3.4 Mitochondrial DNA Methods

To have a representative sample of the harbour seal management areas, a total of five or six individuals were sequenced from each of the following locations: Shetland, Orkney, Moray Firth, Tay and Eden Estuary, Outer Hebrides, Skye and Islay/Jura. In addition Lismore was included as well as there was a high number of samples from this area.

PCR conditions for amplification of Mitochondrial DNA were obtained from Andersen et al. (2011). Primers L15926 (5'-ACACCAGTCTTGTAAACC-3') and PvH00034 (5'-TACCAAATGCATGACACCACAG-3') were amplified with the following conditions: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl 2, 0.8 mmol/L (dNTPs), 1.5 units TaqDNA polymerase, 0.3 mmol/L of each primer, and 70 ng DNA template. The PCR profile consisted of initial denaturation of 90°C for 2.5 min, 37 cycles at 94 °C for 30 s, 48 °C for 60 s, and 72 °C for 60 s. A final cycle included 5 min at 72 °C, then cooling to 4 °C. The PCR products were purified with a QIAGEN-QIAquick gel extraction kit and quantified for further automated sequencing. Chosen individuals were sequenced in both directions (*forward and reverse)* to verify the identity of each. Sequences were edited, checked and aligned by eye with BIOEDIT 7.0.5.3**.** Seventy-four sequences obtained from previous studies (Stanley, Casey et al. 1996 and Andersen, Lydersen et al. 2011) were obtained from the Genbank and compared with the ones found in this study.

MtDNA sequences of other populations of harbor seals were obtained from the GenBank. Duplicate haplotypes across the sample were obtained with the program COLLAPSE 1.2 (Posada © 1998-2006)). Nucleotide (π) and haplotypic (h) diversities (Nei 1987) were calculated for each of the management areas with the program ARLEQUIN 2.0 (Schneider et al. 2000).

A haplotype network was created with the program TCS 1.18 (Clement, Posada et al. 2000), with the objective of displaying the evolutionary steps of the haplotypes in our populations. TCS calculates the frequencies of the haplotypes and creates a matrix of pairwise comparisons among them for which the probability of parsimony is calculated (Clement et al. 2000). The algorithm developed by Templeton et al. (1992) estimates all the possible cladograms with a high probability (>=0.95) of being true. The probabilities are higher when the number of changes between haplotypes is smaller and the probability decreases as the differences between haplotypes increase (Templeton, Crandall et al. 1992). This method is suitable for intra-specific studies and it has been used to infer population genealogies particularly when they show low levels of divergence (Clement et al. 2000).

Mitochondrial DNA Results:

A total of forty-two individuals were sequenced, from each of the harbour seal management areas and Lismore (Table 1) Lismore was chosen because of the large amount of samples obtained from the region. These sequences were compared with a total of 74 d-loop sequences obtained from GenBank originated from Andersen, Lydersen et al.(2011) and Stanley, Casey et al. (1996). Accession numbers and information of all these sequences are shown on Appendix A. A total of eighty different haplotypes were obtained from comparing all the sequences together. The shared haplotypes from this study with those previously published are shown in Table 1 along with an estimation of haplotype (Hd) and nucleotide diversity (Π).

Table 1. Distribution of Mitochondrial DNA haplotypes among eight major Scottish regions. Only 5-6 individuals were sequenced for each region and compared with sequences from other studies. Sample size (N), total number of haplotype (n), haplotype diversity (Hd) and nucleotide diversity (Π) along with their standard deviations (SD) are shown.

Haplotype	Tay /Eden N=6	Skye N=5	Outer Hebrides N=5	Orkney N=5	Moray Firth N=5	Lismore N=5	Isla/Jura N=5	Shetland N=6
PV23 (Andersen et al. 2011)	4	2	3	3	3	3	2	2
G3 (Stanley et al. 1996)	1	2	1		2	1	2	
G24	1							
SK84		1						
OH56388			1	1				2
OR59028				1				
LI76496						1		
SH8								1
SH11								1
IJ3							1	
Total N of Haplotypes	n=3 Π=0.00460 SD=0.00156 Hd=0.600 SD=0.215	n=3 Π=0.00524 SD0.00131 Hd=0.800 SD=0.164	n=3 Π=0.00524 SD0.00157 Hd=0.700 SD=0.218	n=3 Π=0.00429 SD0.00191 Hd=0.700 SD=0.218	n=2 Π= 0.00429 SD0.00125 Hd=0.600 SD=0.175	n=3 Π= 0.00381 SD0.00168 Hd=0.700 SD=0.218	n=3 Π=0.00524 SD0.00106 Hd=0.800 SD=0.164	n=4 Π= 0.00524 SD 0.00104 Hd=0.867 SD=0.129

Our results show that he total number of haplotypes, haplotype and nucleotide diversity in each management region is very similar. Shetland has the highest values and Moray Firth showed only two and the lowest Hd along with Tay/Eden, Lismore showed the lowest value of nucleotide diversity (Table 1). Haplotype PV23 described by Andersen et al., (2011) corresponds to a small number of Norwegian individuals and it is the most common haplotype observed in our sample (20 individuals), followed by G3 described by Stanley et al. (1996) (9 individuals). Stanley et al. (1996) also found two haplotypes in the Moray Firth, one was G3 also found here and G2, absent from this sample, the latter was one of the most common haplotypes found in Scotland and Northern Ireland in a sample of forty-five individuals from those regions. They also found these two haplotypes in the Scottish West

Coast in only seven individuals. A total of seven unique haplotypes were found in this study that was not previously reported (SK84, OH56388, OR59028, LI76496, SH8, SH11 and IJ3).

One hundred and fourteen sequences, including our sample and eighty unique haplotypes from GenBank were arranged in a haplotype network that shows the connections between all of them. (Fig. 1). The most abundant haplotype found in our sample was PV23 found in all the areas, followed by G3 found only in six. Stanley et al. (1996) found G2 (shown in red in Fig. 1) to be the most common haplotype in this region and in the wider North Sea and West Baltic, but it is absent in this study.

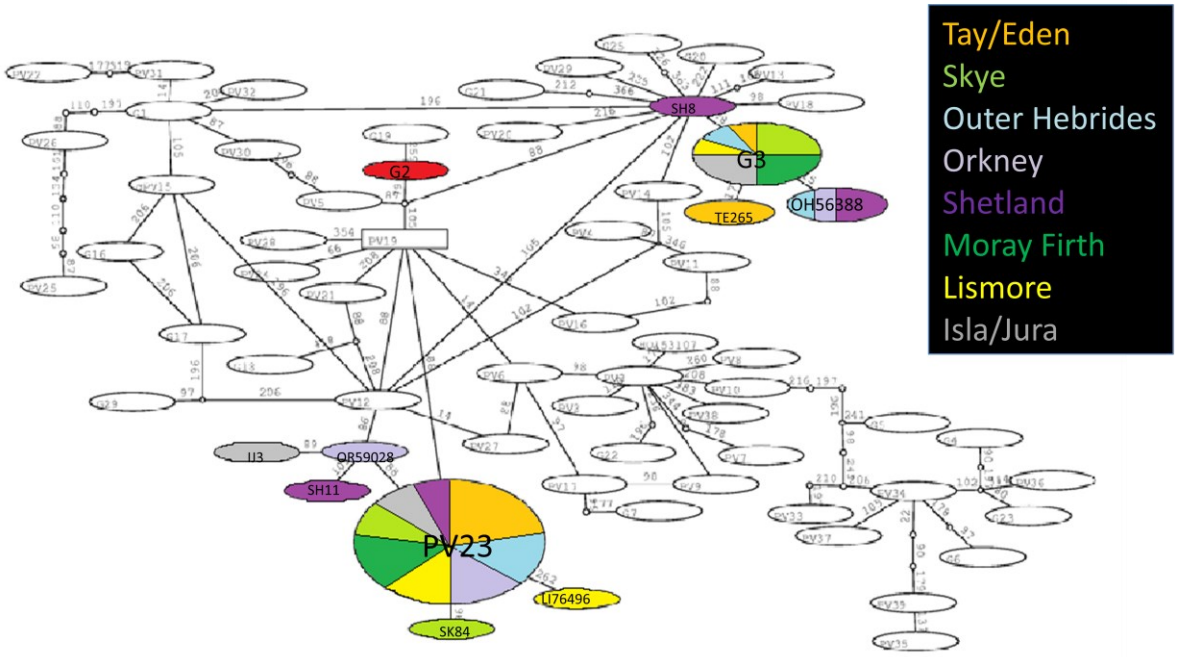

Figure 1. Haplotype network showing the connections between the haplotypes found in the study region. Empty circles represent the haplotypes found in previous studies but that were not found in this study. G2 was one of the most common haplotype found in Scotland and Northern Ireland by Stanley et al. 1996 and it was absent from our sample.

In contrast to the results from the microsatellite alleles, these results show that the mitochondrial genetic diversity found in the management areas analyzed is higher compared to previous studies of the same area. Stanley et al. (1996) only found 3 different haplotypes for Scotland and Northern Ireland in forty-five individuals from Moray Firth, Dornoch, West Coast of Scotland and Strangford Lough. Our study found ten different haplotypes with a similar sample size including Orkney and Shetland that were not included in the previous study and excluding Northern Ireland. Our study also found differences in the frequencies of the haplotypes, while Stanley et al. (1996) found G3 to be the most common haplotype in the area, followed by G2. G2 was not found in the present study but it was the most common haplotype in Stanley's study being present in almost half of the total number of individuals analyzed (101 out of 227) and being present in Scotland and Northern Ireland, the North Sea and the West Baltic Sea. In our present study the most common haplotype is PV23, distributed all

over the area. PV23 was found only in a small number of animals in Norway by Andersen et al. (2011).

While mtDNA evolves rapidly it does not mutate as rapidly as microsatellite alleles, and it may not show a clear population structure (Graves, Helyar et al. 2009). We can observe than in a short period of time (approximately 12 years) the distribution of the haplotype frequencies in the area may have changed considerably; this could be due to the exclusively maternal inheritance of mitochondrial DNA and the random movement of females along the region. The haplotype network (Fig. 1) shows no geographic distribution pattern of mitochondrial genetic diversity and is therefore not a good marker to define management areas.

References:

Andersen, L. W., C. Lydersen, et al. (2011). "A population on the edge: genetic diversity and population structure of the world's northernmost harbour seals (Phoca vitulina)." Biological Journal of the Linnean Society **102**(2): 420-439.

Clement, M., D. Posada, et al. (2000). "TCS: a computer program to estimate gene genealogies." Molecular Ecology **9**: 1657-1659.

Graves, J. A., A. Helyar, et al. (2009). "Microsatellite and mtDNA analysis of the population structure of grey seals (Halichoerus grypus) from three breeding areas in the Baltic Sea." Conservation Genetics **10**(1): 59-68.

Stanley, H. F., S. Casey, et al. (1996). "Worldwide patterns of mitochondrial DNA differentiation in the harbor seal (Phoca vitulina)." Molecular Biology and Evolution **13**(2): 368-382.

Templeton, A. R., K. A. Crandall, et al. (1992). "A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. 3 cladogram estimation." Genetics **132**(2): 619-633.

APPENDIX A

gi|146739413|emb|AM422182.1| Zalophus californianus mitochondrial DNA sequence, isolate S03

gi|1022764|gb|U36371.1|PVU36371 Phoca vitulina mitochondrial control region, genotype HarbourG9

gi|1022766|gb|U36373.1|PVU36373 Phoca vitulina mitochondrial control region, genotype HarbourN17

gi|1022737|gb|U36344.1|PVU36344 Phoca vitulina mitochondrial control region, genotype HarbourG1

gi|1022735|gb|U36342.1|PVU36342 Phoca vitulina mitochondrial control region, genotype HarbourC1

gi|1022768|gb|U36375.1|PVU36375 Phoca vitulina mitochondrial control region, genotype HarbourN41

gi|1022762|gb|U36369.1|PVU36369 Phoca vitulina mitochondrial control region, genotype HarbourG7

gi|1022758|gb|U36365.1|PVU36365 Phoca vitulina mitochondrial control region, genotype HarbourG3

gi|1022756|gb|U36363.1|PVU36363 Phoca vitulina mitochondrial control region, genotype HarbourG28

gi|1022754|gb|U36361.1|PVU36361 Phoca vitulina mitochondrial control region, genotype HarbourG26

gi|1022752|gb|U36359.1|PVU36359 Phoca vitulina mitochondrial control region, genotype HarbourG24

gi|1022750|gb|U36357.1|PVU36357 Phoca vitulina mitochondrial control region, genotype HarbourG22

gi|1022748|gb|U36355.1|PVU36355 Phoca vitulina mitochondrial control region, genotype HarbourG20

gi|1022746|gb|U36353.1|PVU36353 Phoca vitulina mitochondrial control region, genotype HarbourG19

gi|1022744|gb|U36351.1|PVU36351 Phoca vitulina mitochondrial control region, genotype HarbourG17

gi|1022742|gb|U36349.1|PVU36349 Phoca vitulina mitochondrial control region, genotype HarbourG14

gi|1022740|gb|U36347.1|PVU36347 Phoca vitulina mitochondrial control region, genotype HarbourG12

gi|1022760|gb|U36367.1|PVU36367 Phoca vitulina mitochondrial control region, genotype HarbourG5

gi|1022738|gb|U36345.1|PVU36345 Phoca vitulina mitochondrial control region, genotype HarbourG10

gi|1022736|gb|U36343.1|PVU36343 Phoca vitulina mitochondrial control region, genotype HarbourC3

gi|1022767|gb|U36374.1|PVU36374 Phoca vitulina mitochondrial control region, genotype HarbourN21

gi|1022765|gb|U36372.1|PVU36372 Phoca vitulina mitochondrial control region, genotype HarbourN1

gi|1022763|gb|U36370.1|PVU36370 Phoca vitulina mitochondrial control region, genotype HarbourG8

gi|1022761|gb|U36368.1|PVU36368 Phoca vitulina mitochondrial control region, genotype HarbourG6

gi|1022761|gb|U36368.1|PVU36368 Phoca vitulina mitochondrial control region, genotype HarbourG6

gi|1022759|gb|U36366.1|PVU36366 Phoca vitulina mitochondrial control region, genotype HarbourG4

gi|1022757|gb|U36364.1|PVU36364 Phoca vitulina mitochondrial control region, genotype HarbourG29

gi|1022755|gb|U36362.1|PVU36362 Phoca vitulina mitochondrial control region, genotype HarbourG27

gi|1022753|gb|U36360.1|PVU36360 Phoca vitulina mitochondrial control region, genotype HarbourG25

gi|1022751|gb|U36358.1|PVU36358 Phoca vitulina mitochondrial control region, genotype HarbourG23

gi|1022749|gb|U36356.1|PVU36356 Phoca vitulina mitochondrial control region, genotype HarbourG21

gi|1022747|gb|U36354.1|PVU36354 Phoca vitulina mitochondrial control region, genotype HarbourG2

gi|1022745|gb|U36352.1|PVU36352 Phoca vitulina mitochondrial control region, genotype HarbourG18

gi|1022743|gb|U36350.1|PVU36350 Phoca vitulina mitochondrial control region, genotype HarbourG16

gi|1022741|gb|U36348.1|PVU36348 Phoca vitulina mitochondrial control region, genotype HarbourG13

gi|1022739|gb|U36346.1|PVU36346 Phoca vitulina mitochondrial control region, genotype arbourG11

HQ153107PV1

gi|306413201|gb|HQ153108.1| Phoca vitulina haplotype PV2 D-loop, partial sequence; mitochondrial

gi|306413202|gb|HQ153109.1| Phoca vitulina haplotype PV3 D-loop, partial sequence; mitochondrial

gi|306413203|gb|HQ153110.1| Phoca vitulina haplotype PV4 D-loop, partial sequence; mitochondrial

gi|306413204|gb|HQ153111.1| Phoca vitulina haplotype PV5 D-loop, partial sequence; mitochondrial

gi|306413205|gb|HQ153112.1| Phoca vitulina haplotype PV6 D-loop, partial sequence; mitochondrial

gi|306413206|gb|HQ153113.1| Phoca vitulina haplotype PV7 D-loop, partial sequence; mitochondrial

gi|306413207|gb|HQ153114.1| Phoca vitulina haplotype PV8 D-loop, partial sequence; mitochondrial

gi|306413208|gb|HQ153115.1| Phoca vitulina haplotype PV9 D-loop, partial sequence; mitochondrial

gi|306413209|gb|HQ153116.1| Phoca vitulina haplotype PV10 D-loop, partial sequence;mitochondrial

gi|306413210|gb|HQ153117.1| Phoca vitulina haplotype PV11 D-loop,

gi|306413211|gb|HQ153118.1| Phoca vitulina haplotype PV12 D-loop, partial sequence; itochondrial

gi|306413212|gb|HQ153119.1| Phoca vitulina haplotype PV13 D-loop, partial sequence; itochondrial

gi|306413213|gb|HQ153120.1| Phoca vitulina haplotype PV14 D-loop, partial sequence; itochondrial

gi|306413214|gb|HQ153121.1| Phoca vitulina haplotype PV15 D-loop, partial sequence; itochondrial

gi|306413215|gb|HQ153122.1| Phoca vitulina haplotype PV16 D-loop, partial sequence; itochondrial

gi|306413216|gb|HQ153123.1| Phoca vitulina haplotype PV17 D-loop, partial sequence; itochondrial

gi|306413217|gb|HQ153124.1| Phoca vitulina haplotype PV18 D-loop, partial sequence; itochondrial

gi|306413218|gb|HQ153125.1| Phoca vitulina haplotype PV19 D-loop, partial sequence; itochondrial

gi|306413219|gb|HQ153126.1| Phoca vitulina haplotype PV20 D-loop, partial sequence; itochondrial

gi|306413220|gb|HQ153127.1| Phoca vitulina haplotype PV21 D-loop, partial sequence; itochondrial

gi|306413221|gb|HQ153128.1| Phoca vitulina haplotype PV22 D-loop, partial sequence; mitochondrial

gi|306413222|gb|HQ153129.1| Phoca vitulina haplotype PV23 D-loop, partial sequence; mitochondrial

gi|306413223|gb|HQ153130.1| Phoca vitulina haplotype PV24 D-loop, partial sequence; mitochondrial

gi|306413224|gb|HQ153131.1| Phoca vitulina haplotype PV25 D-loop, partial sequence; mitochondrial

gi|306413225|gb|HQ153132.1| Phoca vitulina haplotype PV26 D-loop, partial sequence; mitochondrial

gi|306413226|gb|HQ153133.1| Phoca vitulina haplotype PV27 D-loop, partial sequence; mitochondrial

gi|306413227|gb|HQ153134.1| Phoca vitulina haplotype PV28 D-loop, partial sequence; mitochondrial

gi|306413228|gb|HQ153135.1| Phoca vitulina haplotype PV29 D-loop, partial sequence; mitochondrial

gi|306413229|gb|HQ153136.1| Phoca vitulina haplotype PV30 D-loop, partial sequence; mitochondrial

gi|306413230|gb|HQ153137.1| Phoca vitulina haplotype PV31 D-loop, partial sequence; mitochondrial

gi|306413231|gb|HQ153138.1| Phoca vitulina haplotype PV32 D-loop, partial sequence; mitochondrial

gi|306413232|gb|HQ153139.1| Phoca vitulina haplotype PV33 D-loop, partial sequence; mitochondrial

gi|306413233|gb|HQ153140.1| Phoca vitulina haplotype PV34 D-loop, partial sequence; mitochondrial

gi|306413234|gb|HQ153141.1| Phoca vitulina haplotype PV35 D-loop, partial sequence; mitochondrial

gi|306413235|gb|HQ153142.1| Phoca vitulina haplotype PV36 D-loop, partial sequence; mitochondrial

gi|306413236|gb|HQ153143.1| Phoca vitulina haplotype PV37 D-loop, partial sequence; mitochondrial

gi|306413237|gb|HQ153144.1| Phoca vitulina haplotype PV38 D-loop, partial sequence; mitochondrial

gi|306413238|gb|HQ153145.1| Phoca vitulina haplotype PV39 D-loop, partial sequence; mitochondrial