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

Signed: _____ Candidate

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Date: 9/10/05

Population Differentiation in Blue Whiting (*Micromesistius poutassou)* in the North East Atlantic Ocean.

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Thesis submitted for MSc

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Abstract

Blue whiting is a commercially important gadoid fish found throughout the Atlantic Ocean and the Mediterranean. It is a pelagic, migratory species with feeding grounds in the Norwegian Sea and in the Bay of Biscay. The main spawning area is west of Ireland and takes place in early March and April. Currently, for management purposes, ICES considers the blue whiting population in the NE Atlantic as a single stock, although there is genetic evidence that this grouping may be divided into northern and southern populations.

The aim of this project was to elucidate the stock structure of blue whiting in Irish waters using allozyme and microsatellite molecular markers. Seven samples were collected during the spawning season in April 2003 and March 2004 from the Faroes/Shetlands southwards to the Celtic Sea, a latitude range of ~50°N to 60°N.

At the allozyme locus, *PGM* (Phosphoglucomutase), where four alleles were observed, significant differences were observed between two samples at the extremes of the range. The three microsatellite loci, *MpouBW7*, *MpouBW8* and *MpouBW13*, exhibited 16, 17 and 15 alleles, respectively. Results from the two most polymorphic loci (*MpouBW7* and *MpouBW8*), indicated that samples collected in the Celtic Sea in March 2004 were significantly different from all other samples, including each other. The third locus, *MpouBW13*, demonstrated significant heterogeneity between two samples at the extremes of the range. The results indicate that the significant genetic differentiation between Celtic Sea and other samples could either be due to temporal variation between year classes, or to genetic differences between northern and southern samples.

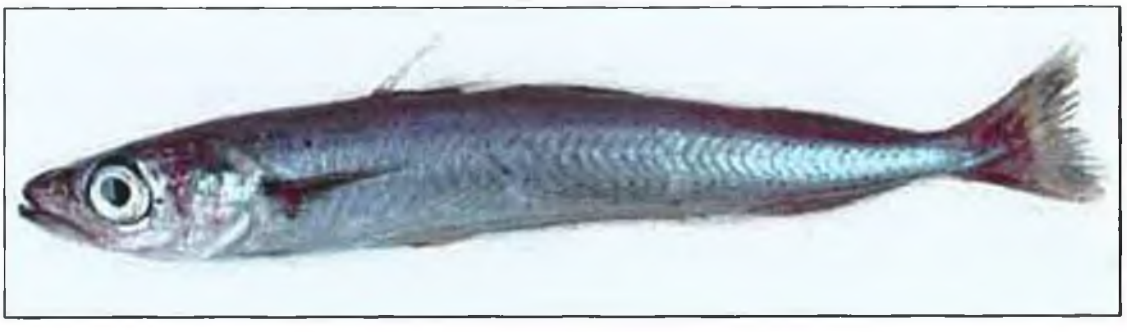
Direct comparison between allozymes and microsatellites showed that microsatellites have greater potential to detect population differentiation than less variable allozyme markers. F_{ST} estimates (a measure of population differentiation) for allozymes were low (0.004) in comparison to estimates for microsatellites (mean 0.019) on the same samples but neither F_{ST} estimate differed significantly from zero.

1. Introduction

1.1 The biology of blue whiting (*Micromesistius poutassou* Risso)

Blue whiting, *Micromesistius poutassou,* is a member of the family Gadidae. The gadoids also include cod, whiting and haddock. It is a relatively small fish with the largest being approximately 40cm in length. It is a narrow fish with a blue/grey dorsal side and a silvery underside. Blue whiting have large eyes, no barbel and a slightly protruding jaw (url 1) (Figure 1.1.1).

Figure 1.1.1 - Blue whiting (*Micromesistius poutassou)*



Blue whiting is a pelagic fish living in relatively deep water from 200-700 m. The eggs, which are found at depths of 200-400 m, hatch at temperatures of 2-14°C after approximately 4-6 days, although lower temperatures retard the process. Blue whiting larvae hatch at a length of 2-3 mm and grow at a rate of 3-5% per day. Juveniles stay in mid to deep water until they are recruited into the spawning population at approximately 2 years old (Bailey, 1982). Previously it was thought that they were recruited into the spawning population at approximately 3-4 years of age, but recently it has been found that the fish reach maturity at an earlier age of 2-3 years. This could be due to the increased fishing pressure on blue whiting populations (Anon, 2003).

Blue whiting feeds on pelagic plankton including euphausiids, *Calanus* and small fish (Bailey 1982). It, in turn, is prey for hake (Cabral and Murta 2002), mackerel (Cabral and Murta, 2002; Pinnegar et al., 2003), cod (Du Buit, 1995), megrim (Pinnegar et al., 2003), lesser spotted dogfish (Olaso et al., 1998) and other

blue whiting (Bailey, 1982). The species is host to many parasites, the most common of which is the nematode *Anisakis* (Bailey, 1982; Fernandez, 2005). This parasite is found in great abundance in the body cavity, gonads and muscle tissue of the fish.

Blue whiting has a large geographic distribution. It ranges from as far south as the Canary Islands and Morocco, northwards into the Bay of Biscay, Celtic Sea, around the Faroe and Shetland Islands, the coast of Norway, and as far north as the Barents Sea and Spizbergen. Blue whiting is also found in the Mediterranean Sea, around Iceland, Greenland and the coast of Canada (Figure 1.1.2)(Bailey, 1982, url 1). There is a closely related species, *Micromesistius australis*, in the southern hemisphere (Ryan et al., 2002).

Figure 1.1.2 - Distribution of blue whiting *Micromesistius poutassou* (in red)

Blue whiting adults undertake long migrations throughout the year from the spawning grounds to the feeding grounds and back again (Bailey,1982; url 2). At present it is unclear as to whether all the fish spawn in the same area at the same time, where exactly they come from, and if they return to the same feeding areas. The main spawning area is off the west coast of Ireland. Fish spawn here between March and April, although further south along the Porcupine Bank and into the Bay of Biscay spawning also occurs, only earlier, beginning around mid February (Kloppmann et al., 2001). There is evidence that spawning continues until May in more northern areas. After spawning, blue whiting migrate northwards towards Norway, Iceland and the Northern Sea (Bailey, 1982), or southwards towards the Bay of Biscay to feed (Carrera et al., 1996). This is not to say that all the fish found to the west of Ireland undertake this migration. There is a residual population that stays around this area and

further south. Suffice to say that the very large numbers of blue whiting found off the west coast of Ireland during the period March to late April diminishes dramatically after this period.

Blue whiting is a commercially important species for many countries, among them Ireland, Norway, Spain, Holland, Iceland, Russia, the UK, the Faroes and Germany. At present Norway, Russia, the Faroes and Iceland are the principal catching countries. In 2002 landings of blue whiting were over 1.5 million tonnes. In 2001 the Irish catch was 30 000 tonnes valued at approximately €2.2 million (Anon, 2003). Most of the Irish catch is landed at Killybegs, Co. Donegal, where it is processed as fishmeal. ICES (the International Council for the Exploration of the Sea) currently classifies the species as being harvested outside safe biological limits, (Anon, 2004a; url 2) and therefore more information on the population structure of the species is essential to assist in the management of the fishery.

1.2 Stocks

Fish species with a wide distribution range are often divided into subdivisions based on political, social, economic or scientific factors. These divisions are known as stocks. However, a stock based on political borders and one based on scientific data may not necessarily correspond. For this reason there are many definitions of the term "stock" when applied to fisheries management. The term stock is used to refer to a group of fish that are separated from the rest of the species based on scientific data; more specifically, a genetic stock is a group of fish that are reproductively isolated from other groups of the species.

Fish species have been divided into such groups on the basis of many biological factors, such as morphometrics (Polonsky, 1969; Zilanov, 1980; Isaev and Silverstov, 1991), meristics (Smith et al., 2002), physiology (Pawson et al., 1978; Karasev, 1989) and genetics (e.g. Mork et al., 1982; Mork and Giaever, 1993; Mork and Giaever, 1995; Mork and Giaever, 1999; Nesbo et al., 2000; also references below). While all of these methods for stock discrimination have their respective merits there are also problems associated with each of them. The main concern when using morphometrics, behaviour and other physical attributes as stock indicators, is that the environment in which the fish is located may influence these characters. For

example, if a group of fish spends most of its time in an area where it is an advantage to be a slightly darker colour than normal, this does not imply that the group is reproductively isolated from the rest of the species. This may also apply to parasite load. If a fish spends most of its time in an area of high parasite concentration it does not necessarily follow that it spawns in the same area and only with individuals from the same location. The fish may spend a lot of time in one area with high parasite concentration, but may move to a communal spawning ground and spawn with individuals from other locations. This is especially true of blue whiting which is a highly migratory species and as such will spend several months of the year feeding in one area before moving to another to spawn. Hence it is desirable that neutral genetic markers, i.e. those not influenced by the environment, be employed to determine if groups are in fact reproductively isolated from one another.

All of the methods for stock discrimination, mentioned above, have been used to investigate the population structure of blue whiting. The first study (Polonsky, 1969) involved the use of morphometrics to ascertain whether there were differences between northern and southern components of the species. Using vertebrae counts the author showed that the number of vertebrae decreased with decreasing latitude, i.e. that fish from southern areas had fewer vertebrae than those found further north. These changes were gradual with no defined stock boundaries, so it can only be said that the extreme north and extreme south of the species range showed differences. The prevalence of a nematode worm was used to differentiate between blue whiting stocks and a stock separation line north and south of 50°N was reported (Sahrhage and Schone, 1975).

Length at age and otolith structure has also been used to study the population structure of the blue whiting (Pawson et al., 1978). A difference in mean length at age and in otolith structure was found between samples collected north and south of 53°N, indicating two different stocks. Twenty-six morphometric characteristics were analysed by Zilanov (1980) and three stocks of blue whiting were found, located north of 72°N, northeast of the Faroe Islands and west of Ireland. Morphometrics were also used by Isaev and Seliverstov (1991) and results indicated four stocks of blue whiting: one in the Mediterranean, one in the Bay of Biscay, one in the West Atlantic and a fourth stock in Norwegian and Hebridean waters, known as the Norwegian-Hebrido stock. In 1984 the stock structure of blue whiting was investigated using morphometrics and eye lens proteins (Bussman, 1984). The author found three stocks,

the first of which was east of Greenland, the second around the Faroe Islands and the third comprised fish from both Spitzbergen and the west of Ireland. Parasite load was used by Karasev (1989) who showed that there were also three, albeit different, stocks, one in the Mediterranean, one in the Barents Sea and one in the rest of the northeast Atlantic.

All these studies showed that there was a difference between northern and southern areas of the blue whiting range. Therefore, for assessment purposes ICES divided blue whiting populations into two hypothetical stocks, northern and southern. The northern stock feeds in the Norwegian Sea and spawns west of Ireland, while the southern stock has its nursery grounds around the Bay of Biscay and is thought to spawn in this area and perhaps further north also. The Porcupine Bank is thought to be a transition zone, i.e. an area where fish from northern and southern "stocks" mix. However, there is also evidence from the above studies that the northern stock may be further subdivided, for example the Barents Sea (Karasev, 1989) and Spitzbergen (Andersen and Jakupsstovu, 1978; Bussman, 1984) aggregations show differences when compared to those in the west of Ireland and the Faroe Islands.

In 1993 the first genetic study was carried out on the population structure of blue whiting (Mork and Giaever, 1993). Using the allozyme loci *PGM* and isocitratedehydrogenase *IDHP* (see later) the authors were unable to show a genetic difference between putative northern and southern stocks, although fish from the Mediterranean and the Barents Sea were both genetically dissimilar from all other areas. These results were later corroborated by Giaever and Stien (1998) who also showed that fish in a Norwegian fjord, Romsdalsfjord, were genetically differentiated. ICES now classifies the blue whiting population in the northeast Atlantic as a single stock but there still remains the possibility that this is not the case and that this 'single' stock comprises genetically differentiated populations. While allozyme analysis did not reveal genetic differentiation of northern and southern groups, this does not necessarily mean that there is just one single panmictic population (see next section for discussion on allozymes).

In 1999 a computer model was used to simulate larval drift over the past twenty years (Skogen et al., 1999). The authors wanted to show that larvae from the hypothetical northern stock would drift northwards and those from the southern stock would drift southwards. Although there was inter-annual variation they found a possible separation line at approximately 54.5°N.

The most recent study to analyse population structure of blue whiting was a genetic study in which four microsatellites and one minisatellite marker (see below) were used to examine both the spatial and temporal structure of NE Atlantic blue whiting (Ryan et al., 2005). The results showed that there were differentiated populations in the Barents and the Mediterranean seas, and also that there was temporal variation between samples collected in 1992 and 1998 on the Hebridean Shelf and Porcupine Bank. However, no strong evidence was found for the more northerly Hebridean samples being genetically differentiated from the more southerly Porcupine Bank samples.

It is obvious from a review of the previous work that there seems to be some separation of blue whiting populations. Meristics, morphometrics and parasite load data support a subdivision of the species into a northern and southern stock, with genetic data supporting genetic differentiation of fish in the extremes of the species range, i.e. Barents Sea and Mediterranean. Genetic data, to date, has not supported further subdivision of the spawning aggregation to the west of Ireland.

1.3 Genetics in Fisheries

The use of genetics in fisheries is not a modern concept. In the 1930s blood group variation began to be used to distinguish between fish stocks. With the advent of protein electrophoresis in the 1960s came an explosion in the use of this technique for stock discrimination. The earliest work was carried out using a non-enzyme protein, haemoglobin, and was spearheaded by Sick who described haemoglobin variants in cod and whiting (Sick, 1961). This protein has recently been used to discriminate between populations of Arctic cod (Dahle and Joerstad, 1993).

Allozyme electrophoresis using enzymes was later developed by Brewer (1970). The technique is one that is very well documented, and has been used to analyse the population structure of many species, from fish (Ridgeway et al., 1970; Allendorf et al., 1977; Cross and Payne, 1978; Giaever et al., 1995; Mork and Giaever, 1995; Wilson et al., 1995; Bourke et al., 1997;; Forthun and Mork, 1997; Giaever and Mork, 1999; Mattiangeli et al., 2000; Igland and Naevdal, 2001) to mammals (Hisheh et al., 2003), including humans (Harris and Hopkinson, 1976). The technique is relatively simple and inexpensive to set up and run. However tissue must

be fresh or frozen as results are dependent on the activity of enzymes, which degrade over time.

Allozymes are different molecular forms of an enzyme, which are products of alternative alleles at a single locus. Allozyme analysis is an indirect method of testing for genetic variation as it measures the variation at the level of gene product. One of the earliest studies to use the method to discriminate between fish stocks was that of Lush (1969) who identified two separate stocks of herring using the Phosphoglucomutase (*PGM*) locus. To date, allozyme analysis has been used to determine the population structure of many fish species including commercially important species such as cod (Cross and Payne, 1976, 1978; Mork et al., 1982; Fevolden and Pogson, 1997; Mork and Giaever, 1999), whiting (Child, 1988; Forthun and Mork, 1997) and herring (Ridgway et al., 1970). In the case of blue whiting there are two loci, Phosphoglucomutase (*PGM*) and Isocitrate dehydrogenase (*IDHP*) that show sufficient polymorphism in this species to be useful markers in analysing population structure (Mork and Giaever, 1993).

Newer techniques using molecular markers, such as mitochondrial DNA, minisatellites and microsatellites, are now being used to examine population structure, and have proved very useful in discriminating between fish stocks (Dahle, 1991, 1994; Pepin and Carr, 1993; Galvin et al., 1995; Rico et al., 1997; O'Connell et al., 1998; Saitoh, 1998; Lage et al., 2001; Buonaccorsi et al., 2001; Mattiangeli et al., 2002; Saitoh, 1998; Ryan et al., 2002; Carlsson et al., 2004; Wilson et al., 2004; Ryan et al., 2005). Microsatellites are particularly useful in differentiating between conspecific populations, and have shown differences where other methods, such as allozymes, have previously indicated a single population (Nesbo et al., 2000; Buonaccorsi et al., 2001; De Innocentiis et al., 2001; Dhuyvetter et al., 2004).

Microsatellites are randomly repeated sequences of 1-6 base-pairs varying in length. They are found in all prokaryotic and eukaryotic cells and are present in high numbers in both coding and non-coding regions of the genome. Microsatellites mutate at rates between 5×10^{-3} and 5×10^{-5} (Estoup and Angers, 1998), which is reflected in their higher polymorphism and their greater potential to detect population differentiation than less variable markers. A prime example of this is Atlantic cod, *Gadus morhua.* Before the advent of microsatellites the only genetic difference that was apparent was between populations separated by great geographical distance, e.g., the east and west Atlantic (however see Cross & Payne 1978 for exception).

However, more recently it has been found, using microsatellites, that there is fine-scale genetic sub-structuring of the species in small geographical areas, e.g., within a single bay (Bentzen et al., 1996; Ruzzante et al., 1997, 1998, 2000; Hutchinson et al., 2001).

The aim of this study was to use one allozyme and three microsatellite marker loci to investigate the population genetic structure of blue whiting in an area of the NE Atlantic between 50°N and 60°N. Since largely the same samples were used for allozyme and microsatellite analysis, a direct comparison could be made on estimates of differentiation.

2. Methods

2.1. Sample Collection

Sampling Sites

Samples of blue whiting were collected from five locations in the North-East Atlantic Ocean while on board the Norwegian research vessel R.V. *Johan Hjort* (Figure 2.1.1) in April 2003 during the Norewgian blue whiting Acoustic Survey. Samples were also collected during the Irish Groundfish Survey on board the R.V. *Celtic Voyager* (Figure 2.1.2) in March 2004 and in the same month on board the R.V. *Celtic Explorer* (Figure 2.1.3) during the Irish blue whiting Acoustic Survey. Figure 2.1.4 shows the locations of samples collected during these three surveys. Haul#10 (H10) was collected on board the R.V. *Celtic Explorer* in March 2004, Haul#56 (H56) was collected on board the R.V. *Celtic Voyager* in March 2004 and the other five samples were collected onboard the R.V. *Johan Hjort* in March 2003.

Sampling procedure

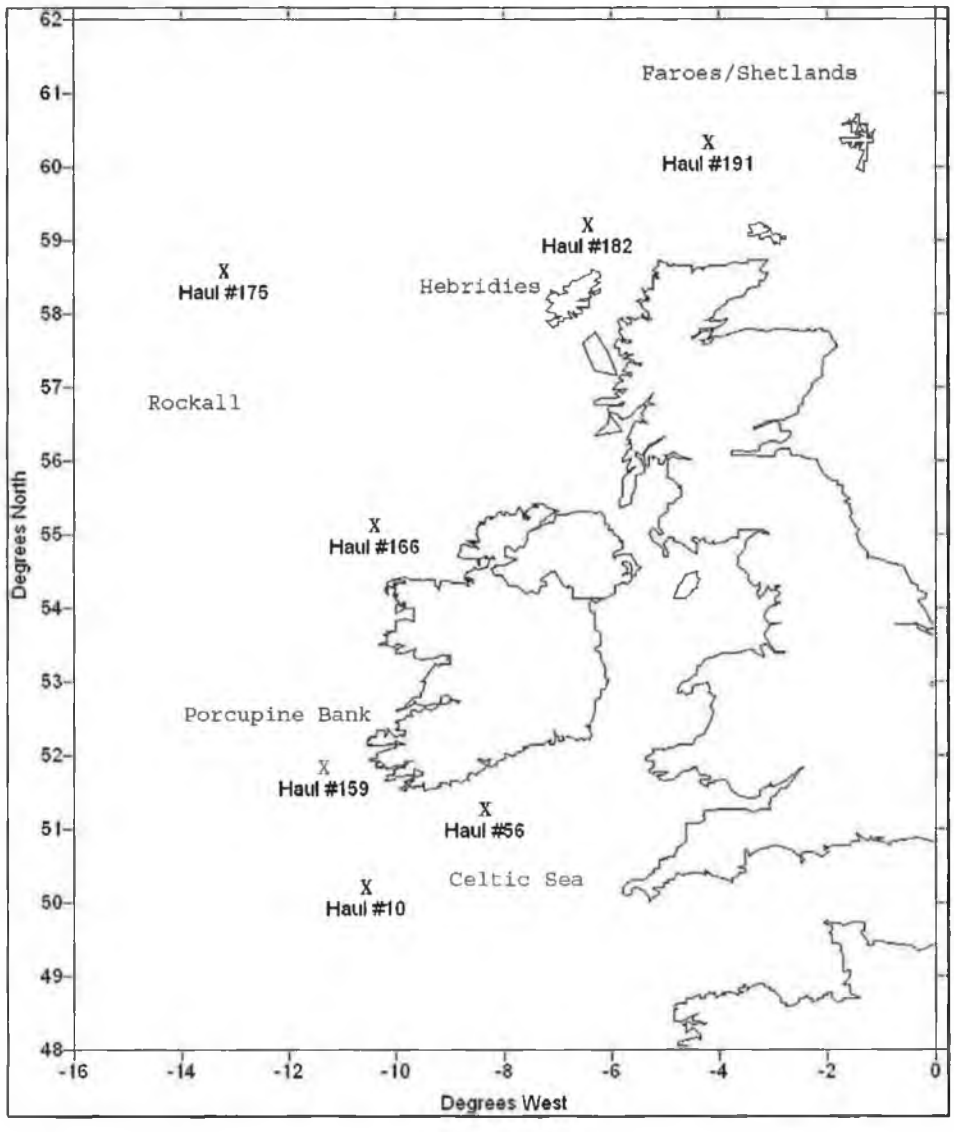
Each sample collected consisted of fifty fish, randomly selected from a single haul. On board the *Johann Hjort* in March 2003 the age and maturity of each fish in the sample were recorded, by Norwegian scientists, for Haul #159 (H159), Haul#166 (H166), Haul#175 (H175), Haul#182 (H182) and Haul #191 (H191). This procedure was also carried out on board the *Celtic Explorer* (H10) by Gavin Power, a postgraduate student in GMIT. The age and maturity were not recorded on board the *Celtic Voyager,* therefore these additional data were not available for H56. White muscle and liver tissue samples, for electrophoretic analysis, were dissected within two hours of the fish being caught. Approximately 8 cm^3 of white muscle tissue was removed from the dorsal side of each fish. If the liver was small the whole organ was removed. If it was large, a section approximately 4 cm^3 was taken. Tissue samples were placed individually in small tubes (Sarsted Germany), labelled and frozen at -20°C on board. The samples were later transferred to a -80°C ultra freezer upon return to the laboratory.

Figure 2.1 1 - R.V. *Johann Hjort*

Figure 2.1 2 - R.V. *Celtic Voyager*

Figure 2.1 3 - R.V. *Celtic Explorer*

Figure 2.1 4 - Locations of samples of *M. poutassou* collected on three surveys in spring 2003 and spring 2004 with names of areas included. All samples were used for allozyme analysis. All samples with the exception of Haul#159 were used for microsatellite analysis.



2.2 Allozyme Analysis

Methodology

A small sample of white muscle tissue (~ 2 cm^3), which had previously been dissected while on board research vessels, was homogenised at ~ 5°C in an equal volume of distilled water using a glass rod. Macerated samples were then spun in a refrigerated centrifuge (EBA 12 R Hettich, Germany) at 0°C for 10 min at 10 000 rpm.

Ten enzymes were initially screened for polymorphism using standard starch gel electrophoretic procedures (Aebersold et al., 1987). Only one, Phosphoglucomutase (*PGM-1** EC 5.4.2.2) was polymorphic.

Defrosted, white muscle tissue was homogenized in distilled water. Samples were run on 11% starch gels (Starch Art Corporation, Texas, USA) using the Tris-Citrate pH 8.5 buffer of Cross and Payne (1976) (CP Gel Buffer: 16.35g Tris, 3.75g Citric Acid in 1l distilled water diluted 1:14) in a 10 x 20 cm gel mould (Biorad, Dublin). A thirty-toothed comb was used to make wells in the gel. A volume of 7.5 µg of homogenised tissue supernatant was loaded into each well using an "Eppendorf" micropipette (Eppendorf AG, Hamburg, Germany) and Bromophenol Blue (3',3',5',5'-tetrabromophenolsulfonphthalein) tracking dye was loaded into the first and last well. A reference sample of known genotype was included on each gel. Electrophoresis was carried out using undiluted CP buffer in the electrode tanks. Gels were run at a constant voltage of 250V using a Consort E833 power supply unit (Medical Supply Company Ltd, Mulhuddart, Dublin). When the dye had reached the anodal end of the gel, usually after 3 h, electrophoresis was terminated. The gel was sliced in two using a thin piece of wire (produced in-house) and the bottom half was placed in a shallow tray for staining. The protocol used for staining *PGM* was that of Mork and Giaever (1995) (Table 2.2.1). The stain was poured over the gel, which was then placed in an incubator at 40°C for approximately 30 min. Stained gels were photographed using a digital camera and preserved frozen at -80°C. An example of a typical gel is shown in figure 2.1.5 below.

Figure 2.1.5 – Typical example of gel stained for PGM locus. Heterozygotes appear as two bands and homozygotes as a single band.

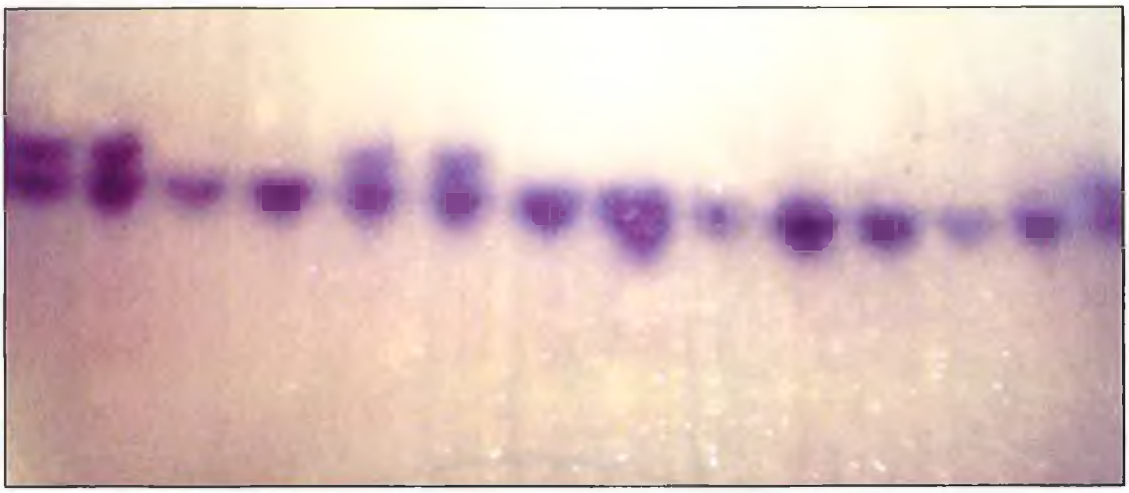


Table 2.2. 1- PGM staining protocol (Mork & Giaever 1995).

Reagent	Amount	Supplier
Glucose-1-phosphate (G-1-P)	600 mg	Sigma Co., Dublin
Glucose-6-phosphate dehydrogenase (G6PDH)	50 Units	Sigma Co., Dublin
β-Nicotinamide adenine dinucleotide phosphate (NADP)	10 mg	Sigma Co., Dublin
Magnesium chloride ($MgCl_2$)	20 mg	Merck KGaA, Germany
Phenazine methosulphate (PMS)	10 mg	Sigma Co., Dublin
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)	10 mg	Sigma Co., Dublin
Tris-HCl Staining Buffer pH 9.0	100 ml	Mork et al. (1995)

Gel Reading and Data Analysis

Alleles were designated according to mobility and direction from the most common (100) allele (Allendorf, 1977). The genotypes were read from the gels and inserted into a spreadsheet. Allelic distribution, expected (H_E) and observed (H_O) heterozygosity estimates for each population were obtained using the GENETIX v. 4.01 computer program (Belkhir et al., 1996). Conformance to Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP v 3.1

program (Raymond and Rousset, 1995), with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. Exact tests of allelic and genotypic distributions between pairs of populations were conducted using GENEPOP. Also, the level of genetic differentiation among populations was quantified using F_{ST} (Weir and Cockerham, 1984). The significance of F_{ST} (different from 0)was determined using permutation tests as implemented in the FSTAT program (Goudet, 2001). Significance of multiple pairwise comparisons was determined using the sequential Bonferroni correction (Rice, 1989).

2.3. Microsatellite Analysis

Methodology

The procedure for microsatellite analysis consists of four main steps:

1. DNA extraction
2. Polymerase Chain Reaction (PCR)
3. Acrylamide Gel preparation
4. Running of Gel

DNA extraction

Two methods were used for DNA extraction. The longer phenol-chloroform method was dropped in favour of the quicker "HotSHOTS" method. However, both procedures are described below.

Phenol/Chloroform Method (Sambrook et al., 1989).

A small sample (approximately 5 g) of muscle tissue was defrosted and blotted on laboratory paper. The tissue was placed in a 1.5 ml Eppendorf microcentrifuge tube

(Eppendorf AG, Hamburg, Germany) with 300 µl hexadecyl-trimethyl-ammonium bromide (CTAB) extraction buffer (containing 100 mM Trizma base, 1.4 M NaCl, 20 mM EDTA and 2% CTAB). Proteinase K (25 µl of 20 mg/ml; Sigma Co.) was added to the tube and shaken briefly. The solution was incubated at 56°C for 1 h to allow cell lysis and release of nucleic acids. The mixture was allowed to cool to room temperature, Ribonuclease-A (25 µl of 20 mg/ml; Sigma Co.) was then added and the tube was shaken briefly. The mixture was incubated at 37°C for 1 h to digest RNA. Phenol (350 µl at pH 8.0; Sigma Co.) was added to digest enzymes and proteins. The solution was shaken for 1 h (Gio Gyrotary Shaker, New Brunswick Scientific Co. New Jersey, USA). Chloroform/isoamyl alcohol (24:1) (350 µl; Sigma Co.) was added to neutralize the phenol and the mixture was upended for 1 h. The solution was centrifuged at 8,000 rpm for 15 min (Centrifuge 5415D, Eppendorf AG). The supernatant was removed to a clean, sterile, labelled Eppendorf microcentrifuge tube using a 1000 µl pipette (Eppendorf AG.). Two volumes of ice-cold ethanol were added and the mixture was placed in a -80°C ultrafreezer for 1 h to precipitate the DNA. The alcohol was removed and discarded using a 1000 µl pipette and 1 ml of 70% ethanol was added. The mixture was upended for 1 h to wash the DNA pellet. The alcohol was removed as before and the DNA pellet was left to air-dry for 10-15 min. The DNA pellet was re-suspended in 50 -100 µl TE solution (10 mM Trizma base, 1 mM EDTA), depending on the size of the pellet.

"Hot SHOTS" Method (Truett et al., 2000).

A small piece of muscle tissue (approximately 5 g) was defrosted and dried on laboratory paper. The tissue was added to a labelled Eppendorf microcentrifuge tube (Eppendorf AG) containing NaOH (150 µl, 12.25 mM Merck KGaA, Germany) at pH 13.0. The tube was shaken and incubated at 99°C for 1 h to allow cell lysis and the breakdown of protein and RNA. The tube was removed from the incubator and 150 µl Tris / HCl buffer pH 5.0 (containing 3.025g Tris and 0.616g $MgSO_4$ in 500 ml distilled H_2O) was added. The DNA was stored at -20°C until required.

DNA Concentration

Five µl of DNA solution was added to 995 µl distilled water (1:200 dilution). The mixture was shaken well and the DNA concentration was read using a spectrophotometer (Spectronic Genesys 2 PC). The following equation was used to estimate DNA concentration:

50 × dilution × ABS value = DNA concentration (µg/ml)

Polymerase Chain Reaction (PCR)

The three microsatellite loci (*MpouBW7*, *MpouBW8* and *MpouBW13*) used in this study were developed by Moran et al. (1999). Primers were labelled with IR700 or IR800 dye, which is detectable by the lasers in the DNA analyser and were purchased from MWG Biotech Ltd UK. The sequences are given in Table 2.3.1.

Table 2.3. 1 - Primer sequences (F: Forward primer; R: Reverse primer)

Primer	Sequence
MpouBW7 F	5' GTG GAC ATG AAC ATG GAC CCA AAC 3'
MpouBW7 R	5' TAG CCG TGT AAT CGT GAT AAC AAC 3'
MpouBW8 F	5' TCG TGC TGT TTT GTG TGT CAT CA 3'
MpouBW8 R	5' ACC CGA TAT ATC ACG TGC CTA AC 3'
MpouBW13 F	5' GTT CCC TCA TCG TAA CCC GGT GAG 3'
MpouBW13 R	5' AAT GCA AGC AAC TGG AAT AAT GAG 3'

DNA (1 µl) was added to the PCR mixture (Table 2.3.2) (9.9 µl) in sterile 0.2 ml PCR tubes (Sarstedt AG) and amplified in a Thermocycler (Biometra GmBH, Germany). Table 2.3.2 below shows concentrations of PCR chemicals required for one individual, and for forty-five individuals for all three loci. The solutions for PCR were made up x45 to reduce sampling error when measuring small volumes.

Table 2.3. 2 - Concentrations and suppliers of chemicals used in PCR

Chemical	× 1 (µl)	× 45 (µl)	Supplier
Distilled sterile water	5.85	263.25	
10× Buffer	1	45	Sigma
$MgCl_2$ 2.0mM	0.8	36	Sigma
dNTPs 2.0mM	2	90	Sigma
Forward labelled primer 1.5µM	0.1	4.5	MWG Biotech
Reverse labelled primer 1.5µM	0.1	4.5	MWG Biotech
Taq polymerase (1 µl = 1 unit)	0.05	2.25	Sigma

The thermocycling protocol consisted of an initial denaturation step at 94°C for 3 min, followed by 7 cycles of: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 5 cycles of: 89°C for 30 s, 55°C for 30 s and 72°C for 30s; and a final extension of 5 min at 72°C. The PCR product was diluted 1:10 with distilled water and then 1:2 with "Stop Solution" (containing formamide solution (0.1 g; Sigma Co.), xylene cyanol FF (0.1 g; Sigma Co.), bromophenol blue (0.1 g; Sigma Co.) and EDTA (4 ml of 400 mM EDTA pH 8.0; BDH Laboratories Supplies, Dublin).

Acrylamide gel preparation

This procedure follows the Li-Cor Biosciences instructions for use of the model 4300 DNA Analyser (Anon). The following amounts relate to a 25cm x 20cm x 0.25mm gel.

Gel apparatus glass plates were carefully washed with distilled water and detergent (Li-Cor Biosciences, UK) and then dried with lint-free tissue (Sigma Co.), washed with 70% ethanol, dried again, given a final wash with distilled water, and left to air-dry. Binding sylene (containing 3(methacryloyloxy)propyl]trimethoxysilane (100 µl; Sigma Co.) and 10% acetic acid (100 µl; Sigma Co.)) was applied to corresponding areas at the top of both plates and left to air dry. Spacers (0.25mm thick) were placed at either side of the glass plates and clamps were applied to hold the plates together.

Ten percent ammonium persulphate (150 µl; Sigma Co.) solution and TEMED (15µl; Biorad) was added to the acrylamide gel matrix (20 ml 6.5% KB^{Plus} Gel Matrix; Li-Cor Biosciences) which had been warmed to room temperature. The mixture was syringed (60 ml syringe; Li-Cor Biosciences) into the space between the glass plates from the top and bubbles that may have formed were removed using a thin piece of hooked wire. A 48-toothed comb (Li-Cor Biosciences) was placed into the top of the gel and the entire gel sandwich was left for approximately 1 h to set.

Running of acrylamide gel

This procedure follows the Li-Cor Biosciences instructions for use of the DNA Analyser (Anon).

The comb was carefully removed from the top of the gel and the top buffer tank was attached. The outside of the glass plates were cleaned with 70% ethanol and dried with lint free tissue. The gel was placed in the DNA analyser (Li-Cor NEN Model 4300 DNA Analyzer; Li-Cor Biosciences, UK) and 1X Tris Borate/EDTA (TBE) buffer (containing 107.8 g Tris (89 mM), 55g Boric Acid (89 mM), 7.4g EDTA (2 mM) and 950 ml ddH_2O) was added to the upper and lower buffer tanks. One µl of diluted DNA in Stop Buffer was loaded into the gel using an 8-channel syringe (Hamilton Bonaduz AG., Switzerland). One µl IR700 or IR800 labelled Molecular Weight Standard (MWS) 50-700 base-pairs (Li-Cor Biosciences) was loaded into the first and last wells of the gel, which was then run for 3 h. Gels can be reloaded and used for three gel-runs, but the third result is often blurred and of poor quality, therefore gels were only re-loaded once.

Gel Reading and Data Analysis

The Li-Cor DNA Analyzer scans the gels in real-time during the gel run. Two lasers in the machine scan the bottom of the gel during the run and pick up infra-red signals from the labelled primers and the labelled Molecular Weight Standard. This creates an image of the gel that can be viewed in real-time while the gel is being run. After the gel run is complete the software ($SAGA^{GT}$) automatically detects lanes, calibrates weights based on the MWS sizes, previously programmed into the software, and

scores alleles. The gel image can be enhanced to visualise bands that are very faint but are still detectable by the IR laser sensors. Allele sizes can also be manually edited, as can lane locations and calibration lines allowing the gels to be manually read.

After the gel was read, and all alleles accurately scored, the genotypes were entered into a Microsoft Excel™, database before being transferred into GENEPOP (Raymond and Rousset, 1995). The genotypes were read from the gels and inserted into a spreadsheet. Allelic distribution, expected (H_E) and observed (H_O) heterozygosity estimates for each population were obtained using the GENETIX v. 4.01 computer program (Belkhir et al., 1996). Conformance to Hardy-Weinberg equilibrium (HWE) was assessed with exact tests implemented in GENEPOP v 3.1 program, with specified Markov chain parameters of 5000 dememorization steps, followed by 1000 batches of 5000 iterations per batch. Excesses of homozygotes or heterozygotes were assessed using F_{IS} values. Exact tests of allelic and genotypic distributions between pairs of populations were conducted using GENEPOP. Also, the level of genetic differentiation among populations was quantified using F_{ST} (Weir and Cockerham, 1984), the significance of which was determined using permutation tests as implemented in the FSTAT program (Goudet, 2001). Significance of multiple pairwise comparisons was determined using the sequential Bonferroni method (Rice, 1989).

3. Results

3.1 Sample Composition

Each of the five samples, made up of approximately fifty fish each, collected on board the R.V. *Johan Hjort* during the 2003 Norwegian blue whiting Acoustic Survey (i.e. H159, H166, H175, H182 and H191) were analysed for age and maturity. Fish from H10 (collected on board the *Celtic Explorer* during the Irish blue whiting Acoustic Survey) were also analysed for age and maturity (Table 3.1.1). H56 was not measured in this way, so maturity and age data were not available for this sample.

Table 3.1. 1 - Sample composition for seven samples of *M. poutassou*. I, Immature; M, Mature

Area	Sample	Mean Age	Maturity	% mature
Porcupine Bank	H159	2	M + I	66%
Hebrides	H166	3	M	100%
	H182	2	M + I	57%
Rockall	H175	3	M + I	82%
Faroe/Shetlands	H191	1	I	0%
Celtic Sea	H56	?	?	?
	H10	2	M + I	98%

As is evident from the table above, not all samples were made up of mature, spawning individuals. H159, H175, H182, H191 and H10 all had immature fish present. H191 contained only immature fish, indicating a possible nursery ground (Anon, 2004; Bailey, 1982; G. Power, pers. comm., GMIT, Galway). All other samples were made up of at least 57% mature fish.

3.2. Allozyme Analysis

Genetic diversity and Hardy-Weinberg Equilibrium (HWE)

An average of 46 individuals per sample was scored using allozyme electrophoresis. Sample sizes ranged from 42 in H166 to 51 in H10. Four alleles were detected at the *PGM* locus. The most common allele was designated *100* and the other three were measured in relation to this allele and given corresponding numbers, i.e. *080*, *112* and *124*. Allele frequencies are shown in Table 3.2.1 and Fig. 3.2.1. Allele *124* was observed only in one sample, H166, at a low frequency of 0.012.

Observed, H_O and expected, H_E, heterozygosities for each sample along with F_{IS} values are given in Table 3.2.2. Four of the seven samples (H159, H182, H191 and H10) had lower observed heterozygosity than expected, although none of the deviations were significant after sequential Bonferroni corrections.

Table 3.2. 1 - PGM allele frequencies in samples of *M. poutassou* from the NE Atlantic Ocean. N=sample size

	H159	H166	H175	H182	H191	H56	H10
N	49	42	49	50	43	42	51
Allele							
080	0.010	0.024	0.000	0.000	0.000	0.061	0.029
100	0.867	0.787	0.867	0.860	0.802	0.893	0.804
112	0.122	0.180	0.133	0.140	0.199	0.049	0.168
124	0.000	0.012	0.000	0.000	0.000	0.000	0.000

Figure 3.2. 1 - Pie charts showing *PGM* allele frequencies in samples of *M. poutassou* from the NE Atlantic

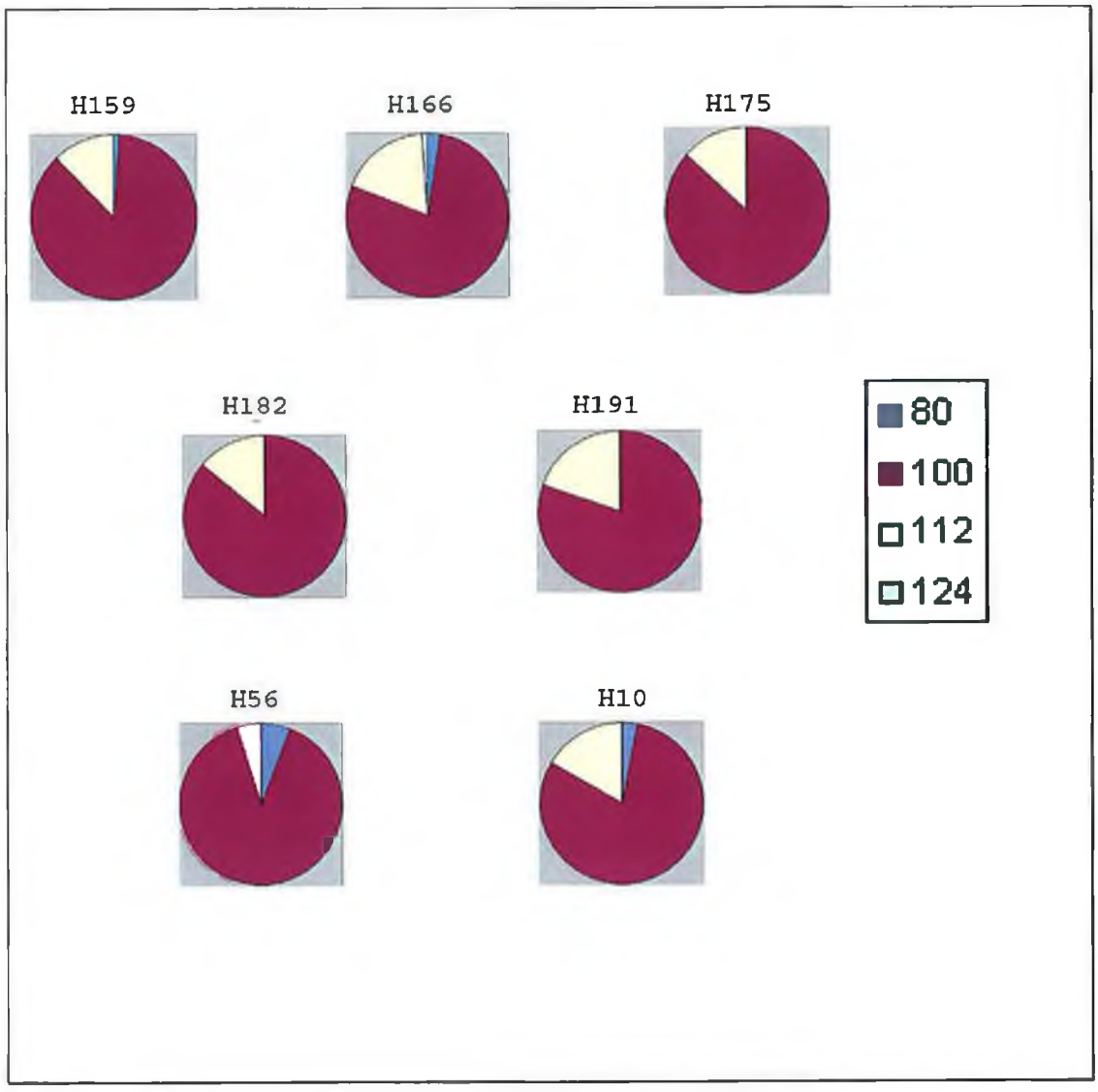


Table 3.2. 2 - Expected (H_E) and Observed (H_O) heterozygosities and F_{IS} values in samples of *M. poutassou* in the NE Atlantic. Positive F_{IS} values indicate a deficiency of heterozygotes and negative F_{IS} values an excess. None of the F_{IS} values were significantly different from zero after sequential Bonferroni correction.

	H159	H166	H175	H182	H191	H56	H10
H_E	0.233	0.350	0.230	0.241	0.317	0.197	0.325
H_O	0.184	0.357	0.265	0.160	0.256	0.214	0.275
F_{IS}	0.220	-0.008	-0.143	0.344	0.205	-0.076	0.165

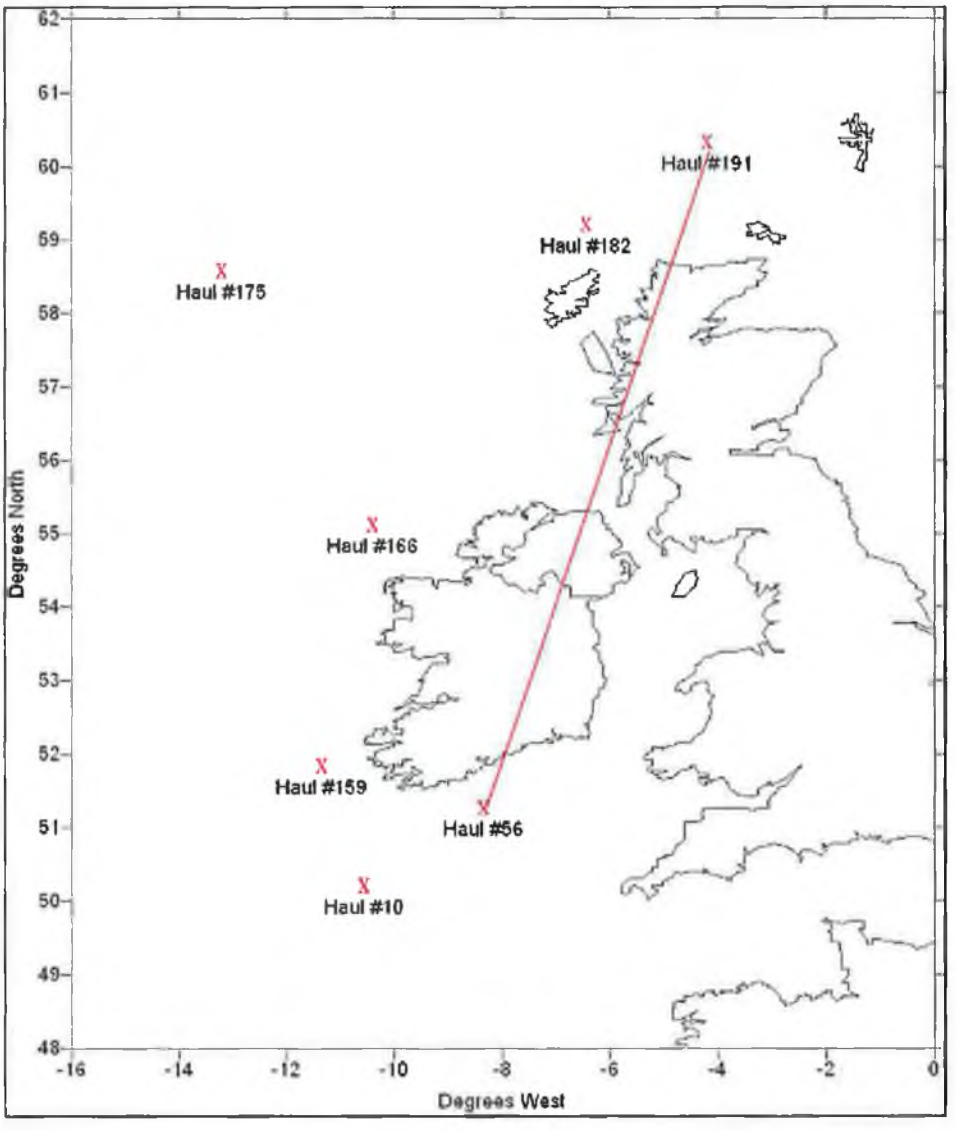
Population differentiation

In pairwise comparisons, using Fisher's exact test of genotypic and allelic differentiation, H56 and H191 was the only pairwise comparison that was significantly different in both allelic and genotypic proportions after sequential Bonferroni correction (Table 3.2.3 and Figure 3.2.2).

Table 3.2. 3 - Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal). Values in bold indicate samples significantly different from zero after sequential Bonferroni correction.

Sample	H159	H166	H175	H182	H191	H56	H10
H159	--	0.435	1.000	0.853	0.276	0.056	0.411
H166	0.419	--	0.130	0.191	0.414	0.019	0.851
H175	1.000	0.156	--	1.000	0.327	0.003	0.193
H182	0.835	0.195	1.000	--	0.387	0.005	0.206
H191	0.226	0.473	0.321	0.329	--	**<0.001**	0.271
H56	0.044	0.014	0.007	0.004	**<0.001**	--	0.032
H10	0.390	0.847	0.234	0.222	0.325	0.025	--

Figure 3.2. 2 - *M. poutassou* samples that are significantly different from each other in both allelic and genotypic proportions at the *PGM* locus are joined by a red line.



Pairwise F_{ST} estimates indicated an overall lack of significant differentiation except for the pairwise comparison H56 and H191 (Table 3.2.4), which agree with the results of Fisher's exact tests (Table 3.2.3).

Table 3.2. 4 - P values for pairwise F_{ST} tests (above line) and F_{ST} values (below line). Values in bold indicate samples significantly different from zero after sequential Bonferroni correction.

Sample	H159	H166	H175	H182	H191	H56	H10
H159	-	0.434	1.000	0.856	0.273	0.057	0.411
H166	0.0054	-	0.127	0.193	0.411	0.018	0.852
H175	-0.0103	0.0061	-	1.000	0.330	0.003	0.192
H182	-0.0121	0.0005	-0.0111	-	0.392	0.005	0.208
H191	0.0049	-0.011	0.004	-0.0019	-	**0.001**	0.268
H56	0.0077	0.041	0.0163	0.0163	**0.05**	-	0.032
H10	-0.0007	-0.0109	0.0003	-0.0041	-0.01	0.0298	-

3.3 Microsatellite Analysis.

Between 36 and 48 (mean 43) individuals were analysed at the three microsatellite loci, *MpouBW7, MpouBW8* and *MpouBW13*. All three loci were polymorphic in all samples. H159 was not analysed for microsatellites. The number of alleles per locus was 16, 17 and 15 for *MpouBW7, MpouBW8* and *MpouBW13,* respectively, and the frequency of any one allele was no higher than 0.36 for any locus (Table 3.3.1). The high polymorphism recorded was reflected in high mean H_E values of 0.83-0.86 across all loci. However mean H_O values were lower than expected for all loci. Allele size range was 231-261 for *MpouBW7,* 263-297 for *MpouBW8,* and 162-192 for *MpouBW13*.

Table 3.3. 1 – Allele frequencies, H_E, H_O and F_{IS} values for three microsatellite loci in six samples of *M. poutassou* in the NE Atlantic. (-, zero values; *, alleles not observed at that particular locus; values in bold, private alleles; values in red, significant deviation from HWE after sequential Bonferroni correction).

Locus	Sample Name	Sample Size	H_E	H_O	F_{IS}	Number of Alleles	Allele Frequencies																
							1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MpouBW7	H166	46	0.82	0.89	0.07	12	-	0.01	0.01	0.03	0.09	0.29	0.22	0.05	0.04	0.16	0.04	0.02	-	-	-	**0.02**	*
	H175	40	0.82	0.70	0.15	10	-	0.01	0.03	0.01	0.19	0.28	0.18	0.01	0.14	0.15	0.01	-	-	-	-	-	*
	H182	39	0.87	0.74	0.16	12	**0.01**	0.01	0.04	0.04	0.14	0.23	0.14	0.09	0.09	0.06	0.08	0.06	-	-	-	-	*
	H191	40	0.85	0.65	**0.25**	11	-	0.01	-	0.06	0.19	0.26	0.10	0.05	0.13	0.10	0.06	0.03	0.01	-	-	-	*
	H56	44	0.82	0.64	0.24	10	-	-	0.01	0.01	-	0.10	0.28	0.23	0.11	0.13	0.06	-	0.06	**0.01**	-	-	*
	H10	42	0.81	0.67	0.19	10	-	-	0.04	0.08	0.35	0.11	0.14	0.05	0.14	0.06	0.02	-	-	-	**0.01**	-	*
Means		42	0.83	0.72	0.18	10.83																	
MpouBW8	H166	46	0.86	0.74	0.15	12	0.01	0.12	0.25	0.07	0.01	-	0.09	0.11	0.13	0.12	0.07	0.02	-	0.01	-	-	-
	H175	45	0.89	0.80	0.11	14	0.04	0.12	0.19	0.02	-	0.02	0.13	0.1	0.11	0.09	0.1	0.01	0.03	0.01	0.01	-	-
	H182	42	0.84	0.83	0.03	11	-	0.16	0.11	0.08	-	0.01	0.12	0.04	0.29	0.11	0.06	-	0.01	0.02	-	-	-
	H191	36	0.84	0.72	**0.15**	12	0.01	0.15	0.29	0.03	-	0.01	0.08	0.15	0.14	0.04	0.03	-	0.03	0.03	-	-	-
	H56	45	0.84	0.82	0.03	11	-	0.2	0.26	-	-	0.02	0.09	0.14	0.16	0.03	0.07	0.01	0.01	-	-	-	**0.01**
	H10	39	0.88	0.69	0.23	14	0.01	0.03	0.14	0.18	0.13	-	-	0.03	0.09	0.17	0.08	0.06	0.03	0.01	0.04	**0.01**	-
means		42	0.86	0.77	0.12	12.33																	
MpouBW13	H166	44	0.82	0.82	0.04	10	-	0.09	0.06	0.05	0.03	0.15	0.31	0.21	0.05	0.05	0.02	-	-	-	-	*	*
	H175	45	0.83	0.91	0.06	10	-	0.11	0.06	0.09	0.03	0.12	0.33	0.10	0.06	0.06	0.04	-	-	-	-	*	*
	H182	47	0.82	0.79	0.08	13	-	0.10	0.06	0.05	0.04	0.11	0.36	0.13	0.02	0.06	0.03	0.01	-	0.01	0.01	*	*
	H191	41	0.79	0.80	0.13	13	-	0.06	0.05	0.04	0.05	0.27	0.35	0.02	0.07	0.01	0.02	0.02	0.01	0.01	-	*	*
	H56	48	0.84	0.90	0.06	13	-	0.05	0.01	0.15	0.03	0.08	0.32	0.09	0.08	0.10	0.03	0.01	-	0.01	0.02	*	*
	H10	43	0.89	0.81	0.17	13	**0.04**	0.06	0.06	0.08	0.05	0.17	0.19	0.14	0.07	0.06	0.06	0.02	0.01	-	-	*	*
means		45	0.83	0.84	0.09	12.00																	

MpouBW7

Genetic diversity and HWE

Sixteen alleles were observed at this locus, with allele 251 at the highest frequency in Hauls H166, H175, H182 and H191 and alleles 239 and 243 highest in H10 and H56, respectively (Table 3.3.1). Private alleles, i.e. alleles that are only found in a single sample, were observed in four of the six samples: allele 261 in H166, allele 231 in H182, allele 257 in H56 and allele 259 in H10 (Table 3.3.1).
A significant departure from HWE was observed in a single sample, H191 (Table 3.3.1) and was due to a deficit of heterozygotes.

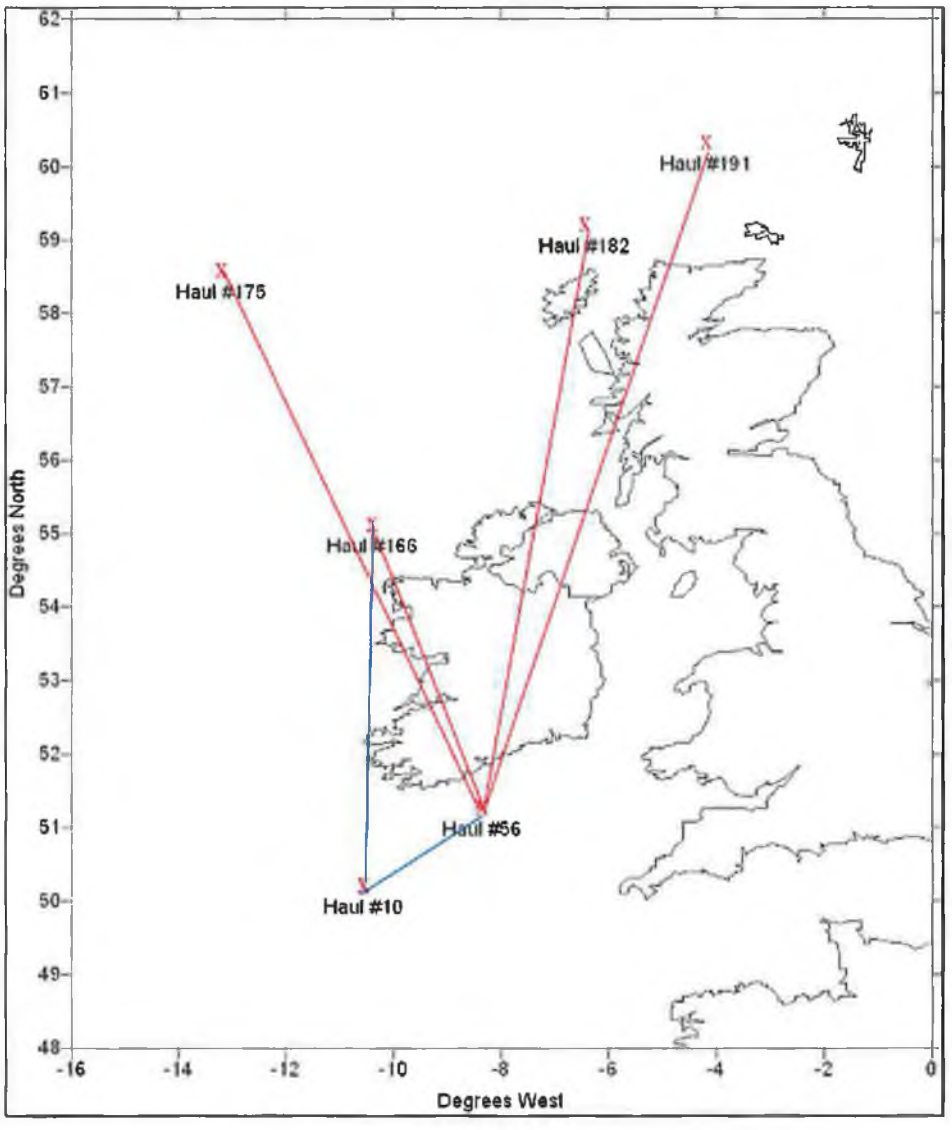
Population differentiation

In pairwise comparisons H56 was significantly different in both allelic and genotypic proportions from all other samples, as was H10 from H166, after sequential Bonferroni correction. This is illustrated in Table 3.3.2 and Figure 3.3.1 below.

Table 3.3. 2 - Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal) for locus *MpouBW7*. Values in bold indicate samples significantly different after sequential Bonferroni correction.

Sample	H166	H175	H182	H191	H56	H10
H166	-	0.108	0.184	0.116	**<0.001**	**<0.001**
H175	0.108	-	0.025	0.207	**<0.001**	0.007
H182	0.184	0.025	-	0.659	**<0.001**	0.009
H191	0.116	0.207	0.659	-	**<0.001**	0.038
H56	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**
H10	**<0.001**	0.007	0.009	0.038	**<0.001**	-

Figure 3.3. 1 - *M. poutassou* samples that were significantly different in allelic and genotypic proportions at the *MpouBW7* locus are joined by a red line involving H56 and a blue line involving H10



MpouBW8

Genetic diversity and HWE

Seventeen alleles were observed at this locus, with allele 267 at the highest frequency in H166, H175, H191 and H56, and alleles 279 and 269 highest in H182 and H10, respectively (Table 3.3.1). Private alleles were observed in two of the six samples; allele 295 in H10 and allele 297 in H56, although both of these were at low frequency.

Significant departure from HWE was observed in H191 due to a deficit of heterozygotes.

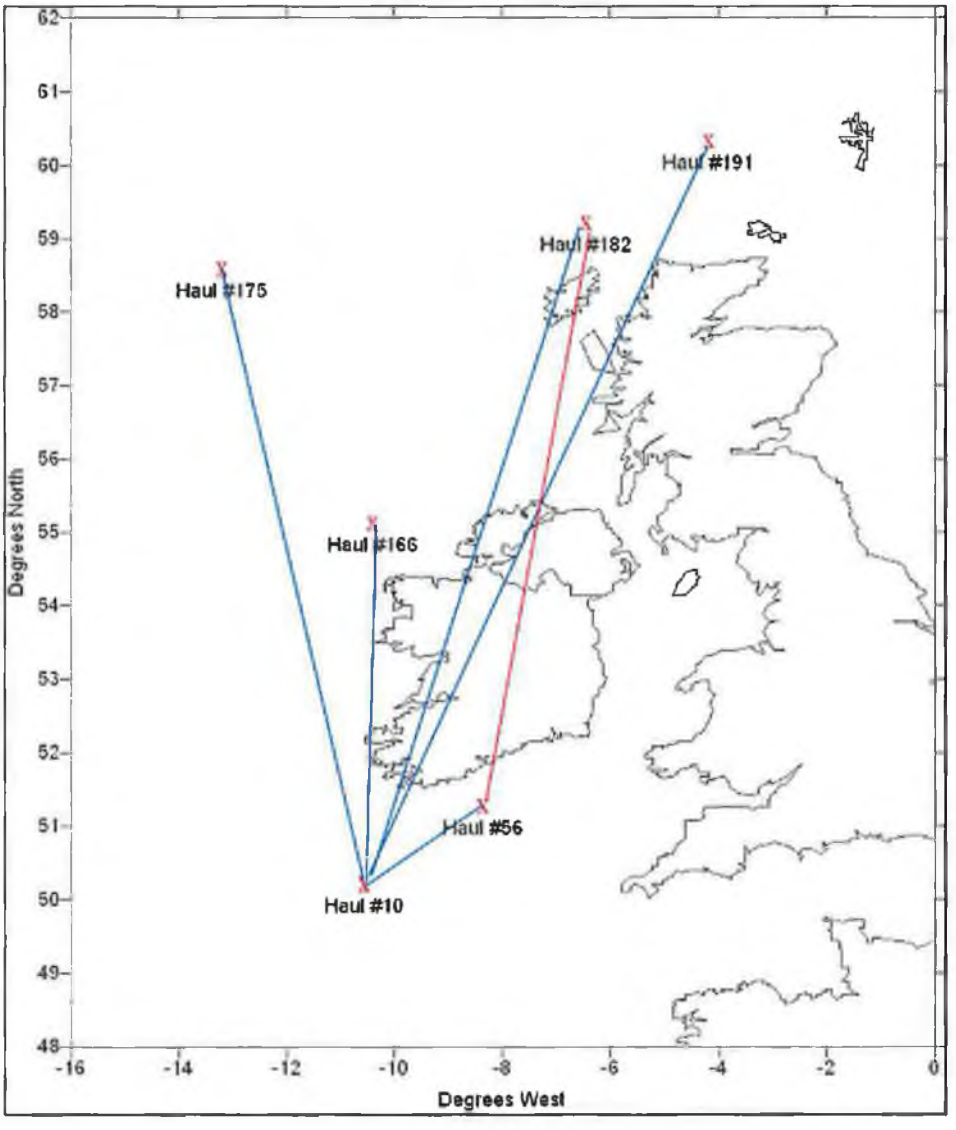
Population differentiation

In pairwise comparisons H10 was significantly different in both allelic and genotypic proportions from all other samples, as was H56 from H182, after sequential Bonferroni correction. This is illustrated in Table 3.3.3 and Figure 3.3.2 below.

Table 3.3. 3 - Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal) for locus *MpouBW8*. Values in bold indicate samples significantly different after sequential Bonferroni correction.

Sample	H166	H175	H182	H191	H56	H10
H166	-	0.505	0.085	0.502	0.067	**<0.001**
H175	0.483	-	0.054	0.699	0.246	**<0.001**
H182	0.043	0.024	-	0.027	**0.001**	**<0.001**
H191	0.458	0.518	0.006	-	0.805	**<0.001**
H56	0.075	0.184	**<0.001**	0.759	-	**<0.001**
H10	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-

Figure 3.3. 2 - *M. poutassou* samples that are significantly different in allelic and genotypic proportions at the *MpouBW8* locus are joined by a red line involving H56 and a blue line involving H10.



MpouBW13

Genetic diversity and HWE

Fifteen alleles were observed at this locus, with allele 176 at the highest frequency in all samples (Table 3.3.1). Only one sample, H10, had a private allele, allele 164, present at low frequency. All samples were in HWE.

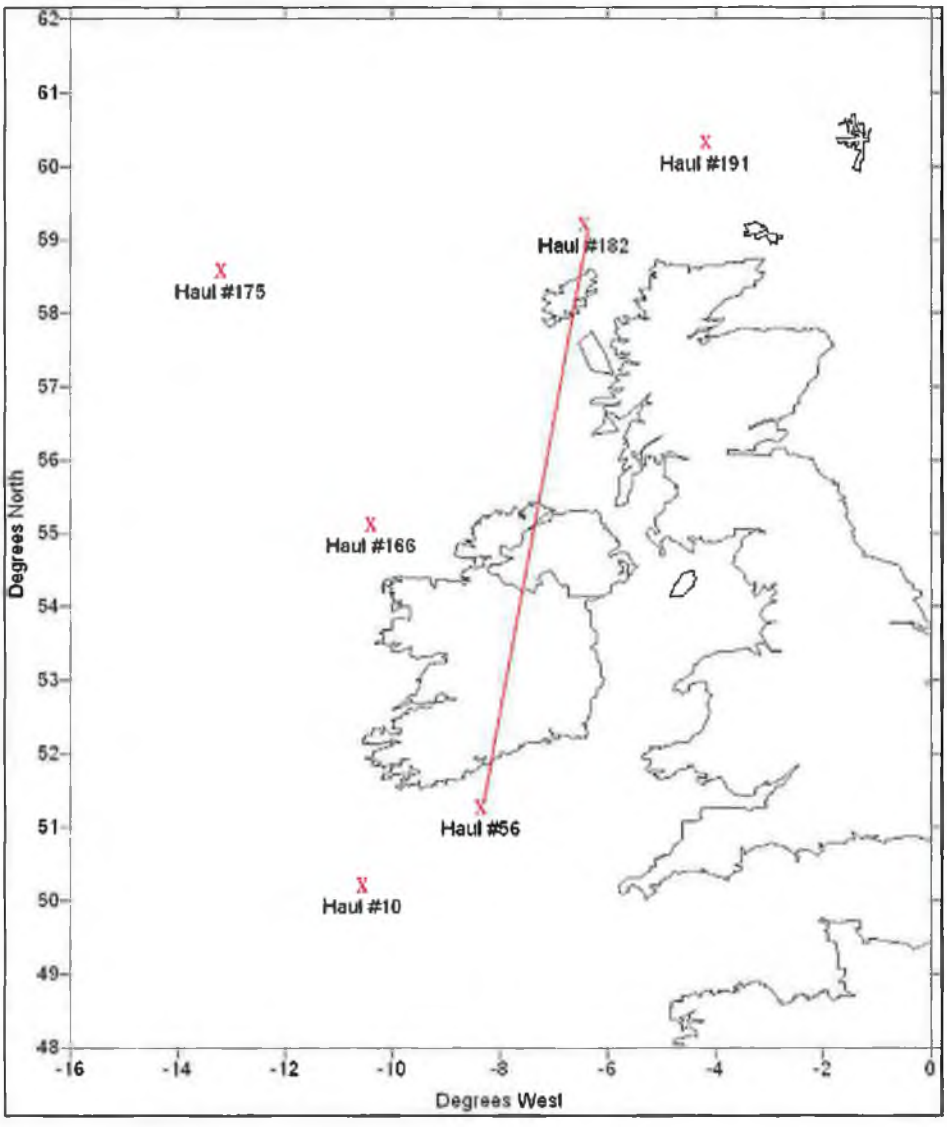
Population differentiation

In pairwise comparisons significant differences in both genotypic and allelic proportions were only observed between H56 and H191 after sequential Bonferroni corrections. This is illustrated in Table 3.3.4 and Figure 3.3.3 below.

Table 3.3. 4 - Probability values for Fisher's exact test of allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal) for locus *MpouBW13*. Values in bold indicate samples significantly different after sequential Bonferroni correction.

Sample	H166	H175	H182	H191	H56	H10
H166	-	0.777	0.947	0.025	0.038	0.316
H175	0.769	-	0.965	0.057	0.329	0.299
H182	0.925	0.966	-	0.079	0.325	0.243
H191	0.011	0.064	0.030	-	**0.002**	0.058
H56	0.033	0.409	0.204	**0.001**	-	0.066
H10	0.351	0.381	0.206	0.040	0.068	-

Figure 3.3. 3 - *M. poutassou* samples that are significantly different in allelic and genotypic proportions at the *MpouBW13* locus are joined by a red line involving H56.



Multilocus comparisons

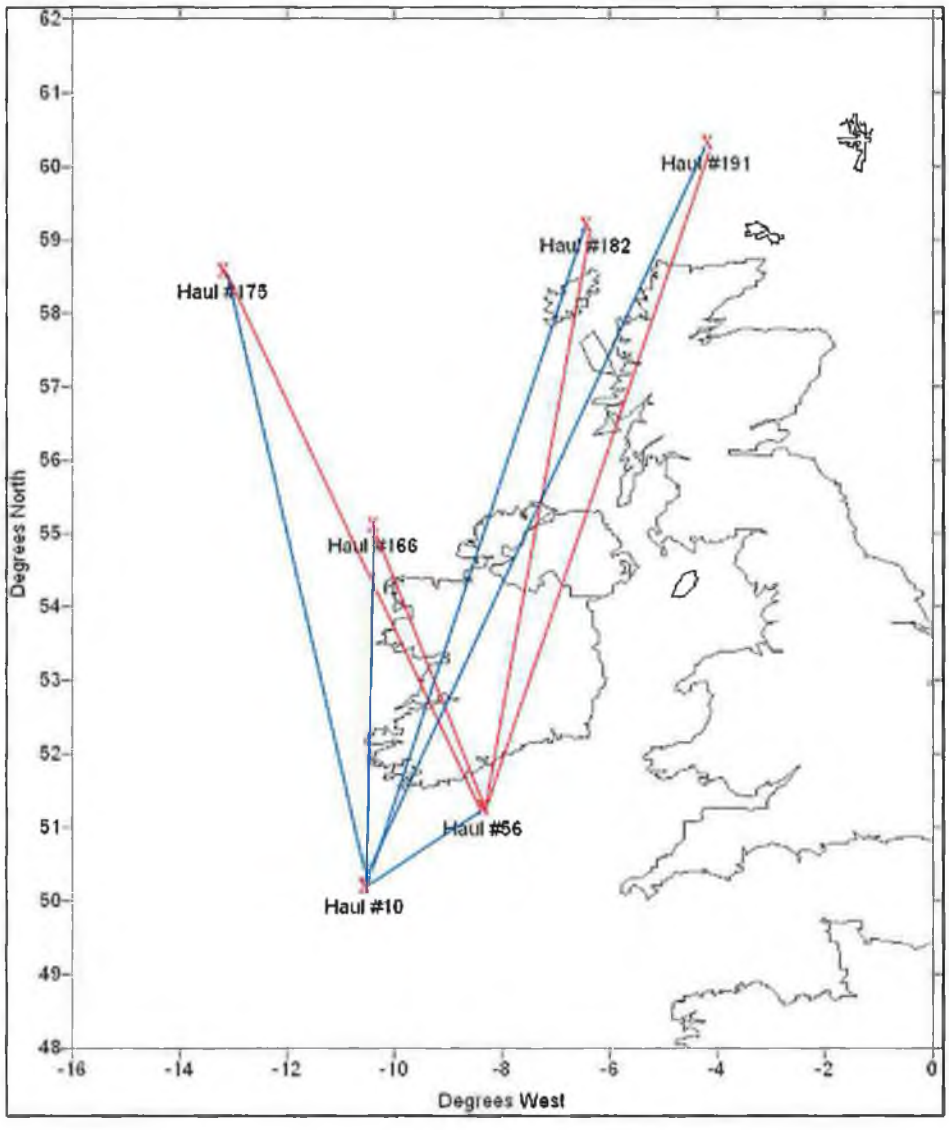
Fisher's exact tests

In pairwise multilocus comparisons of genotypic and allelic proportions significant differences were observed between H56 and all other samples and also between H10 and all other samples after sequential Bonferroni correction. This is illustrated in Table 3.3.5 and Figure 3.3.4 below.

Table 3.3. 5 - Probability values for Fisher's exact test of multilocus allelic differentiation (below the diagonal) and genotypic differentiation (above the diagonal). Values in bold indicate samples significantly different after sequential Bonferroni correction.

Sample	H166	H175	H182	H191	H56	H10
H166	-	0.474	0.263	0.065	**<0.001**	**<0.001**
H175	0.376	-	0.074	0.169	**<0.001**	**<0.001**
H182	0.131	0.021	-	0.043	**<0.001**	**<0.001**
H191	0.022	0.127	0.006	-	**<0.001**	**<0.001**
H56	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**
H10	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-

Figure 3.3. 4 - *M. poutassou* samples that are significantly different in multilocus allelic and genotypic proportions are joined by a red line involving H56 and a blue line involving H10



F_{ST} estimates

In this study F_{ST} values were 0.03, 0.02 and 0.01 (multilocus mean 0.019) for *MpouBW7, MpouBW8* and *MpouBW13* respectively. None of these values were significantly different from zero after sequential Bonferroni correction.

In pairwise multilocus comparisons of F_{ST} values significant differences were observed between H56 and all other samples and also between H10 and all other samples, after sequential Bonferroni corrections (Table 3.3.6). These results are in agreement with Fisher's exact tests for population differentiation.

Table 3.3. 6 – Probability values for pairwise F_{ST} (Weir & Cockerham, 1984) values from permutation tests in FSTAT program. (values in bold indicate samples significant at the 0.01 level after sequential Bonferroni correction.

Sample	H175	H182	H191	H56	H10
H166	0.393	0.272	0.087	**<0.01**	**<0.01**
H175		0.069	0.276	**<0.01**	**<0.01**
H182			0.032	**<0.01**	**<0.01**
H191				**<0.01**	**<0.01**
H56					**<0.01**

3.4 Summary

Pairwise comparisons using F_{ST} estimates and Fisher's exact tests of genotypic and allelic differentiation at three microsatellite loci showed that two samples, H56 and H10, were significantly differentiated from each other and from all other samples. In contrast, significant differentiation was only observed between H56, the most southerly sample and H191, the most northerly sample, using the *PGM* allozyme marker.

4. Discussion

4.1 Overall Results

Significant genetic heterogeneity was observed throughout the sample range, using allozyme and microsatellite markers. Allozyme analysis at the *PGM* locus showed that H56 and H191 differed significantly, a result that was also observed for one of the microsatellite loci, *MpouBW13*. The other two microsatellite loci revealed a much greater degree of differentiation between samples. Using *MpouBW7*, H56 was significantly different from all other samples and H10 differed significantly from H56 and H166. H10 was significantly different, at the *MpouBW8* locus, from all other samples and H56 was significantly different from H10 and H182. When the results for the three microsatellites were combined the overall picture was that H10 and H56 differed from all other samples, including each other (Figure 3.3.4). When the allozyme results were considered, the one significant pairwise comparison, H56 and H191, supported the results observed for *MpouBW7* and *MpouBW13*. As might be expected, both Fisher's exact tests of population differentiation and F_{ST} estimates gave the same results, with the same samples being differentiated in each case.

4.2 Ocean circulation model and larval drift theory in relation to stock structure

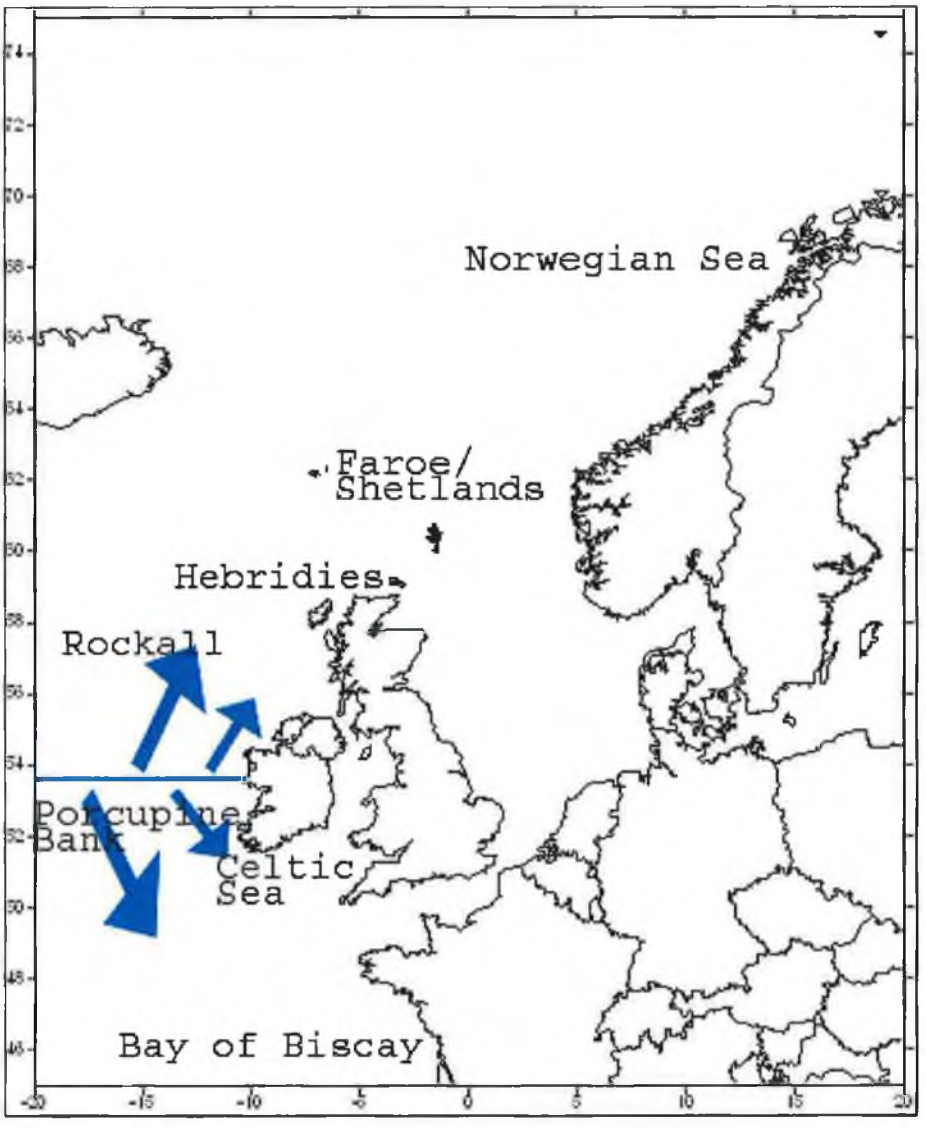
Species, such as blue whiting, with planktonic eggs and larvae, have a great capacity for dispersal. This means that the area in which the eggs were spawned may not be the area in which the eggs and larvae stay. Ocean currents, gyres, winds etc will cause larval drift. Previous work has shown that blue whiting eggs and larvae spawned off the west coast of Ireland will move north or south depending on the area in which they were spawned (Bartsch and Coombs, 1996; Kloppmann et al., 2001; Skogen et al., 1999).

Based on computer simulations, Skogen et al. (1999) suggested a separation line at 54.5°N, although this line can differ annually. The authors found that larvae on the north shelf of the Porcupine Bank seemed to move very little, while those

found in the Rockall Gyre tended to either stay in the immediate area or drift northwards. Larvae found on the Porcupine Bank itself generally tend to move southwards, far from their original spawning site. These observations were supported by earlier findings by Bartsch and Coombs (1996), who also found that larvae from the west of Ireland and the Rockall area were displaced north to north-eastwards, or else stayed on the Porcupine Bank. Some larvae from the Porcupine Bank were also displaced southwards. Kloppmann et al. (2001) found that water circulation over the Porcupine Bank can hold blue whiting eggs and larvae in the area, due to that fact that they are concentrated in the top 50m of the water column (Adlandsvik et al., 2001). However, since water circulation is dependent on winds, strong winds can disrupt the currents holding the larvae on the bank and so larvae can be displaced southwards. The latitude and longitude at which larvae either drift northwards or southwards was suggested by as being 53°30′ N and 12°30W (Kloppmann et al., 2001). Larvae found north and east of the line will be retained in the Rockall gyre or move northwards, those spawned south and west of the line will be retained above and around the Porcupine Bank (Figure 4.2.1). Of course there will be some displacement of larvae southwards from above the separation line, and some northwards from the southern component also. Analysis of otolith microstructure also agrees with the circulation model of Bartsch and Coombs (1996) and Skogen (1999). Fish that grow in southern waters have a faster growth rate than those that grow in the colder more northerly waters. In an analysis of blue whiting from the same geographic area as the present study Brophy and King (2004), using otholith structure analysis, have found that blue whiting from northern areas were distinct from those from southern areas, and that this difference became more obvious with age. It is suggested that there is a mixing or a transition zone off the west coast of Ireland, around the Porcupine Bank, where the two groups overlap.

Although there is some disagreement between authors on the placement of a separation line (Isaev and Seliverstov, 1991; Kloppmann et al., 2001; Skogen et al., 1999), there is evidence that a discrete stock structure for blue whiting in the NE Atlantic exists, with a southern component found south of the Porcupine Bank, a northern component found around the Faroe/Shetland area, and a "mixing zone" located on the Porcupine Bank itself.

Figure 4.2. 1 - Separation line of hypothetical northern and southern stocks of *M. poutassou* in the NE Atlantic. Arrows indicate direction of larval drift to north and south of line.



4.3 Catch composition in relation to results

Results from the present study are in agreement with results from the previous studies mentioned above (Isaev and Seliverstov, 1991; Kloppmann et al., 2001; Skogen et al., 1999). The samples collected at the extremes of the range, H56 and H191, differed significantly at three of the four marker loci. H191 was made up entirely of juvenile immature fish. Although catch data was not available for H56, other catches in the same area taken at the same time indicated a large percentage of mature fish with some juveniles among them (Anon, 2004b; Power, pers.comm. 2003). The genetic difference seen between these two samples might indicate two separate stocks of blue whiting, but it is apparent that further sampling of fish in these areas is necessary to clarify the situation.

H10 and H56 also differed from each other at two out of four loci, indicating that there could also be separate stocks of blue whiting in the Celtic Sea. H10 was similar to H56 in that it was almost entirely (98%) made up of mature fish.

Since no samples were analysed between 51°N and 55°N using microsatellite loci, it was not possible at this stage to say where the separation line may lie between northern and southern putative stocks. It is clear that further sampling over this latitude is required to ascertain this. However, H159 (51.84°N, 11.33°W), which was analysed at the *PGM* locus but not for microsatellites, was collected at the southern end of this gap but no significant differences were found between this sample and any other. Since the results of this study, two additional samples from the NE Atlantic along with H159 have been analysed using the same microsatellite loci as used in the present study (Was, unpublished data). These samples were treated in the same manner as described in the methods section above, and gels were read by Dr. A. Was, a Post-Doctoral Researcher who assisted in the reading of gels in the present study to ensure intercalibration of the two sets of data. These two samples were collected further west than the samples in the present study (Fig 4.3.1). They were also collected in Spring 2003, at the same time as H159, H166, H175, H182, and H191, from commercial fishing vessels fishing for blue whiting. The results show that sample 2 is different from all other samples except sample 11 and H159, and that H159 is only dissimilar to H56 and H10 (Figure 4.3.2). It is clear from these results that there is significant heterogeneity of blue whiting in the NE Atlantic. Samples at

the southernmost end of the sampling range were not genetically differentiated from each other (i.e. samples 2, 11 and H159). However each of these samples were genetically differentiated from H56 and H10, adding weight to the suggestion that the differences between these two samples and other seven may be due to temporal variation. The lack of genetic differentiation between H159 and the other, more northern samples collected in the same year (i.e. H166, H175, H182 and H191) would suggest that this area is a mixing ground for the putative northern and southern stocks. The fact that sample 2 is not differentiated from H159 but is from all of the other northern samples mentioned above could mean that this area forms part of the spawning ground of the "southern" stock, whereas the "northern" stock is found further north of this, with the area around H159 acting as a mixing zone, thus explaining the lack of differentiation between H159 and any of the other samples.

Figure 4. 3.1- Map showing locations of samples of *M. poutassou* in the NE Atlantic. Samples 2, 11 and H159 were analysed by Dr. Ania Was at GMIT.

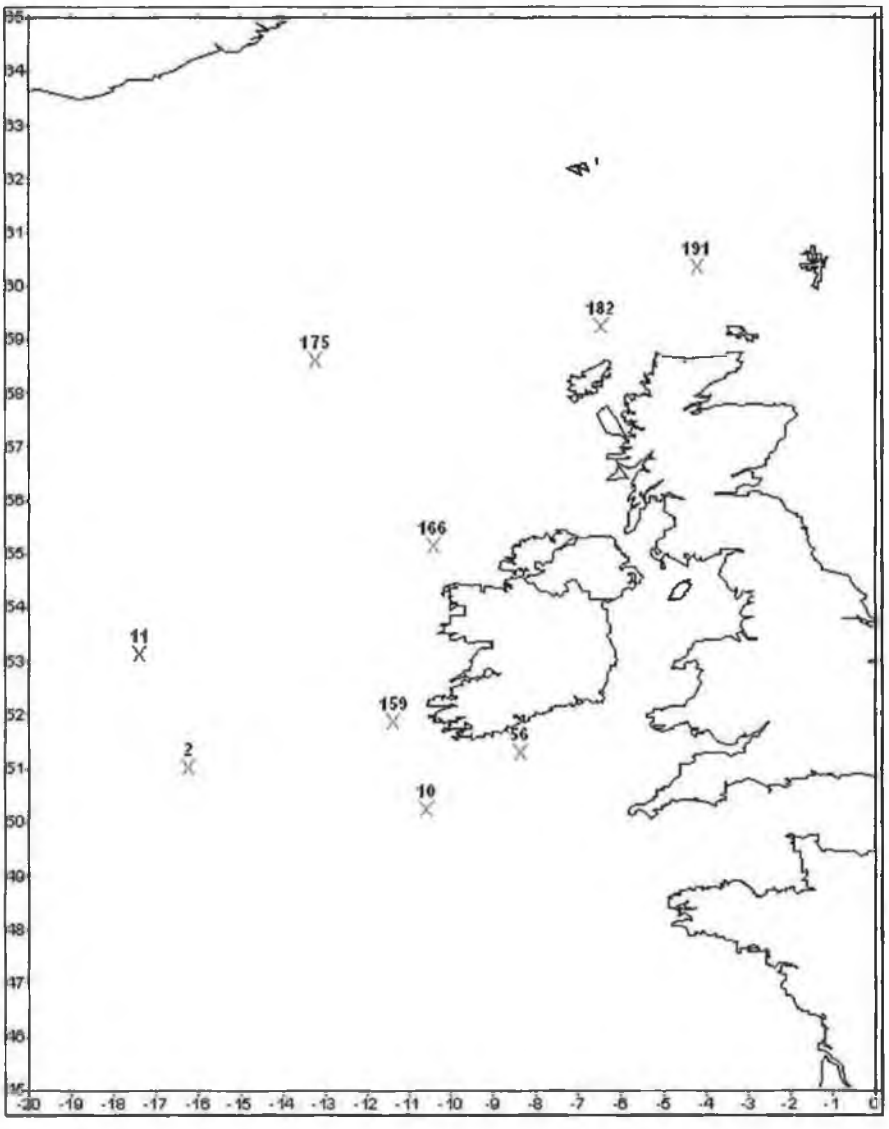
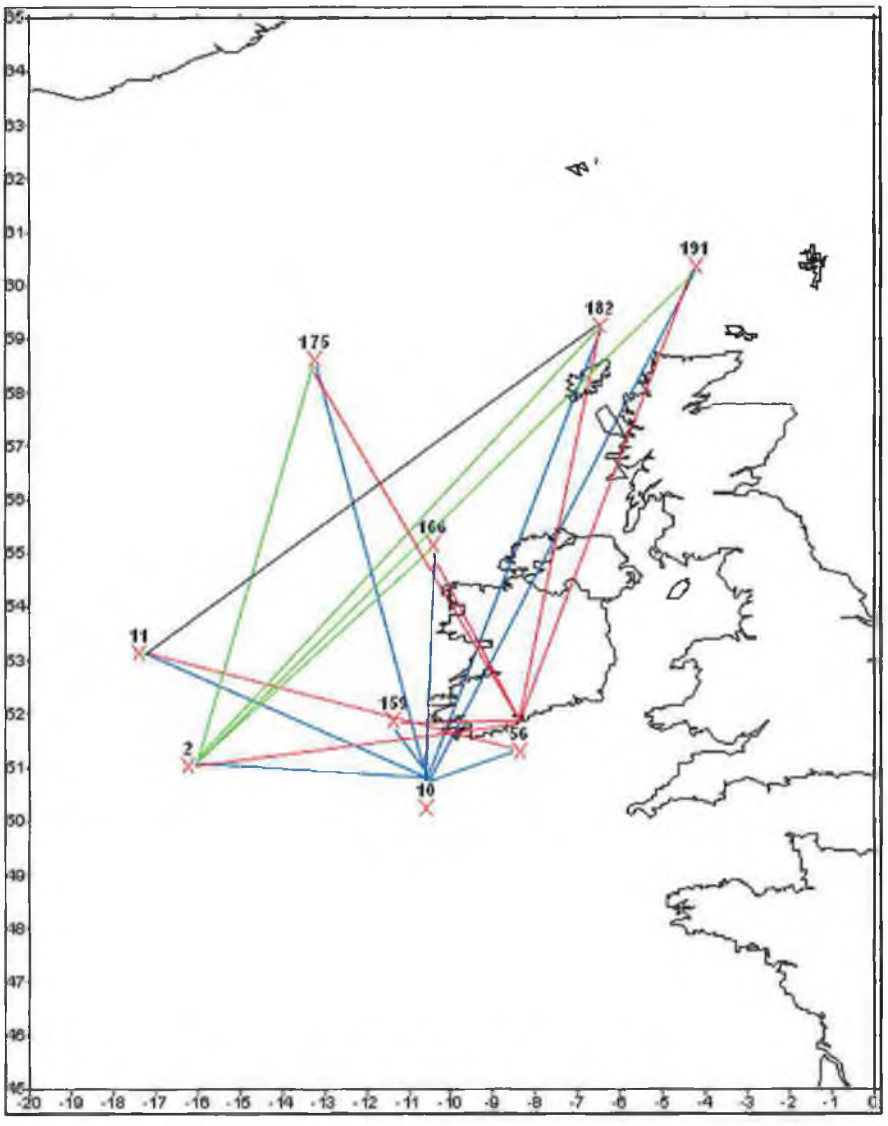


Figure 4.3. 2 - Nine samples of *M. poutassou* showing genetic differentiation. Green lines, differences involving Sample 2; black line, differences involving sample 11; red lines, differences involving H56; blue lines, differences involving H10.



4.4 Previous studies using microsatellites on stock structure of blue whiting

In a recent paper on genetic differentiation in blue whiting in the Eastern Atlantic Ocean, Ryan et al. (2005) using five microsatellites (three of which were used in the present study) and one minisatellite locus, reported genetic heterogeneity in the Porcupine/Hebridean spawning area. However, the differences found were stronger between years (1992 and 1998) than between regions. They did, however, find significant differences between samples at the latitudinal extremes of the range (Barents Sea and the Mediterranean).

The results of the present study and that of Ryan et al. (2005) show several differences. One of the major differences is the fact that in the present study differences were found between samples from the Celtic Sea and all other samples, whereas in Ryan et al. (2005) strong significant differences were observed between samples at the extremes of the range. Other differences included the number of alleles, which was lower for each locus in the present study than in the Ryan study (Table 4.1). This could be due to their sampling a larger area, which included samples from the Barents Sea, Mediterranean and Iceland, whereas in the present study the samples were from ~60°N to 50°N. This large sample range makes it difficult to compare results between the two studies as the two studies do not have overlapping sample sites.

Sample size was also smaller in the present study, which most likely led to fewer alleles being observed. Ryan et al. (2005) reported significant deviations from HWE at two loci, one of which was *MpouBW13*which they attributed to null alleles. But in the present study this locus was the only microsatellite locus not to show a deficit of heterozygotes at any sample site. Another difference was the higher multilocus F_{ST} value of 0.019 observed in the present study compared to the value of 0.0047 across six loci obtained by Ryan et al. (2005). When they only considered the four loci which were in HWE the F_{ST} value was still lower (0.0066) than presented here.

Direct comparison between results is not possible at this stage due to lack of raw data, however it would be interesting to combine the results of the two studies

into a large dataset, the present study "filling in the gaps" in the sampling area of Ryan et al. (2005).

Compared to work on other marine species the current study used few genetic markers. Most other studies use at least five genetic markers including microsatellites, mitochondrial DNA sequence, minisatellites or more than one allozyme marker, hence giving a more robust dataset and a clearer picture of stock structure (Bentzen et al., 1996; Mattiangeli et al., 2003; Carlsson et al., 2004)). Because the species is highly migratory there are very little barriers to gene flow. The hake *Merluccius merluccius* is also a migratory species. Significant differences have been found between samples taken from the Mediterranean and the Atlantic, and also between the Bay of Biscay and Portuguese waters. No difference was found between the hake of the Celtic sea and those in the south of the Bay of Biscay. This level of genetic substructuring is comparable to that reported here, with what looks like isolated populations around the Celtic Sea area and the rest of the sample range and also between the Porcupine Bank and other areas (Fig 4.3.2).

Other marine species show the same level of genetic differentiation as reported in this study . Relatively low F_{ST} values are common in marine species. Hauser and Ward (1998) found a mean F_{ST} value of 0.062 for marine species with a median of 0.02, which is similar to the F_{ST} value reported here for blue whiting.

Table 4. 1 - Number of alleles observed at three loci in present study and that of Ryan et al. (2005)

Locus	Number alleles	
	Ryan et al. (2005)	Present study
MpouBW7	20	16
MpouBW8	31	17
MpouBW13	18	14

4.5 Concluding remarks

The results show that samples taken from the Celtic Sea differed from each other and from samples taken north of 55°N. However there was one year between collections of the two sets of samples, with all but H10 and H56 collected in 2003, and the rest in 2004. When three other samples collected in 2003, from south of ~54°N were added to the present data it was found that H56 and H10 still differed from all other samples. However one of the additional samples, sample 2 also differed from all other samples with the exception of those closest to it (H159 and sample 11). This adds evidence for the argument that there may be more than one stock of blue whiting in the NE Atlantic.

The present study highlights the importance of studying temporal variation in population genetics of a highly mobile marine species. Temporal variation has previously been recorded for this species (Giaever and Stein, 1998; Ryan et al., 2005) and although the time frame in the present study was less than that of both the other studies (one year here compared with four years for Giaever and Stein (1998) and six years for Ryan et al. (2005) between samples) caution is required when making inferences from the results. Extensive sampling of the spawning stock with replicates from different years is required to gain insight into the stock structure of this species. Ongoing work at GMIT will allow this to occur. Samples from the same areas as the present study were collected in March of this year (2005), so genetic comparisons may be made between years as well as between locations. A larger sample size is also desirable as the relatively low numbers of fish in the samples may not have allowed detection of all alleles at these highly polymorphic microsatellite markers.

This study also highlights the importance of using variable genetic markers to elucidate stock structure in marine fishes. Two of the genetic markers employed, the allozyme locus *PGM* and the microsatellite *MpouBW13,* showed only one significant pairwise comparison, whereas the other two microsatellites, *MpouBW7* and *MpouBW8* both indicated six significant comparisons. The two markers showing greatest genetic differentiation had the highest number of alleles, 16 for *MpouBW7* and 17 for *MpouBW8*, compared with 14 for *MpouBW13* and 4 for the allozyme *PGM*. Without the use of these two highly variable markers subtle differences

between samples would not have been seen and only H191 and H56 would have been found to be genetically differentiated. The use of allozymes, while useful in detecting differences at the extremes of species range (Giaever and Forthun, 1999; Giaever and Stien, 1998; Mork and Giaever, 1995; Mork et al., 1985), is not suitable for elucidating more discreet population differences. Therefore, it is important to complement allozyme use with a more variable marker, such as microsatellites.

Future work could involve the use of other types of variable markers, such as mitochondrial DNA or minisatellites coupled with more microsatellites to help elucidate the population structure of this highly migratory species. Ongoing work underway in GMIT is currently employing the use of a minisatellite marker developed for whiting, *Merlingus merlangus*, and four other microsatellites, developed for pollock, *Pollachius pollachius,* to try and achieve this. Also, additional samples over a wider geographic range are also being analysed.

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