANG	112	30.8	101.1	89.41	50	12
ANG	117.4	31.4	99.9	96.48	57.6	13
ANG	108.8	28.8	96.4	81.97	-	14
ANG	118.2	32.1	105.2	111.2	53.8	13
STO	135.5	34.7	120.4	141.2	66.1	13
STO	116.3	34	106.5	118	63	13
STO	99.3	26.8	90.4	73.3	48.8	15
STO	117.5	31	107.1	99.6	54.4	14
STO	125.5	31.4	111.7	126.7	61.3	13
STO	122.1	31.4	111.1	131.4	52.7	13
STO	105.5	31.1	95.7	96.2	45	13
STO	98.1	26.9	89.9	75.15	-	13
STO	113.5	30.3	105.6	110.3	58.8	12
STO	117.4	36	109.1	128.75	60.1	12
STO	121	30.5	107.7	118.1	56.2	13
STO	122.5	30.4	108.2	110.5	59.1	13
STO	123.9	30.7	113.7	130.65	61.2	13
STO	115	29.8	103.7	104.7	54.8	13
STO	113	31	100	116.4	51.6	13

STO	129.8	33	117.2	133.45	60.4	13
STO	118.5	32.1	108.3	107.9	59.7	13
STO	127.2	35.1	117	138.9	64.6	13
STO	132.9	34.2	123.3	147.85	61.7	13
STO	113.8	29.7	104.7	98	50.1	13
STO	113.2	30.2	106.7	99.5	53.3	12
STO	100	24.7	89.1	66.3	47.3	12
STO	101.5	26.7	93.2	78.6	48.5	13
STO	113.5	32.8	102.4	107.3	45.3	13
MUL	120.5	32.4	106.7	96.73	60.9	13
MUL	122.5	29.6	102.7	89.2	60.4	13
MUL	119.5	30.3	102.2	90.72	59.2	14
MUL	112.5	30.4	103.6	85.12	58.2	14
MUL	112.2	27.3	101.9	79.15	-	13
MUL	113	27.5	107.1	79.69	55.1	13
MUL	118	29.8	102.4	83.09	58.8	13
MUL	121.3	29.7	104	85.08	57.1	13
MUL	111.8	28.9	100.8	88.1	57.5	13
MUL	116.8	30.9	105.2	88.47	60.9	13
MUL	118.5	29.4	105.3	90.98	-	13
MUL	114.5	29.9	98.2	83.9	53.5	13
MUL	122.3	32.1	107.8	110.7	62.7	13
MUL	121.3	30.8	109.4	101.55	59.7	13
MUL	121.5	32.2	108.5	107.76	62.4	14
MUL	119.1	31	107.8	94.2	60	13
MUL	110.9	29.4	101.5	85.6	57	13
MUL	119.5	30.3	105.2	90.73	58.5	14
MUL	120	30.6	107.7	90.2	57.3	13
MUL	121.5	29.5	104.7	87.62	61.8	14
MUL	119.4	33.4	107.3	104.38	61.5	12
MUL	113.5	29.2	103.2	87.89	57.3	12
MUL	115.5	30	107	98.61	58.9	13
MUL	122.4	30.5	111.4	108.6	60.2	13
MUL	119.2	30.7	105.1	92.42	55.3	13
MUL	112.7	31.3	99.2	82.25	56.3	13
MUL	115.4	30.2	102.6	83.11	56	12
MUL	117.4	30.2	108	105	59.7	13
MUL	115.1	30.6	105.4	93.34	58.1	13
MUL	121.9	32.9	106.5	101.61	60	13
LYM	99.8	26.2	90.6	65.47	49.3	13
LYM	114	29.9	98.3	94.13	59	14
LYM	102.1	29	93.8	79.69	48.9	13
LYM	99	27.4	88.9	79.3	47	12
LYM	106.4	29.8	92.2	84.09	52	14
LYM	113.2	30.1	97	90.95	58.4	13
LYM	116.5	29.9	103.1	100.09	56.6	14
LYM	87.3	24.6	80.2	53.84	47.7	13
LYM	90	24.8	79.1	63.98	48.7	13
LYM	101.5	27.1	88.9	70.93	53.3	13
LYM	95.1	24.9	83.4	63.61	50.3	12
LYM	91.4	24	79.9	57.44	47.8	13
LYM	99.2	28.1	89.5	75.49	52.8	13
LYM	106.8	29.2	96	88.3	52.8	13
LYM	95	26.3	84.6	71.52	53.6	14
LYM	95.6	25.8	86	68.52	54.6	13
LYM	116.1	32.4	104.5	125.3	67	13
LYM	90.6	24.8	80.2	55.87	48.4	13
LYM	90.2	23.4	82.1	59.19	46.1	13
LYM	119.4	29.9	106.3	118.18	61.2	12

LYM	105.8	30.2	93	95.12	52.5	13
LYM	103.2	28.9	94.8	84	57.1	13
LYM	110	28.6	99.7	89.62	56	14
LYM	106.5	30.5	99	98.82	55.1	12
LYM	92.5	25	80.8	62.6	51.2	13
LYM	94.5	25.8	86	69.97	48	12
LYM	110	29.3	96.4	98.91	61.4	13
LYM	114.2	31.8	104.1	135.54	67	14
LYM	107.2	25.4	93.7	75.6	52.3	13
LYM	119.1	32.2	105.4	110.78	61	12
LYM	89	24.9	77.4	55.3	51.9	13
LYM	111	30.5	98.7	94.62	57.2	13
LYM	88.7	23.8	81.5	59.51	46.6	12
LYM	89.5	22.4	79.1	53.8	43.9	13
LYM	96.5	24.6	83.8	63.21	49.6	13
LYM	92.5	26.3	85.5	70.82	52.1	12
LYM	105	29.3	92.7	83.83	51.5	12
LYM	108.5	29.1	98.9	96.21	61	12
LYM	113.4	31.8	100.3	108.61	57.3	14
LYM	119.1	33.6	108.6	129.68	57.8	14
LYM	113.4	31.2	100.9	109.01	62.5	13
LYM	109	29.9	99.2	102.08	57.8	12
LYM	96.1	27.7	85.7	73.57	50.8	13
LYM	116.2	29	100	97.8	61.7	13
LYM	111.7	30.7	102.9	112.91	57.8	12
LYM	106.4	29.8	93.9	92.79	58	12
LYM	91.9	26.2	85.5	65.48	44.7	14
LYM	114.2	30.4	104.9	98.7	61.8	12
LYM	91.8	25.1	84.7	66.96	54.6	13
LYM	98.1	29.8	87.2	81.89	51.9	13
LYM	96.4	25.6	84.1	73.49	54.5	13
LYM	88.5	23	79.6	55.33	48	13
LYM	97.8	28.9	85.9	77.62	49.3	13
LYM	87.2	23.3	79.2	59.91	43.4	13
LYM	101.4	30.7	98.4	96.77	54	13
LYM	84.5	23.8	77	59.8	44.5	15
LYM	111.9	31.3	96.4	94.34	56.8	14
LYM	101.8	31.1	92.7	89.42	52.5	13
LYM	93	25.3	81.1	59.83	51.7	12
LYM	67.9	20.9	64.1	36.64	37.7	13
POL	102.1	25.2	85	53.04	50.3	13
POL	94.6	25.2	84.7	58.61	45.8	13
POL	90.4	26.3	85.4	67.44	46	13
POL	95.3	26.4	82.5	60.28	-	13
POL	93.9	25.4	83.9	61.37	53.9	13
POL	90.3	23.3	82.8	48.9	51	13
POL	89.9	24	81.3	53.4	43.3	13
POL	110.7	30.8	97.6	96	56.6	14
POL	95.1	22.8	82.3	52.41	49.2	14
POL	90.1	25.6	82.2	57.61	49.7	13
POL	111.1	29.4	101.4	99.26	-	13
POL	91.5	25.5	78.7	51.69	45.4	13
POL	92.8	24.4	84.1	61.47	48.8	13
POL	87.2	23.4	80	47.69	45.4	13
POL	88.4	23.3	77.7	48.09	45.8	12
POL	95.7	25.7	85	62.6	49.2	13
POL	91.7	26.5	85.5	65.55	47.1	12
POL	81.9	22.3	77.5	45.72	-	12
POL	90.8	23.6	81.1	53.7	47.5	14

POL	87.9	25.4	78.6	55.4	45.8	13
POL	101.9	28.9	92.4	80.37	51.9	13
POL	116.1	29.6	100.6	94.75	52	14
POL	89.4	25.7	81.6	56.99	44.4	13
POL	90.9	25.6	82.7	64.01	47.8	13
POL	89.4	24.2	81.7	51.46	44.5	16
POL	89.6	24.7	79.9	51.06	38.9	13
POL	90	26.7	81.5	53.09	43.3	12
POL	93.4	24.5	82.2	56.58	48.3	13
POL	95.3	26.8	84.7	61.8	48.4	14
POL	86	24.6	78.8	49.71	42.6	13
POL	89.7	25.6	80.7	56.48	43.9	13
POL	97.2	25.8	86	63.88	-	13
POL	90	25.3	79.5	53.49	43.2	14
POL	102.4	28.4	91.2	79.16	50.6	13
POL	88.6	24.1	80.3	52.2	44	13
POL	93.4	24.7	85.7	60.16	49.5	15
POL	97.2	26.2	85.2	63.69	51.7	13
POL	105.6	28.1	94.9	80.69	50.6	13
POL	94.7	24.9	84.3	57.76	47	13
POL	86.3	23.7	77.9	51.9	43.8	12
POL	91.4	25	83	54.15	-	13
POL	107	29.9	93.9	85.95	-	12
POL	104.5	27.2	91.5	71.49	50.4	15
POL	84.1	22.3	75.8	48.21	41.8	13
POL	91	24.9	83	58.38	48.6	13
POL	91.1	25.1	85.6	60.5	45.7	13
POL	95.5	25.5	85.2	64.93	53.7	13
POL	91.2	25.9	84	61.78	-	12
POL	87.2	24.5	79	53.11	44.4	12
POL	90.5	25.3	84.1	53.47	44.9	12
POL	91.6	27.2	79.5	62.78	50.4	13
POL	88	23.1	80.9	51.94	46	13
POL	89.8	24.2	81.3	51.65	45	13
POL	86.7	25.9	79.1	56.56	45	13
POL	93	26	82.2	56.7	48.3	13
POL	93.3	25.7	83.3	60.4	45.4	13
POL	86.2	23.6	78.2	54.45	46.7	12
POL	87.7	23.5	80	52.8	45.9	13
POL	98.2	27.3	87.8	66.74	49.7	13
POL	90.8	24.3	82	51	46.3	13
POL	93.8	26.8	80.2	61.8	46.5	12
JAC	59.6	18.2	54.4	16.2	33.5	14
JAC	62.6	18.1	54.1	17.18	33.8	16
JAC	59.1	17.8	53.1	15.1	33.1	13
JAC	104.1	25.1	92	67.9	56.7	13
JAC	67.1	19.4	58.9	22	38.3	15
JAC	55.9	16.3	49.7	12.1	30.8	13
JAC	52.5	15.7	48.1	11.4	27.4	13
JAC	58.2	18.6	51.4	13.8	31.8	13
JAC	52.6	16.5	48.2	10.15	30.9	13
JAC	65.7	16.9	59	18.9	34.1	14
JAC	61.9	20	55.2	16.7	34	15
JAC	59.8	18.4	51.1	15.5	33.2	14
JAC	55.8	16.6	50	11.32	34.5	14
JAC	56.9	17.5	51.2	16.48	31	13
JAC	56	17.8	49.1	13.9	31.3	15
JAC	52.2	16.4	46.7	10.8	31.3	13
JAC	56.4	17	49.2	14	33.7	14

JAC	52.2	16.1	46.3	10.3	28.6	13
JAC	66.6	21.5	60.4	22.78	37.5	13
JAC	53.5	16.8	48.1	12.2	27.7	14
JAC	54.3	16.6	48.9	11.32	31.1	14
JAC	102.3	29.1	88.2	60.57	55.5	15
MRY	101.1	27.4	93.5	66.18	51.9	13
MRY	103.6	26.3	93.4	66.19	52.9	13
MRY	101.4	27.4	88.4	64.15	50.7	13
MRY	105.1	28.7	91.2	63.31	54.6	13
MRY	103	27.9	90.3	57.87	50.2	14
MRY	99.9	30.1	89.7	69.54	53.8	14
MRY	113.7	29.9	99.7	81.11	55.5	13
MRY	109.2	28.8	93.6	67.98	52.6	13
MRY	107.3	26	95.3	66.4	53.2	13
MRY	103	28.7	90.6	65.17	53.3	13
MRY	106.1	25.4	95.1	69.58	54.2	13
MRY	105.8	29.3	94.2	82.83	54	13
MRY	107.6	26.7	92.3	63.9	55	13
MRY	102	28.6	91.5	71.51	49.4	13
MRY	105.3	28	93.3	67.43	55.6	13
MRY	101	26.1	91.5	63.22	53	12
MRY	116	28.5	100.4	77.47	52.4	13
MRY	109.6	27.6	94.8	71.35	57.8	13
MRY	109.2	28.1	96.4	71.31	52.8	13
MRY	105.3	27.9	94.7	70.82	55.8	13
MRY	99.9	28.8	88.6	68.82	50.1	13
MRY	108.5	28.4	93.3	76.09	57.3	13
MRY	106.1	27.3	92.2	65.55	53.6	13
MRY	94.6	25.9	87.2	53.5	52.7	13
MRY	108	27.5	94.5	69.86	54.2	13
MRY	106.4	27.5	92.3	71.89	56	12
MRY	114.8	28.2	101.6	82.8	59.2	13
MRY	106.4	28.7	91.6	73.82	52.2	14
MRY	113.2	29.3	99.8	76.85	55.9	13
MRY	108.3	28.1	96.4	74.01	58.7	12
KIL	126.3	33.6	111.8	130.14	64.7	13
KIL	127.1	32	114.6	119.99	58.9	13
KIL	152.3	36.9	129.8	148.01	68.7	13
KIL	127.4	34	113.8	125.56	65.6	12
KIL	144.8	39.2	126.5	196.48	79.1	13
KIL	134.5	37.6	120.9	140.83	60.7	12
KIL	138.7	37.3	121.2	131.58	64.9	13
KIL	149.5	40.3	133.3	178.8	75.3	13
KIL	146.9	37.7	128.5	153.78	73.3	13
KIL	128.8	32.9	113.7	118.41	60.2	13
KIL	141.5	34	126.4	140.6	66.8	12
KIL	144.6	39	123	179.9	69.3	13
KIL	141.9	36.8	123	158.83	73.8	13
KIL	138	36.6	123.8	144.75	65.4	14
KIL	151.2	37.5	134.2	154.73	70.3	13
KIL	144.5	37.1	126.5	144.18	67.4	14
KIL	148.8	36.9	131.9	148.14	71.9	13
KIL	146.4	35.2	125.5	158.3	72.8	13
KIL	124.9	33.2	108.3	114	63.6	14
KIL	136	35.9	122	156.58	72.5	13
KIL	154.3	38.2	135	178.18	72.1	13
KIL	148.5	37.1	136	169.38	69.6	13
KIL	146.5	35.3	131.8	145.5	72.6	13
KIL	120	29.8	104.5	95.53	61.9	14

KIL	133.5	34.5	119.3	120.62	60.3	13
KIL	128	33.2	115.4	127.21	69.1	12
KIL	139.4	35.1	119.5	134.59	70.9	13
KIL	134.3	34.3	120.9	119.78	65.3	14
KIL	127.5	34	113.6	132.07	61.2	13
KIL	142.5	37.2	127.8	155.41	69	15
STB	118.3	31	108.4	113.88	67.2	13
STB	113.4	31.5	103.8	121.09	66	12
STB	110.1	27.6	97	80.15	53.9	13
STB	122.7	29.4	109.1	104.78	61.4	14
STB	114.7	29.3	104.2	89.29	62.5	12
STB	112.8	31.2	100.9	110.02	57.5	12
STB	119.2	29.6	107.8	108	61.6	12
STB	110.8	27.8	100.1	89.76	58.3	12
STB	110.9	29.3	111.1	94.23	59.7	12
STB	113.3	30.1	112.4	99.31	57.3	11
STB	112.9	31.1	100.2	100.88	55.6	12
STB	105.6	26.9	93.3	82.9	52.3	13
STB	111	29.9	98.1	108.5	55.1	12
STB	113.5	28.7	101.1	95.99	59	12
STB	117.8	27.2	100.5	82.81	56	12
STB	104.6	27	94.5	74.08	51.5	12
STB	115.2	31.3	102.4	112.93	57.4	12
STB	102.5	25.3	93.5	73.93	54.2	13
STB	112.2	29.5	100.1	79.96	56	13
STB	109	28.4	98.6	-	56	13
STB	114	30.4	102.3	112.12	56.4	12
STB	116.7	28.5	101.5	94.38	63.1	13
STB	120.2	30.2	105.7	98.41	62	13
STB	112.3	28.5	104.1	92.93	58.8	12
STB	116.1	29.5	102.7	90.76	60.8	12
STB	105.1	28.7	94.3	80.78	52.8	12
STB	121.4	30.6	106.6	102.63	63.1	13
STB	115	29	101.3	103.1	59.8	14
STB	116.2	29.2	102.4	87.87	59.1	12
STB	119.4	28.6	103.5	107.33	59.1	12
STB	110	29.1	95.9	81.69	55	12
STB	118.1	30.2	107.7	111.53	60.7	13
STB	114.2	32.2	101	103.52	55.3	12
STB	101.6	26.7	91.7	78.82	52.8	13
STB	109.6	29.2	99	91.1	56.4	12
STB	108	27.5	96.9	79.9	53.1	12
STB	108.9	29.4	98	88.11	55.1	12
STB	110.9	27.8	98.8	87.99	55	12
STB	115.9	27.6	103.3	84.01	54.4	13
STB	115.6	27.8	100	90.03	55	12
STB	106.3	28.2	96.5	94	58.5	12
STB	111.6	29.6	100.8	94.82	56.5	12
STB	115.2	29.5	104.4	91.81	64.4	12
STB	113.8	28.3	99.7	87.8	61.1	14
STB	115.8	30.2	100	91.49	59.7	13
STB	104.2	25.3	94.1	71.51	51.9	12
STB	104.7	29.2	99.5	98.07	58.2	12
STB	108.7	29.6	96.9	84.43	55	13
STB	113.9	30.7	102.8	98.5	61.8	12
STB	109.9	30.6	97.2	95.39	56.1	12
LAT	108.4	-	97.6	102.9	59	12
LAT	132.3	35.6	112.2	149.71	70	13
LAT	111	29.8	97.3	103.4	62.5	12

LAT	129	34.7	114.3	138.68	83	13
LAT	127.8	34.1	112.8	141.62	76.5	13
LAT	133	36.6	116.9	154.88	73	13
LAT	131	35.8	112.2	120.28	71.2	13
LAT	118.2	34.4	105.2	114.2	69.8	13
LAT	123.5	33	113.5	138.42	67.5	12
LAT	122.8	32.7	107.5	127.38	66.5	12
LAT	143.2	39.1	126	193.62	82.5	12
LAT	122.4	37.1	111.7	133.74	70.7	11
LAT	139.5	37.8	123.9	175.58	77.3	13
LAT	139.2	39.6	122.5	192.6	80	11
LAT	126	35.4	109.2	135.2	76.7	13
LAT	119	31.9	105.4	130.36	69.5	12
LAT	113.8	29.2	101.3	108.22	56.7	13
LAT	126.6	36.7	115.4	166	71.3	12
LAT	134	36.8	118.8	157.46	74.3	12
LAT	80.2	21	70.4	43.62	48.1	14
LAT	85.5	23.5	79.4	50.8	54.4	13
LAT	119.7	33.5	107.8	124.94	71.3	13
LAT	118.7	34.4	109.8	131.71	70.5	12
LAT	124.8	34.7	112	131.72	71.2	12
LAT	137	36	120.7	154.43	71.4	13
LAT	128.5	34.2	111.7	137.9	70.6	12
LAT	118.7	31.5	110.8	111.7	64.7	12
LAT	133.6	35.8	115.4	138.8	72.1	13
LAT	146.4	39	131.8	191.69	81.6	13
BRE	118.3	31.3	103.8	86.8	64.4	13
BRE	100.6	27.5	89.5	71.62	58.3	13
BRE	103.8	28.7	93.3	81.71	55.8	13
BRE	99.4	27.2	88.7	77.5	56.9	13
BRE	113.1	31	99.8	95.36	65.3	12
BRE	117.6	30.7	101.5	93.45	62	13
BRE	104.8	29.1	95.1	75.01	58.7	13
BRE	109.6	30.5	95.2	83.35	56.4	13
BRE	107.6	28.9	96.1	86	55.1	13
BRE	118.6	31.2	103.3	99.45	68.7	12
BRE	103.1	26.8	91.5	72.1	60.2	13
BRE	103	26	90.5	74.02	58	13
BRE	105	27.2	90.5	65.39	57.3	13
BRE	108.1	27.4	95.4	85.28	56.7	12
BRE	117.4	30	103	93.62	65.7	13
BRE	108.7	27.3	95.3	88.05	61.4	12
BRE	87.5	24.9	77.9	51.13	48.2	13
BRE	108.2	28.5	95.4	83.21	59.7	12
BRE	104.7	29	90.9	77.17	61.3	13
BRE	118.5	31.1	103.9	109.51	68.7	13
BRE	107.5	30	95.6	91.01	63.9	12
BRE	118.6	34	105.3	113.92	62.2	13
BRE	111.2	30.6	94.8	89.69	60.3	13
BRE	102.4	28.9	91.9	76.9	57.5	12
BRE	102.3	27.7	91.1	69.09	54.5	13
BRE	102.9	29.9	91.2	81.98	64.2	12
BRE	105	27.9	93.7	77.29	58.4	13
BRE	110.1	29.5	96.5	82.78	63.3	14
BRE	105.5	28.2	94.8	79.6	58.5	13
BRE	107.5	28	93.6	68.7	61.8	12

Appendix C: Details of solutions used in this study.

ALLOZYME BUFFER RECIPES:

1). Tris-Maleic EDTA:

	g/l
Tris	12.1
Maleic acid	11.6
EDTA	3.72
$MgCl_2$	4.06
NaOH	5.6

Adjust pH to 7.4 with NaOH.
Use at full strength as electrode buffer, 1:10 (with dH_2O) as gel buffer.

2.) Tris-Citrate-EDTA pH7.0 (Saavedra *et al.*, 1993):

	g/l
Tris	16.35
Citric acid (free acid, monohydrate)	9.45
EDTA	0.45

Use at full strength as electrode buffer, 1:9 as gel buffer.

3.) N-(3-aminopropyl)-morpholine (Clayton and Tretiak, 1972):

	g/l
Citric acid (free acid, monohydrate)	8.41

Add N-(3-aminopropyl)-morpholine to pH7.4
Use full strength as running buffer. 1:20 in gel.

4.) TME/trisodium citrate.

0.1M Trisodium citrate (Citric acid Na_3 salt)
Reduce to pH7.4 with NaH_2PO_4.
Use for gel diluted 1:10. Electrode buffer 1.)TME pH7.4

5.) Tris-citrate (Ahmad *et al.*,1977):

Electrode	g/l
Tris	16.35
Citric acid	9.032

adjust to pH7.1
Gel: 1:15 dilution.

6.) Tris-borate-EDTA pH8.7:	g/l
Tris	109.0
Boric acid	31.0
EDTA	7.4

Use at 1:7 for electrode buffer, 1:10 for gel buffer.

7.)Tris-citrate pH6.3/6.7 (Huelvan, 1985):

Electrode:	g/l
Tris	27

Citric acid	16.8
Adjust to pH 6.3 with tris or citirc acid	
Gel:	
Tris	0.97
Citric acid	0.64
Adjust to pH6.7 with 1M tris or 1M citric acid	

8.) Phosphate buffer XIV (Shaw and Prasad, 1970):

Electrode:	g/l
K_2HP0_4	29.1
Citric acid	5.7
Gel:	
K_2HP0_4	1.06
Citric acid	0.254

9.) Tris-citrate pH8.0 (Huelvan, 1985):

Electrode	g/l
Tris	75.6
Citric acid	30
Adjust to pH8 with tris or citric acid	
Gel:	
1:29 dilution of electrode buffer.	

10.) Poulik.

Gel:	g/l
Tris	9.2
Citric acid	1.05
Electrode:	g/l
Boric acid	18.55
NaOH	2.4

ALLOZYME STAIN BUFFERS:

L-Malic acid

L-Malic acid	1g
0.1M Tris-HCl pH8.0	100ml
Adjust to pH8 for *Mdh* and pH7 for *Me* with NaOH.	

Tris-Maleate pH5.3

Tris	1.2g
Maleic acid	1.2g
Water	100ml
Adjust pH with NaOH.	

ALLOZYME STAIN RECIPES:

Acid Phosphotase (*Acp*; 3.1.3.2)

α-napthyl phosphate (Na salt)	50mg
Fast Blue BB	50mg

0.05M citrate buffer pH 4.5	50ml
Apply as fluid stain	

Adenylate Kinase (*Ak*; 2.7.4.3)

0.5M Tris-HCl pH8.0	4ml
ADP (Na_2 salt)	10mg
$MgCl_2$	5mg
Glucose	40mg
Water	5ml
NADP	5mg
Glucose-6-phosphate dehydrogenase (140u/ml)	25µl
Hexokinase (280u/ml)	25µl
MTT ($5mg.ml^{-1}$)	1ml
Meldola Blue (0.8%)	50µl
2% agar	10ml

Alcohol dehydrogenase (*Adh*; 1.1.1.1)

NAD	25mg
0.05M Tris-HCl pH8.6	20ml
Isopropanol	4.5ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Aldehyde oxydase (*Ao*; 1.2.3.1)

Note: double stain with *Sod*.	
EDTA	100mg
0.1M Tris-HCl pH8.4	15ml
Heat to dissolve.	
Riboflavin	5mg
Benzaldehyde	15µl
MTT ($5mg.ml^{-1}$)	1ml
PMS ($2mg.ml^{-1}$)	50µl
2% agar	25ml

Leave in light to darken then incubate in dark at 37 C. SOD appears as light bands and AO as dark bands.

Aldolase (*Ald*; 4.1.2.13)

NAD	20mg
Fructose-1-6-diphosphate ($Na_3.8H_2O$)	100mg
Sodium arsenate	60mg
0.1M Tris-HCl pH8.0	25ml
Glyceraldehyde-3-phosphate-dehydrogenase (800U/ml)	50µl
MTT ($5mg.ml^{-1}$)	1ml
Meldola Blue (0.8%)	50µl
2% agar	25ml

Alkaline phosphotase (*Akp*; 3.1.3.1)

β-Napthyl phosphate (Na salt)	25mg

$MgSO_4.7H_2O$	60mg
Fast blue BB	25mg
0.06M borate pH9.7	50ml

Arginine kinase (*Ark*; 2.7.3.3)

$MgCl_2$	5mg
ADP	10mg
NADP	5mg
Glucose	40mg
0.5M Tris-HCl pH8.0	10ml
Glucose-6-phosphate dehydrogenase	25µl
Hexokinase	25µl
Arginine phosphate	30mg
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	10ml

Aspartate aminotransferase (*Aat*; 2.6.1.1)

Fast blue BB	250mg
α-ketoglutaric acid	73mg
L-aspartic acid	266mg
Polyvinylpyrolidone	1g
EDTA	100mg
Na2HPO4	2.84g
Water	100ml

β-Galactosidase (-Gal; 3.2.1.23)

4-methylumbelliferyl- -galactoside	4.5mg
0.1M Tris-HCl	10ml

Dissolve, soak onto filter paper and lay on gel. Incubate at 37 C. View under short wave UV.

β-N-Acetylglucosamidase (*Hex*; 3.2.1.30)

0.1M Citrate buffer pH4.5	20ml
4-methylumbelliferyl-N-acetyl- -D-glucopyranoside	20mg

Apply on filter paper. View under short wave UV.

Catalase (*Cat*; 1.11.1.6)

Stage 1:

H_2O_2	500µl
water	100ml

Incubate 15min at room temperature

Stage 2:

Pour off solution. Rinse gel under tap water.

2% potassium ferricyanide, 2% ferric chloride	50ml

pour on. Catalase appears as light bands on dark background.

Carbonic anhydrase (*Ca*; 4.2.1.1)

Fluorescein diacetate	10mg
0.1M phosphate buffer pH6.5	100ml

Apply as fluid stain. Inspect under long wave UV.

D-Amino acid oxidase (*Damox*; 1.4.3.3)

DL-?(30mM)	
0.5M Tris-HCl pH8.0 (re-adjust pH)	50ml
FAD	8mg
Peroxidase (100U/mg)	5mg
3-amino-9-ethyl carbazole	25mg

NADH Diaphorase (*Dia*; 1.6.2.2)

NADH	10mg
2,6 Dichlorophenol indophenol	0.1mg
0.025M Tris-HCl pH8.0	20ml
MTT ($5mg.ml^{-1}$)	1ml
2% Agar	25ml

Esterase (*Est*; 3.1.1.1)

Stage 1:	
Tris-maleate pH5.3	30ml

Pour on. Leave for 10min at 37 C.

Stage 2:	
4-methylumbelliferyl acetate (Sigma M0883)	10mg
Acetone	5ml
0.1M Tris-HCl pH5.3	25ml

Dissolve substrate in acetone, add buffer. Soak onto filter paper and lay over gel. View under UV.

Formaldehyde dehydrogenase (*Fdh*; 1.2.1.1)

NAD	20mg
Reduced glutathione	40mg
Formaldehyde	0.3ml
0.2M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola Blue (0.8%)	50µl
2% agar	25ml

Fructose 1-6 Bisphophatase (*Fbp*; 3.1.3.11)

Fructose-1-6-diphosphate	50mg
NADP	5mg
0.1M Tris-HCl	25ml
Glucose-6-phosphate dehydrogenase	20µl
Glucose phosphate isomerase	20µl
MTT ($5mg.ml^{-1}$)	1ml
Meldola Blue (0.8%)	50µl
2% agar	25ml

Glycolate oxidase (*Gox*; 1.1.3.1)

Glycolic acid	50mg
0.5M Tris-HCl pH7.5	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Glucose phosphate isomerase (*Gpi*; 5.3.1.9)

$MgCl_2$	5mg
NADP	5mg
Fructose-6-phosphate	20mg
0.1M Tris-HCl pH8.0	25ml
Glucose-6-phosphate dehydrogenase (140U/ml)	10µl
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Glucose-6-phosphate dehydrogenase (*G6pdh*; 1.1.1.49)

Glucose-6-phosphate	10mg
$MgCl_2$	5mg
NADP	5mg
0.2M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola Blue (0.8%)	50µl
2% agar	25ml

Glutamate pyruvate transaminase (*Gpt*; 2.6.1.2)

DL-Alanine	500mg
2-Oxoglutaric acid	146mg
NADH	20mg
0.1M Tris-HCl pH7.5	20ml
Lactate dehydrogenase (2750U/ml)	100µl

Apply on filter paper. View under UV.

Glutathione reductase (*Gr*; 1.6.4.2)

Oxidised glutathione	40mg
NADPH	5mg
2,6 dichlorophenol indophenol	0.1mg
0.1M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
2% agar	25ml

Glutathione-S-transferase (*Gst*; 2.5.1.18)

Stage 1:

1-chloro-2,4-dinitrobenzene	20mg
ethanol	5ml

Dissolve substrate by vigorous stirring (15mins)

phosphate pH7.5	25ml
Reduced glutathione	30mg
2% agar	25ml

Incubate for 40-50mins at 37 C then remove agarose.	
Stage 2:	
0.1% KI	25ml
2% agar	25ml

α-Glycerophosphate dehydogenase (Gpd; 1.1.1.8)

NAD	20mg
Pyruvic acid (Na salt)	200mg
DL-α-glycerophosphate (Na_2 salt)	650mg
0.06M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Guanylate kinase (*Guk*; 2.7.4.8)

ATP	10mg
GMP	25mg
Phosphoenolpyruvate (K salt)	10mg
NADH	10mg
$MgCl_2$	5mg
0.5M KCl	2ml
0.5M $CaCl_2$	0.2ml
Pyruvate kinase (400U/ml)	25µl
Lactate dehydrogenase (2750U/ml)	50µl
Apply as filter paper overlay.	

Hexokinase (*Hk*; 2.7.1.1)

NADP	5mg
Glucose	9mg
ATP	40mg
Glucose-6-phosphate dehydrogenase	40µl
0.1M Tris-HCl pH7.5	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Inorganic pyrophosphatase (*Ipp*; 3.6.1.1)

Stage 1:	
0.05M Tris/0.01M sodium pyrophosphate pH7.8	25ml
$MgCl_2$	5mg
2% agar	25ml
Incubate 50min at 37 C	
Stage 2:	
Molybdic acid	50mg
water	25ml
3N sulphuric acid	0.5ml
L-ascorbic acid	3g
2% agar	25ml

Isocitrate dehydrogenase (*Icd*; 1.1.1.42)

Isocitric acid	20mg
$MgCl_2$	5mg
NADP	5mg
0.5M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Lactate dehydrogenase (*Ldh*; 1.1.1.27)

L-lactic acid	50mg
$MgCl_2$	5mg
NAD	10mg
0.05M Tris-HCl pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	20ml

Leucine aminopeptidase (*Lap*; 3.4.11.*)

Stage 1:

L-leucyl- -napthylamide	20mg
Distilled water	20ml

Apply as liquid stain. Observe under UV until bands appear. Wash off.

Stage 2:

Fast garnet	5mg
0.1M tris-HCl	20ml
2% agar	20ml

Malate dehydrogenase (*Mdh*; 1.1.1.37)

$MgCl_2$	5mg
NADP	10mg
L-Malic acid pH8.0	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Malic enzyme (*Me*; 1.1.1.40)

$MgCl_2$	5mg
NADP	5mg
L-malic acid pH7.0	25ml
MTT ($5mg.ml^{-1}$)	1mg
Meldola blue (0.8%)	50µl
2% agar	25ml

Mannose phosphate isomerse (*Mpi*; 5.3.1.8)

$MgCl_2$	5mg
NADP	10mg
Mannose-6-phosphate	20mg
Glucose phosphate isomerase	20µl

Glucose-6-phosphate dehydrogenase	25µl
0.2M Tris-HCl pH7.5	25ml
MTT (5mg.ml^{-1})	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Octopine dehydrogenase (*Odh*; 1.5.1.11)

$MgCl_2$	5mg
NAD	5mg
D+ Octopine	20mg
0.1M Tris-Hcl pH8.0	25ml
MTT (5mg.ml^{-1})	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Peptidase (*Pep*; 3.4.11.*)

o-Dianisidine	10mg
L-amino acid oxidase	10mg
Peroxidase	20mg
$MnCl_2$	20mg
L-Al-P or LGGP	20mg
0.1M phosphate buffer pH7.5	100ml

Phosphoglucomutase (*Pgm*; 2.7.5.1)

$MgCl_2$	5mg
NADP	5mg
Glucose-1-phosphate	50mg
Glucose-6-phosphate dehydrogenase	10µl
0.1M Tris-HCl pH8.0	25ml
MTT (5mg.ml^{-1})	1ml
Meldola Blue (0.8%)	50µl
2% agar	25ml

6-Phosphogluconate dehydrogenase (*Pgd*; 1.1.1.44)

6-Phosphogluconate	20mg
NADP	5mg
$MgCl_2$	5mg
MTT (5mg.ml^{-1})	1ml
Meldola Blue (0.8%)	50µl
0.5M Tris-HCl pH8.0	25ml
2% agar	25ml

Phosphofructokinase (*Pfk*; 2.7.1.11)

Fructose-6-phosphate (Na_2 salt)	12mg
ATP	12mg
NAD	7mg
$MgCl_2$	40mg
Sodium arsenate	200mg
0.1M Tris-HCl pH8.0	20ml

β-mercaptoethanol	20µl
Aldolase (90U/ml)	0.4ml
Triose phosphate isomerase (10,000U/ml)	50µl
Glyceraldehyde phosphate dehydrogenase (800U/ml)	50µl

Apply on filter paper. View under UV.

Phosphoglycerate kinase (*Pgk*; 2.7.2.3)

$MgCl_2$	40mg
NADH	10mg
ATP	30mg
3-phosphoglycerate (Na_3 salt)	15mg
Glyceraldehyde-3-phosphate dehydrogenase	40µl
Glycerol-3-phosphate dehydrogenase	40µl
Triose phosphate isomerase	10µl
0.5M Tris-HCl pH8.0	5ml

Apply on fliter paper. View under UV.

Pyruvate kinase (*Pk*; 2.7.1.40)

Phosphoenol pyruvate (K salt)	15mg
ADP	30mg
NADH	10mg
Fructose-1-6-diphosphate ($Na_3.8H_2O$)	15mg
$MgSO_4$ ($7H_2O$)	40mg
KCl	40mg
Lactate dehydrogenase (2750U/ml)	20µl
0.5M Tris-HCl pH7.4	8ml

Apply on filter paper. View under UV.

Sorbitol dehydrogenase (*Sdh*; 1.1.1.14)

Sorbitol	125mg
Sodium pyruvate	50mg
Pyrazole	50mg
$MgCl_2$	5mg
0.05M Tris-HCl	25ml
MTT ($5mg.ml^{-1}$)	1ml
Meldola blue (0.8%)	50µl
2% agar	25ml

Superoxide dismutase (*Sod*; 1.15.1.1)

See Aldehyde oxidase

Tyrosinase (Tyr)

DL-Dopa	55mg
0.1M phosphate buffer pH7.0	50ml

General protein

Amido black	50mg

In 1:4:5 acetic acid:water:methanol. Stain 15min. Wash off. Destain in mixture without amido black.

DNA ELECTROPHORESIS BUFFERS

TAE (50x):

Tris	242g
Glacial acetic acid	57.1ml
0.5M EDTA pH8.0	100ml

Make to 1l.

TBE (5x):

Tris	54g
Boric acid	27.5g
0.5M EDTA pH8	20ml

Make to 1l.

SOLUTIONS FOR DNA EXTRACTION

Homogenisation buffer:

Sucrose	171.15
KCl	11.18
0.2M EDTA pH7.5	10ml
0.5M Tris-HCl	50ml

1.0M Sucrose l^{-1}

Sucrose	342.3g
0.2M EDTA pH7.5	25ml
0.5M Tris-HCl pH7.5	20ml

1.5M Sucrose l^{-1}

Sucrose	513.45g
0.2M EDTA pH7.5	25ml
0.5M Tris-HCl pH7.5	20ml

STE:

NaCl	4g
0.5M Tris-HCl pH8.0	100ml
0.2M EDTA pH8.0	50ml

TE: l^{-1}

0.5M Tris-HCl pH8	20ml
0.1M EDTA pH8	50ml

CTAB:

NaCl	4.1g
CTAB	10g

Disolve in 100ml water

BACTERIOLOGICAL MEDIA

Luria agar:

Peptone	10g
Yeast extract	5g
NaCl	0.5g

Add approx 800ml distilled water. Adjust pH to 7. Make to 1l. Add:

Agar	15g

Autoclave

When cooled add 20ml 10% sterile (filtered) glucose.

LB Medium:

Peptone	10g
Yeast extract	5g
NaCl	10g

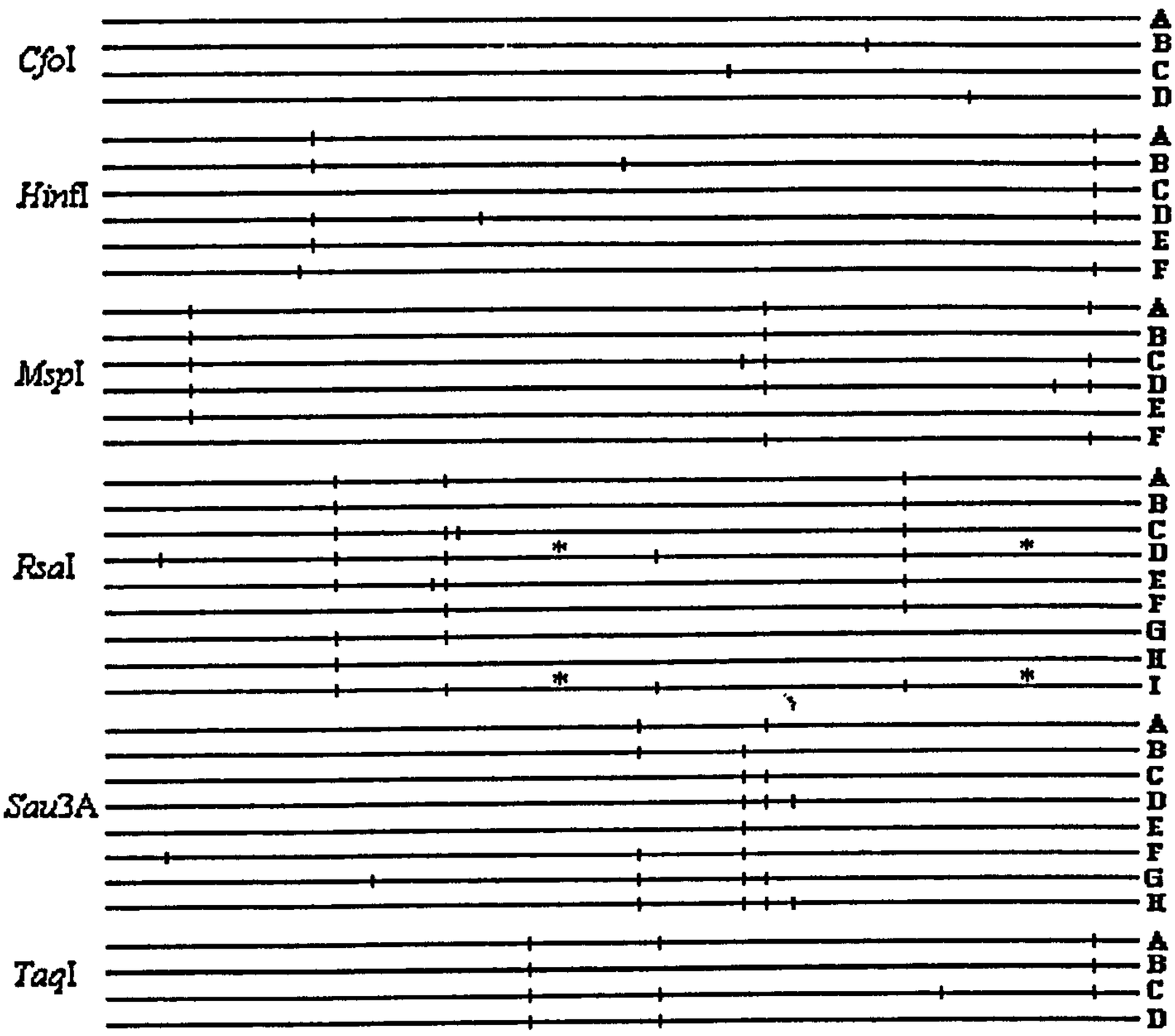
Make to 1l. Adjust pH to 7. Autoclave in universal tubes.

Appendix C. Caesium chloride ultracentrifugation.

Mitochondrial DNA was purified using the standard CsCl protocol for plasmid purification of the School of Biological Sciences, University of Wales, Bangor.

7.45g CsCl (Molecular Biology Products) was added to each of two 13.5ml polyallomer tubes (Kontron Instruments). After addition of 7ml TE to each tube, the CsCl was left to dissolve. Following dissolution, each of two mtDNA samples isolated from separate, individual animals (in 50µl, prepared as in Chapter 4) were added, one to each tube, with 200µl (5mg.ml^{-1}) ethidium bromide. Liquid paraffin was pipetted on top and used to balance the tubes to a final weight of 19.14g. Lids were placed on (ensuring the rubber ring was flush) and tightened with grub screws. The tubes were then centrifuged in an L8-80M Beckman ultracentrifuge overnight (17.5hrs) at 55,000r.p.m., 17°C (200,000g), then reduced to 35,000r.p.m. for 45mins then left to stop for 45mins. On completion of centrifugation, bands were viewed under UV light. The base of the tube was then pierced and liquid drained. Liquid containing the lower band (mtDNA) was collected. To remove the ethidium bromide from this mtDNA solution an equal volume of isopropanol, saturated with CsCl was added and the mixture inverted several times. The top layer (into which the ethidium bromide partitions) was removed and discarded. This was continued until the ethidium bromide stopped producing a purple colour in the top layer. Caesium chloride was removed from the DNA by dialysis. Dialysis tubing ($1^{8}/_{32}$'') was prepared by boiling for 20mins in distilled water. Liquid containing the purified DNA band was then pipetted into the tubing which was closed with dialysis tubing clips. This was dialysed against TE buffer (changed after 1h, 3h, overnight, +1h). Following dialysis, purified mtDNA was precipitated overnight at-70°C after addition of 0.1vol 3M sodium acetate and 3vol ethanol.

Appendix D. Inferred positions of restriction sites for 6 enzymes applied to the Pma1 amplified fragment of *P.maximus* mtDNA. Sites were not mapped and therefore relative positions may not be exact. Letters A-I refer to haplotypes used in construction of composite haplotype. Asterisks indicate fragments assumed to comigrate and therefore appear as a doublet.



Appendix E. Binary matrix of restriction site presence/absence (1/0) in 63 composite haplotypes generated from restriction digestion of Pma1 amplified mtDNA of *P. maximus*. Numbers 1-63 refer to order of haplotypes as in table 6.4. Sites as in appendix (left-right).

	*Cfo*I	*Hin*fI	*Msp*I	*Rsa*I	*Sau*3AI	*Taq*I
1	000	01001	10101	0101001	001010	1101
2	000	01001	10101	0101001	001010	1001
3	000	01001	10101	0101001	001100	1101
4	000	01001	10101	0101001	000110	1101
5	000	01001	10101	0101001	000110	1001
6	000	01001	10101	0101001	000111	1101
7	000	01001	10101	0101001	000100	1101
8	000	01001	10101	0101001	101100	1101
9	000	01001	10101	0101001	011110	1101
10	000	01001	10101	0101001	001100	1001
11	000	01001	10100	0101001	001010	1001
12	000	01001	10100	0101001	001010	1101
13	000	01001	10101	0100001	001010	1101
14	000	01001	10101	0100001	000110	1001
15	000	01001	10101	0101101	001111	1101
16	000	01001	10101	0101101	000110	1101
17	000	01001	10101	0101000	000100	1101
18	000	01001	10101	0101000	000110	1101
19	000	01001	10101	0101000	001010	1101
20	000	01001	10101	0101000	001100	1101
21	000	01001	10101	0001001	000110	1111
22	000	01001	10101	0101101	001010	1101
23	000	01001	10101	0101101	001100	1101
24	000	01001	10100	1101011	000110	1101
25	000	01001	10100	0101001	001100	1001
26	000	01001	10100	0001001	001010	1001
27	000	01001	10100	0101001	000110	1001
28	000	01001	10100	0101001	001010	1100
29	000	01001	10111	0101001	001010	1101
30	000	01001	10100	0100001	001010	1001
31	000	01001	11101	0101000	000110	1101
32	000	01001	10100	0101001	001100	1101
33	000	01001	10100	0101011	001010	1101
34	000	01001	10100	0111001	001010	1001
35	000	01001	10111	0100001	001010	1001
36	000	01001	10100	0101001	001100	1001
37	000	01001	10100	0101000	001010	1001
38	000	01001	10000	0101001	001010	1101
39	000	01001	00101	0101001	001010	1101
40	000	01001	00101	0101001	000110	1101
41	000	01011	10101	0101001	001010	1101
42	000	01011	10100	0101001	000110	1101
43	000	10001	10100	0101001	001010	1101
44	000	00001	10101	0101001	001010	1101
45	000	00001	10101	0100001	000100	1101
46	000	00001	10100	0101001	001010	1001
47	000	00001	10101	0101001	000110	1101
48	000	00001	10101	0101000	000110	1101

49	000	00001	10101	0101101	000110	1101
50	000	01101	10100	0101001	001010	1001
51	000	01000	10101	0101001	001010	1101
52	000	01000	10101	0100001	001010	1101
53	010	01001	10101	0101001	001010	1101
54	010	01001	10100	0101001	001010	1101
55	010	01001	10100	0101001	001010	1001
56	010	00001	10101	0101001	001010	1101
57	010	00001	10101	0101001	000110	1101
58	010	01101	10101	0101001	000110	1101
59	100	01001	10101	0101001	001010	1101
60	100	01001	10101	0111001	001010	1101
61	100	01001	10100	0101001	001010	1101
62	100	01001	10100	0101001	001010	1001
63	001	01001	10100	0101001	001010	1001

Appendix F. Binary matrix of restriction fragment presence/absence (1/0) in 63 composite haplotypes generated from restriction digestion of Pma1 amplified mtDNA of *P. maximus*. Numbers 1-63 refer to order of haplotypes as in table 6.4. Fragments as in Figure 6.3 (largest first).

	*Cfo*I	*Hin*fI	*Msp*I	*Rsa*I	*Sau*3AI	*Taq*I
1	1000000	0001000101	00100101	000100010110	0100100010	0011001
2	1000000	0001000101	00100101	000100010110	0100100010	1001000
3	1000000	0001000101	00100101	000100010110	0101000001	0011001
4	1000000	0001000101	00100101	000100010110	1000100000	0011001
5	1000000	0001000101	00100101	000100010110	1000100000	1001000
6	1000000	0001000101	00100101	000100010110	1000010000	0011001
7	1000000	0001000101	00100101	000100010110	1001000000	0011001
8	1000000	0001000101	00100101	000100010110	0011000000	0011001
9	1000000	0001000101	00100101	000100010110	0000101101	0011001
10	1000000	0001000101	00100101	000100010110	0101000001	1001000
11	1000000	0001000101	00101001	000100010110	0100100010	1001000
12	1000000	0001000101	00101001	000100010110	0100100010	0011001
13	1000000	0001000101	00100101	001000010100	0100100010	0011001
14	1000000	0001000101	00100101	001000010100	1000100000	1001000
15	1000000	0001000101	00100101	000010010110	0100010000	0011001
16	1000000	0001000101	00100101	000010010110	1000100000	0011001
17	1000000	0001000101	00100101	010000010010	1001000000	0011001
18	1000000	0001000101	00100101	010000010010	1000100000	0011001
19	1000000	0001000101	00100101	010000010010	0100100010	0011001
20	1000000	0001000101	00100101	010000010010	0101000001	0011001
21	1000000	0001000101	00100101	000101000100	1000100000	0001111
22	1000000	0001000101	00100101	000010010110	0100100010	0011001
23	1000000	0001000101	00100101	000010010110	0101000001	0011001
24	1000000	0001000101	00101001	000000101110	1000100000	0011001
25	1000000	0001000101	00101001	000100010110	0101000001	1001000
26	1000000	0001000101	00101001	000101000100	0100100010	1001000
27	1000000	0001000101	00101001	000100010110	1000100000	1001000
28	1000000	0001000101	00101001	000100010110	0100100010	0101001
29	1000000	0001000101	00100011	000100010110	0100100010	0011001
30	1000000	0001000101	00101001	001000010100	0100100010	1001000
31	1000000	0001000101	00010101	010000010010	1000100000	0011001
32	1000000	0001000101	00101001	000100010110	0101000001	0011001
33	1000000	0001000101	00101001	000000110110	0100100010	0011001
34	1000000	0001000101	00101001	000100010101	0100100010	1001000
35	1000000	0001000101	00100011	001000010100	0100100010	1001000
36	1000000	0001000101	00101001	000100010110	0101000001	1001000
37	1000000	0001000101	00101001	010000010010	0100100010	1001000
38	1000000	0001000101	10000001	000100010110	0100100010	0011001
39	1000000	0001000101	01000100	000100010110	0100100010	0011001
40	1000000	0001000101	01000100	000100010110	1000100000	0011001
41	1000000	0000011101	00100101	000100010110	0100100010	0011001
42	1000000	0000011101	00101001	000100010110	1000100000	0011001
43	1000000	0010000011	00101001	000100010110	0100100010	0011001
44	1000000	1000000001	00100101	000100010110	0100100010	0011001
45	1000000	1000000001	00100101	001000010100	1001000000	0011001
46	1000000	1000000001	00101001	000100010110	0100100010	1001000
47	1000000	1000000001	00100101	000100010110	1000100000	0011001
48	1000000	1000000001	00100101	010000010010	1000100000	0011001
49	1000000	1000000001	00100101	000010010110	1000100000	0011001
50	1000000	0000100111	00101001	000100010110	0100100010	1001000
51	1000000	0100000100	00100101	000100010110	0100100010	0011001
52	1000000	0100000100	00100101	001000010100	0100100010	0011001
53	0010010	0001000101	00100101	000100010110	0100100010	0011001
54	0010010	0001000101	00101001	000100010110	0100100010	0011001
55	0010010	0001000101	00101001	000100010110	0100100010	1001000
56	0010010	1000000001	00100101	000100010110	0100100010	0011001

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57 0010010 1000000001 00100101 000100010110 1000100000 0011001
58 0010010 0000100111 00100101 000100010110 1000100000 0011001
59 0001100 0001000101 00100101 000100010110 0100100010 0011001
60 0001100 0001000101 00100101 000100010101 0100100010 0011001
61 0001100 0001000101 00101001 000100010110 0100100010 0011001
62 0001100 0001000101 00101001 000100010110 0100100010 1001000
63 0100001 0001000101 00101001 000100010110 0100100010 1001000
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Appendix G. Binary matrix of restriction fragment presence/absence (1/0) in 36 composite haplotypes generated from restriction digestion of Pma2 amplified mtDNA of *P. maximus*. Numbers 1-36 refer to order of haplotypes as in table 6.6. Fragments as in Figure 6.4 (largest first).

	*Dra*I	*Hae*III	*Hin*fI	*Msp*I	*Rsa*I	*Sau*3AI
1	0011001011	0110100000010111	100101101011 00	0101000000001010	000110001110 11	11011000
2	0011001011	0110100000010111	100101101011 00	0101000000001010	000110011110 10	11011000
3	0011001011	0110100000010111	110100101000 00	0101000000001010	000110001110 11	11011000
4	0001101111	0110000011000010 1	100101101011 00	0101000000001010	100010001110 10	11011000
5	0011001011	0011100000010111	100101101011 00	0001010000011 10	000110001110 11	11011000
6	0001101111	0110100001000010 1	100101101011 00	0101000000001010	100010001110 10	11011000
7	0011001011	0110100000010111	100101101011 00	0000101000110 10	000110001110 11	11101000
8	0001101111	0010100101100010 1	100101101011 00	0101000000001010	100010001110 10	11011000
9	0011001011	0110100000010111	100101101011 00	0101000000001010	000110111010 00	11011000
10	0011001011	0110100000010111	100101101011 00	0100010000010 11	000110011110 10	11011000
11	0011001011	0110100000010111	100101101011 00	0001010000011 10	000110001110 11	11011000
12	1001001011	0110100000010111	100101101011 00	0101000000001010	000110011110 10	11011000
13	0011001011	0010100101100010 1	100101101011 00	0001000011010 10	000110001110 11	11011000
14	0011001011	0110100000010111	100000111111 10	0101000000001010	000110001110 11	11011000
15	0011001011	0110100000010111	110100101000 00	0100010000010 11	000110001110 11	11011000
16	0011001011	0110100000010111	100101101011 00	0001000100110 10	000110001110 11	11011000
17	0011001011	0110100000010111	100101101011 00	0101000000001010	000111000110 11	11011000
18	0011001011	0110100000010111	100101101011 00	0001010000011 10	000110011110 10	11011000
19	0000111100	0110100001000010 1	100101101011 00	0101000000001010	100010001110 10	11011000
20	0001101111	0110100001000010 1	110100101000 00	0101000000001010	010010001110 10	11011000
21	0011001011	0110100000010111	100000111111 10	0101000000001010	000110011110 10	11011000
22	0011001011	0010100000001111 1	100101101011 00	0101000000001010	000110001110 11	11011000
23	0011001011	0110100000010111	100101101011 00	1100000000001010	100010001110 10	11011000
24	0011001011	0110100000010111	100101101011 00	0011000000001010	000111000110 11	11011000
25	0001101111	0010100101100010 1	100101101011 00	0101000000001010	100010001110 10	11101000
26	0011001011	0110100000010111	100101101011 00	0101000000001010	000110011110 10	11010100
27	0011001011	0110100000010111	110100101000 00	0101000000001010	000110011110 10	11011000
28	0011001011	0110100000010111	100101101011 00	1000010000010 10	001000001110 10	11011000
29	0001101111	0110100001000010 1	100101101011 00	0101000000001010	000110001110 11	11011000
30	0011001011	0110100001000010 1	100101101011 00	0101000000001010	000110011110 10	11011000
31	0001101111	0100011000001011 1	110100101000 00	0101000000001010	000110001110 11	11011000
32	0011001011	0010110001011000 0	100100101111 10	0101000000001010	000110011110 10	11011000
33	0011001011	0100100001001111 1	100101101011 00	0101000000001010	000110011110 10	11011000
34	0001101111	1010100001000000 0	100101101011 00	0101000000001010	100010001110 10	11011000
35	0011001011	0100100001001111 1	100101101011 00	0101000000001010	000110001110 11	11011000
36	0001101111	0110100001000010 1	100101101011 00	0101000000001010	010010001110 10	11011000

Appendix H. *P. maximus* sequences in Genbank. l = sequence length. ID = identification of sequence. Ac = accession number.

12S rRNA.	l = 856bp	ID = PM12SRRNA	Ac = X67246.
Repeat region	l = 1592bp	ID = MTPMRPT	Ac = X71091.
$tRNA^{ala}$	l = 66bp	ID = PMMTALA	Ac = X74743.
$tRNA^{asp}$	l = 67bp	ID = PMMTASP	Ac = X74744.
$tRNA^{gly}$	l = 66bp	ID = PMMTGLY	Ac = X74745.
$tRNA^{ile}$	l = 70bp	ID = PMMTILE	Ac = X74746.
$tRNA^{lys}$	l = 69bp	ID = PMMTLYS	Ac = X74747.