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An analysis of Highlands Hill Loch Trout (*Salmo trutta*, *L, 1758) Phylogeography

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1. Abstract

Knowledge of the genetic structure of Highlands Hill Loch brown trout (*Salmotrutta*) is limited, but is crucial to ensure its future genetic conservation. This study examined the genetic variation of brown trout at four microsatellite loci. Samples from 56 brown trout from 9 different but associated Scottish highland hill lochs were analysed. Several variables were measured when considering genetic distance and genetic diversity; altitude, area, distance from car park, distance from the nearest road and geographical distance. Results indicated that genetic distance increased with altitude (r- value 0.556). In contrast there was a negative correlation between genetic distance and distance from the nearest car park; similarity between the genetic diversity and distance from the nearest road. In addition genetic diversity increased as water body area decreased. Results suggest that physical barriers have an influence on brown trout population and suggest additional, anthropogenic variables which may have influenced the genetic structure of the populations under study (such as site distance from road and car park). The indication that smallest lochs have a higher genetic diversity is a reinforcement of the anthropogenic influences on the brown trout genetic structure. In conclusion, the results from this suggest that the genetic structure of the Highlands Hill Loch brown trout it is possibly influenced by both, anthropogenic and environment factors; this raises questions regarding a more suitable methods of considering the phylogeography of the Highlands Hill Loch brown trout populations.

Acknowledgements

I would like to thank my lecturer and supervisor Steve Kett for this great opportunity to work with him, for all his supporting patience and most of all for introducing me to the world of trout.
I would like to thank Mahmoud Naase for the use of the molecular lab, Kevin Crawford for his kindness and support in providing lab equipment when it was needed. I like to also thank my partner Paul Gandolfi, my dear friend Ayaan Farah for their help and my lab partner Tim Jones. Lastly but not least I would like to thank the Wester Ross Fisheries Trust Biologist (Peter Cunningham) and angling volunteers for providing all the brown trout samples used in the experiment.

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2. Introduction

2.1 Brown trout (Salmo trutta) population genetics

Research regarding brown trout (*Salmo trutta L*.1758) population genetics has been ongoing since 1976, as part of wider research programmes assessing salmonid fish genetics and molecular ecology (Ferguson, 1997)

Brown trout is a geographically widespread and phenotypically plastic member of the family Salmonidae. This family is being extensively studied as most members are of significant economic and recreational importance (Prodohl *et al 1994;* Youngson *et al.,* 2003*)*. The brown trout is indigenous to Europe, North Africa and western Asia, but have been introduced into at least an additional 24 countries outside Europe and now have a world-wide distribution (Klemetsen *et al*., 2003). Since rivers, lakes and streams took their present form in the aftermath of the last glacial retreat Brown trout have been integral parts of the natural biodiversity of the freshwater ecosystems of temperate and sub-arctic regions bordering the north- east Atlantic, (Jonsson *et al.,* 2001 and McKeown*et al* 2010). Partly because of this geographic range and variation in habitat occupied, brown trout show extensive variability and plasticity in morphology, ecology and behaviour (Prodohl *et al,* 2007). They exhibit ontogenetic niche shifts partly related to size and partly to developmental rate, switching when there is surplus energy available for growth (Klemetsen *et al*.2003). Habitat characteristics influence life-history traits or tactics that enable a species to cope with a range of ecological problems of animals (Jonsson *et al.,* 2001; Mims *et al.,* 2010), and the brown trout exploits a large ecological niche. Equally, as brown trout show two different life cycles, exploiting both fresh and salt waters for feeding, and fresh for spawning, mean populations are often partially migratory; this could also have had a significant long term influence on their diversity(Klemertsen *et al* 2003).

A number of different types of markers (morphological, karyotypical and molecular) have been applied to the characterization and management of brown trout genetic resources (Presa and Guyomard, 1996). On the basis of protein electrophoretic studies, brown trout have been shown to have among the highest reported levels of polymorphism of any vertebrate species (Prodoh l*et al,* 2007 cited Ferguson, 1989). They display a level of differentiation approximately 10 times greater than that observed in Atlantic salmon (Youngson *et al.,* 2003)

2.2 The relevance of Phylogeography

Over the past decade, phylogeography has grown as a discipline because allelic phylogenies provided explicitly historical tools for the study of geographical subdivision among populations (Hare, 2001). Avise (2000) describes Phylogeography as the study of principles and processes governing the geographic distribution of genealogical lineages, especially those within the closely related species. Phylogeography looks at the historical and phylogenic components of a spatial distribution of genes lineages (Buckley, 2009; Teske *et al.,* 2011).
Phylogeography aims to look at the time and space of the organism concerned. To gain a full understanding and analyse the interpretation of lineage distribution it usually requires a clear conclusion of molecular, population genetics, ethology and historical geography. (Hickerson *et al*., 2010).

Phylogeography plays a very important role in the study of evolution as it can be used as a means by which to examine both the diverse micro-evolutionary and macro-evolutionary disciplines. In phylogeography, an empirical focus on gene lineages enables the history of population processes to be inferred from the simultaneous analysis of temporal and spatial patterns (Hare, 2001).
The screening of microsatellite variation is the most efficient procedure for assaying genetic variation in applied fisheries (Lerceteu-Kohler and Weiss, 2006 and Nielsen & Sage, 2002 Many studies in brown trout have used microsatellite data as a means by which to examine phylogeographic relationships (Lerceteau-Koler and Weisse (2006); Kallio-Nyberg *et al.,(*2010) and Presa and Guyomard(1996).
Microsatellite data also had markedly smaller standard errors compared to other techniques; observed heterozygosity levels (Koljonen *et al* 2002) were much more significant between microsatellite and geographic distances (p-value 0.001) than between Amplified Fragment Length Polymorphism and geographic distances (p-value 0.02)(Gaudeul *et al* 2004). Individual assignment test accuracy was higher for microsatellites (73.1%) than SNPs (single nucleotide polymorphisms - 66.6%) (Narum *et al.,*2008).

Ferguson (2006) last reviewed the studies on genetic variation in natural brown trout populations from Britain and Ireland, Finland, France, Greece, Iceland, Norway, Sweden, U.S.A. and U.S.S.R., revealing abundant geographical variation in gene

frequency, with individual populations containing only a limited part of the gene diversity of the species.

2.3 Highlands Hill Lochs

The coastline of western Scotland is characterised by the presence of a series of sea lochs and glacial formations. Geologically, the loch basins are developed within a mixture of igneous and metamorphic rocks, dominated by the Etive igneous complex, which lies beneath the entire inner loch (Mente *et al*, 2008).Due to compressional tension along faults, the rocks along such features are prone to developing fractures. Where such faults and their consequent fractures meet the surface of the land, water infiltrates the fractures. The freezing and thawing of this water, coupled with its flow down slope, contributes to the acceleration of erosion that causes the development of the lochs of Scotland which display the characteristic southwest to northeast relative orientation(Cunningham, 2009). Many of these lochs have sufficiently isolated catchments as to preclude natural genetic exchange between their trout populations, but this does not necessarily preclude genetic input via other means. Cunningham (2009) explains that some local anglers believe that periodic stocking of lochs with trout from out of the area improved the quality of fishing through the introduction of 'new blood' for the same reason that new rams were brought in to maintain the quality of hefted sheep herds. As a consequence, there is anecdotal and material evidence to show that anglers have moved brown trout from one loch to another within the same area. Such movements can influence the genetic structure of their populations, although environmental and ecological factors continue to play major roles in determining genetic structure.

3. Aims:

- To consider the influences of geological and environmental structures on the genetic structure of Highlands Hill Loch brown Trout.

- To investigate how anthropogenic influences may influence the brown trout population of the Scottish highlands.

4. Materials and Method

4.1 Population studied and map of the location where samples were collected

Figure 1: Map of location of each population; each studied population is pointed in the map by a blue circle. Table 1 indicates name of each abbreviations used in the map. (http://www.streetmap.co.uk/map.srf?x=184200&y=880060&z=126&sv=184200,880060&st=4&ar=Y&mapp=map.srf&searchp=s.srf&dn=666&ax=185500&ay=873500&lm=0).

Table 1: Location and number of brown trout (*Salmo trutta*) samples used in the specimens

Name of loch/ Sampled localities	Abbreviations	Map references	Sample size
Loch Laraig	LL	NG 7679	7
Loch Laraig 'minor'	LLM	NG 8477	4
Elf's Loch	ELF	NG 8773	7
Sands River	SR	NG 7679	7
Loch nan Buainichean	LB	NG 8573	7
Loch an AirdShielg	LAS	NG 86 75	7
Loch na h-Oidhche outflow	LNO	NG 8866	7
Loch naFeitheMingaig	LFM	NG 8674	4
Loch AtrighMhicCriadh	LAM	NG 8376	7

4.2 Microsatellite Analysis

4.2.1DNA extraction procedure

Fresh fin tissue samples were collected by WRFT (Wester Ross Fisheries Trust) angling volunteers and preserved by air drying and stored at room temperature. DNA was extracted following the isolation of genomic DNA from animal tissue procedure (Wizard® Genomic DNA Purification kit).

For extracting DNA from fin samples, a small piece (approximately 2.5 cm^2) of fin tissue was added to 100 µl of chilled NucleiLysis solution and homogenized.Later another 500µl of chilled Nuclei Lysis solution was added. The samples were incubated for 15-30 minute at 65ºC.

Lysis and protein precipitation was followed by adding 3µl of RNase solution to each sample and the samples were mixed. Samples were incubated again at 37ºC for 15-30 minutes and cooled to room temperature. This was followed by the addition of 200 µl of protein precipitation solution in each sample. The sample mixtures were placed in a

vortex and chilled on ice for 5 minutes. To finalise this step the tubes were centrifuged for 5 minutes at 16,000 g.

The DNA was precipitated by transferring the supernatant to a fresh tube containing 600 µl of room temperature isopropanol and centrifuging for 2 min at 16 000 g. The supernatant was removed and 600µl of room temperature 70% ethanol was added. The samples were placed again in a centrifuge for 2 min at 16 000 g. To finalize the ethanol was aspirated and the pellet was air dried. Each DNA sample was rehydrated in 100µl of DNA Rehydration solution.

4.2.2 Polymerase chain reaction

Primers were selected by searching the relevant literature (REFS) for past experiments that showed positive results with the species used in the experiment.

Table 2: *Characteristics of the microsatellite loci used in this study.*

Locus	**Size Range**	**No. Alleles**	**Sequences (5'- 3')**	**References**
SSa197	107-177	18	5'GGGTTGAGTAGGGAGGCTTG-3' 5'TGGCAGGGATTTGACATAAC-3'	Aurelle*et al (*2002); Hansen *et al*(2001)
T3-13	175-235	21	5'-CCAGTTAGGGTTCATTGTCC-3' 5'-CGTTACACCTCTCAACAGATG-3'	Hansen *et al*(2002b); Hansen *et al*(2001
STR60INRA	87-111	9	5'-CGGTGTGCTTGTCAGGTTTC-3' 5'-GTCAAGTCAGCAAGCCTCAC-3'	Hansen *et al*(2001)
Strutta12	127-206	27	5'-AATCTCAAATCCGATCAGAAG AGCTATTTCAGACATCACC	Poteaux *et al*(1999)

Sequences obtained from: http://www.qub.ac.uk/bb-old/prodohl/TroutConcert/fr_molecularmarkers.htm#MtDNA%20markers

PCR mixture contained a final concentration of:
1X Reaction Buffer,
0.2 mMdNTPs mix,
1.5 mM MgCl2,
0.025u/µl Taq DNA polymerase,
Sterile Water (to a final volume of 25µl)
1µM of each Primers mix

23 µl of PCR mix was added to 0.5mL microfuge tubes; using a sterile tip 2 µl of each individual sample was added to the PCR mix in each tube. For each set of samples water was added to one microfuge tube containing PCR mix which was used as a negative control.
All microfuge tubes were added to the thermo cycler and the procedure was repeated for the following programmes; Initial start at 95^{o}Cfor 5 min, followed by annealing temperatures depending on the microsatellite (table 3).

Table3: *Annealing temperature of each microsatellite*

Primer	**Primers annealing**
SSa197	60^{o}C
T3-13	54^{o}C
STR60INRA	60^{o}C
Strutta12	56^{o}C

The Primer extension was set, 72^{o}C for 30 seconds.The above cycles were repeated for 30 times. In the final extension, 72^{o}C was applied for 5 minutes and stopped by chilling to 4^{o}C.

4.2.3 Detection of PCR products:

A 2% agarose gel was made using 0.5×TAE buffer. The gel was then poured into a mould and placed in an electrophoresis tank. Using a Gilson pipetman 2 µl of loading dye (blue) was added to the microfuge tube containing the ladder (HyperLadder™ IV) and each of the microfuge tubes containing the PCR mixture. 15 µl of each tube was loaded on the agarose gel. The amplified DNA fragments were visualized by UV illumination.

4.3. Genetic and statistical analysis:

Each band size was determined by using a standard curve to relate the distance the DNA migrated to the fragment size. The standard curve was created using a modification of regression analysis with a fitted line plot using MINITAB 15 English, method adapted from Rochelle *et al.,* (1985).

Amplified products were scored in a binary mode (1, 0), where "1" represented the presence of a marker and "0" its absence. In the case of microsatellites with more than one allele, the smallest range sized allele was designated 'A' increasing alphabetically (table4).

The obtained results were used to calculate the Genetic diversity (Fst), using the equation adapted from Stone *et al.,* (*2007)*:

$F_{ST} = (P_A - P_B)^2/2P(1-P)$

P_A= population A

P_B = Population B

P= global population

For genetic distance (Simpson index - D) the following formula:

$$D = \frac{\Sigma\, n(n-1)}{N(N-1)}$$

n = the total number of alleles found of a particular loch or river
N = the total number of alleles found in all sites (lochs and river)

- Simpson index(D)– genetic diversity was correlated with:

Altitude (metres) of each site was calculated using Google earth (a “virtual globe” map and geographical information program)

Area (Km2) of each loch was calculated by measuring the longest width and longest length of loch.

Distance from nearest road (km) by using a Os map a ruler was used to measure the distance between each loch and the nearest road. Programme Google earth was also used to compare the accuracy of the Os map.

Distance from nearest car park (km) by using a Os map a ruler was used to measure the distance between each loch and the from nearest car park (km) Programme Google earth was also used to compare the accuracy of the Os map.

Map scales conversation: 1:25,000 Scale OS Explorer Maps are 4cm to 1km and 1:50,000 scale OS Maps are 2cm to 1km

- Genetic distance (Fst) was correlated with variables: Altitude difference (meters), difference in area (km^2), difference in proximity to road (km) and Difference in proximity to car park (km). Variables were all calculated between two points (lochs). By calculating the value of each variable of each loch as describe above, the variable values of each loch was subtracted from another individual loch. This was done subsequently with each variable of every loch, by calculating all possible pairs of lochs (population) and variables.

Geographical distance (metres), was also calculated between two points, the geographical coordinates of the two points.

Correlations between variables were made using parametric Pearson tests and Spearman rank correlation respectively; Pearson's correlation coefficient (r) is a measure of the strength of the association between the two variables. A measure of +1 would indicate perfectly positive correlation a measure of -1 would indicate perfectly negative correlation (Pestorius, 2006)

With two variables X and Y, with means XBAR and YBAR respectively and standard deviations S_X and S_Y respectively. The correlation is computed as:

$$r = \frac{\sum_{i=1}^{n}\left(X_i - \bar{X}\right)\left(Y_i - \bar{Y}\right)}{\left(n-1\right)S_X S_Y}$$

Multiple regression analysis was used to explore the relationships between Genetic distance and each variable and also between genetic diversity and each variable.

A general multiple equations follows:

$y = a + b_1x_1 + b_2x_2 + b_3x_3 + ... + b_nx_n$

$b_n = b_1$ is called the coefficient of x_1, b_2 is the coefficient of x_2, and so forth

x_n =independent variables

A = the constant that is added to the sum.

The R provides a measure of how well Y can be predicted from the set of X scores (Berger 2007). R^2 of above 75% as very good; 50-75% as good; 25-50% as fair; and below 25% as poor and perhaps unacceptable (Lea,2007)

The significance level for all statistical tests was set at $p \leq 0.05$.All tests were carried out using the statistical software package MINITAB 15 English

5. Results:

5.1 Gel electrophoresis

A gel that best expressed the characteristics of the microsatellite was selected for this section. The rest of the results are found in the appendix A.

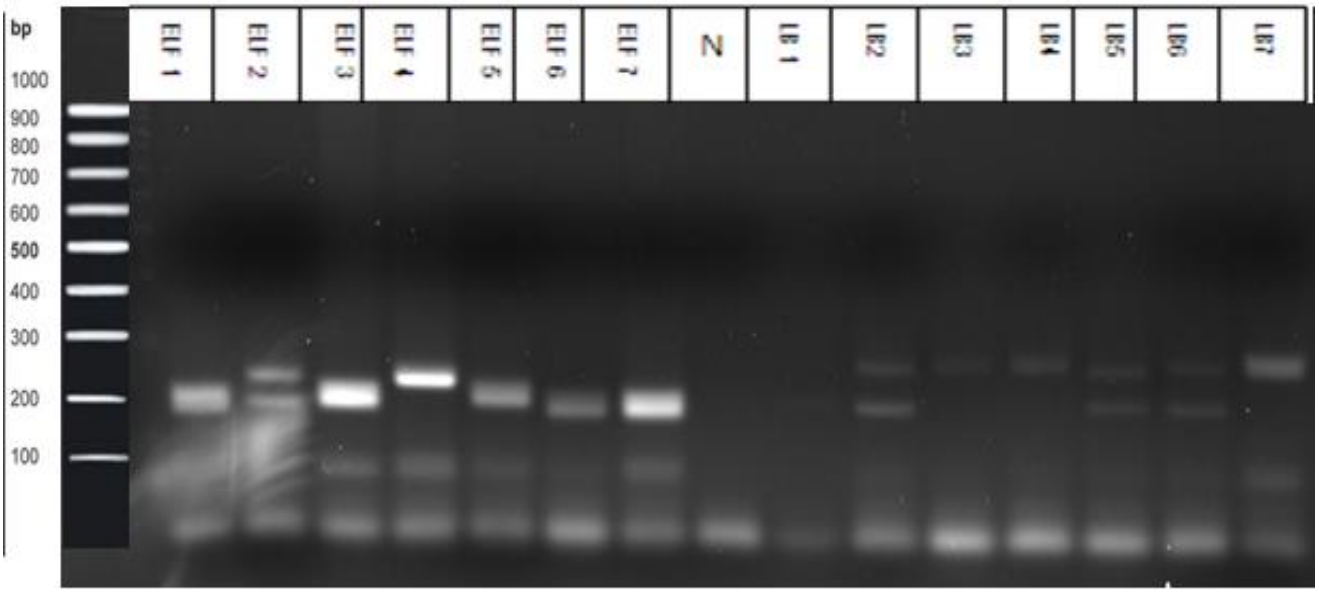


Figure2: Gel electrophoresis of PCR showing presence or absence of alleles in brown trout in Elf's Loch and Loch nan Buainicheanrelating to their genetic relationship using

Microsatellite SSa197.Lane 1 ladder (HyperLadder™ IV)lane 2 to 8Elf's Loch samples 1-7 (ELF 1-7),lane 9: negative control;lane 10 to16 from Loch nan Buainichean samples 1-7 (LB 1-7).

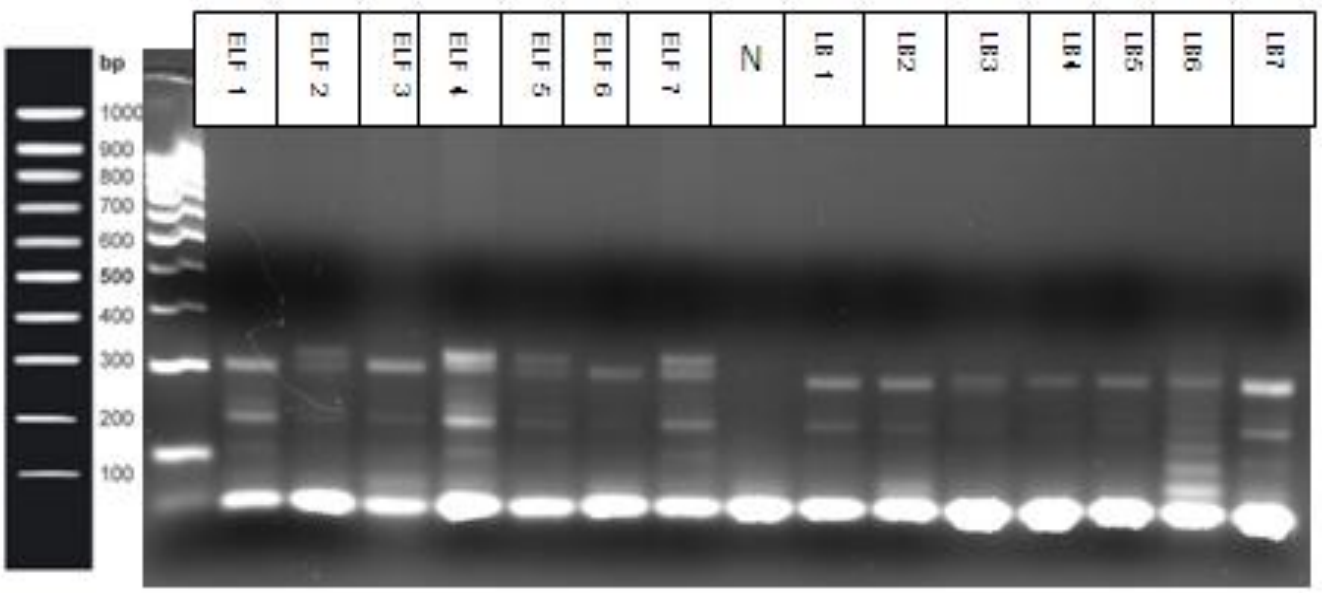


Figure 3: Gel electrophoresis of PCR showing presence or absence of alleles in brown trout in Elf's Loch and Loch nan Buainicheanrelating to their genetic relationship using Microsatellite T3 -13. Lane 1 ladder (HyperLadder™ IV) lane 2 to 8Elf's Loch samples 1-7 (ELF 1-7) ,lane 9 negative control. Lane 10 to16 from Loch nan Buainichean samples 1-7 (LB 1-7).

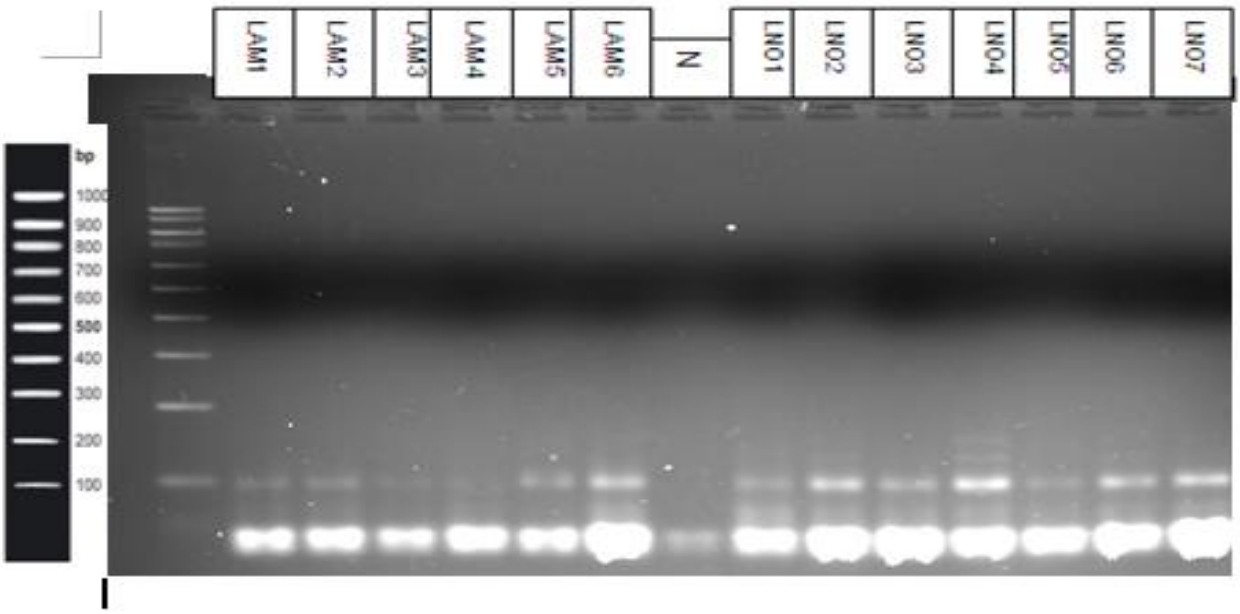
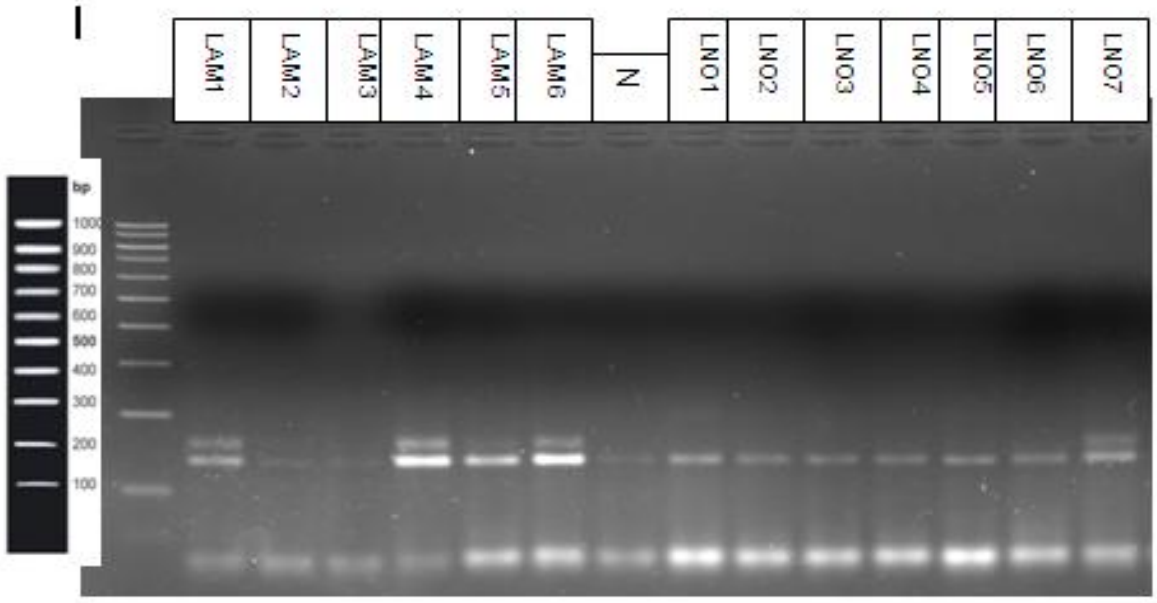


Figure 4: Gel electrophoresis of PCR showing presence or absence of alleles in brown trout in Elf's Loch and Loch nan Buainichean relating to their genetic relationship using Microsatellite strutta60. Lane 1 ladder (name of ladder) lane 2 to 7Loch AtrighMhicCriadh1-7 (LAm 1-7) ,lane 8 negative control. Lane 9 to15 from Loch na h-



Oidhche outflowsamples 1-7 (LNO 1-7).

Figure 5: Gel electrophoresis of PCR showing presence or absence of alleles in brown trout in Elf's Loch and Loch nan Buainicheanrelating to their genetic relationship using Microsatellite strutta12. Lane 1 ladder (HyperLadder™ IV) lane 2 to 7Loch AtrighMhicCriadh 1-7 (LAM 1-7) ,lane 8 negative control. Lane 9 to15 from Loch na h-Oidhche outflowsamples 1-7 (LNO 1-7).

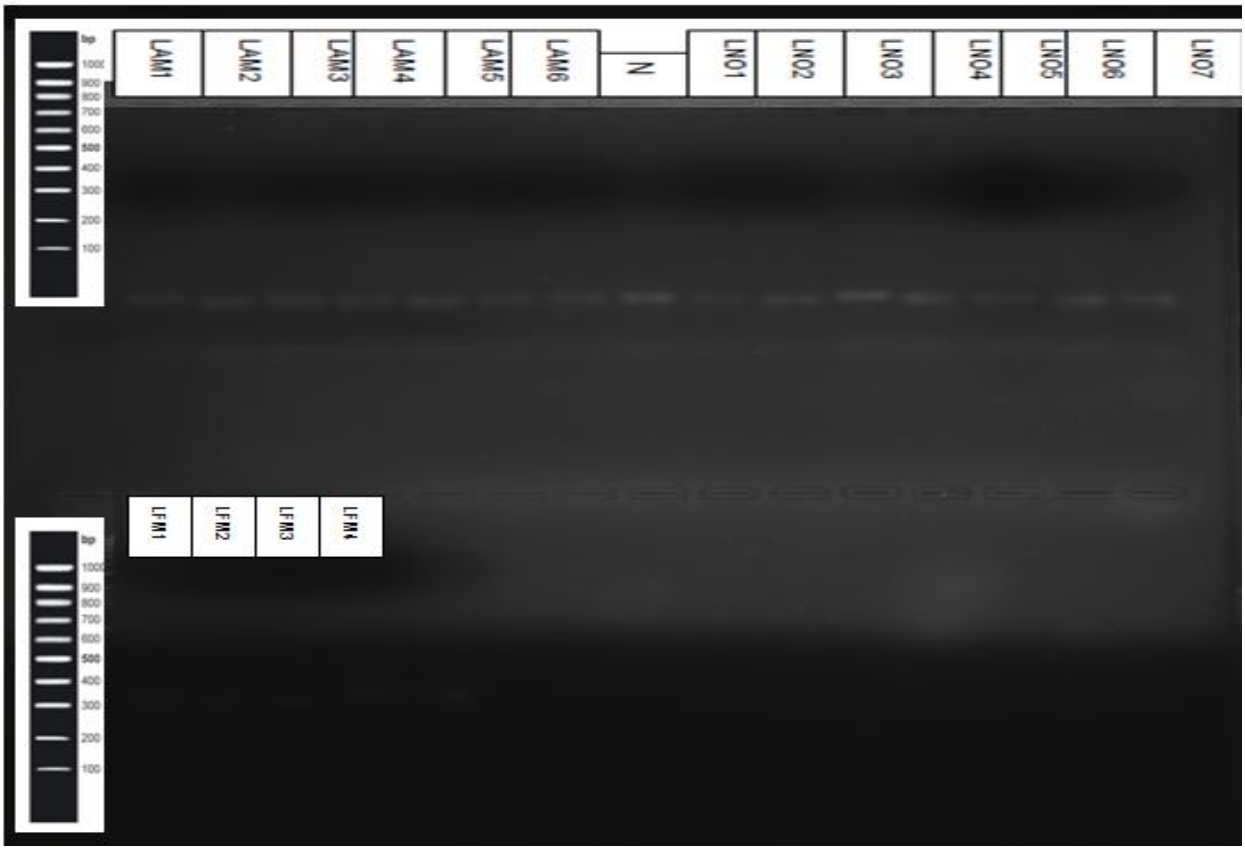


Figure 6: Gel electrophoresis of PCR showing the absence of alleles in brown trout using Microsatellite SSa197 in Loch AtrighMhicCriadh (LAM),Loch na h-Oidhche outflow

(LNO) and Elf's Loch(ELF).

5.2 Allelic size range

Table 4: *Size range of allele present in each microsatellite*

	Microsatellites			
Allele	SSa197	T3-13	Strutta12	Str60
A	164-171	215-226	150-154	66-111
B	149-156	189-208	128-134	----
C	132-136	----	----	----
D	122-126	----	----	----

Table showing the range of genetic bands found in the studied populations. In total there was four different designated alleles used in this study (alleles, A, B,C,D). Each

allele was designated according to how closely related the DNA migrated to the fragment size.

5.3 The absence and presence of alleles

Table 5a:*The presence and absence of each allele in all the four microsatellites*

CODE	SSA17 A	SSA17 B	SSA17 C	SSA17 D	STR60A	T3-13 A	T3-13 B	STRUTTA12 A	STRUTTA12 B
LL1	0	0	0	0	0	0	0	0	1
LL2	0	0	0	0	1	1	1	0	1
LL3	1	0	0	0	1	0	1	0	0
LL4	0	1	0	0	1	0	1	1	0
LL5	0	0	0	0	1	0	1	0	1
LL6	1	1	0	0	0	0	1	1	1
LLM1	0	0	0	0	1	0	1	1	1
LLM2	1	0	1	0	1	0	1	1	1
LLM3	1	0	0	0	1	1	0	1	1
LLM4	1	0	0	0	1	0	1	0	1
ELF1	0	0	1	0	1	0	1	1	1
ELF2	1	0	1	0	1	1	1	0	1
ELF3	0	0	1	0	1	0	1	0	1
ELF4	0	1	0	0	1	1	1	0	1
ELF5	0	0	1	0	1	1	1	0	1
ELF6	0	0	0	1	1	0	1	0	1
ELF7	0	0	0	1	1	1	1	1	1
SR1	0	0	0	0	1	0	0	0	0
SR2	0	0	1	1	1	0	0	1	1
SR3	0	1	0	0	0	0	0	1	1
SR4	0	0	0	0	1	0	0	0	0
SR5	0	0	0	0	1	0	0	0	0
SR6	0	0	0	0	1	0	0	0	1
SR7	0	0	0	1	0	0	1	0	1
LB1	0	0	0	0	1	0	1	0	1
LB2	1	0	0	1	1	0	1	0	1
LB3	1	0	0	0	1	0	1	0	1
LB4	1	0	0	0	1	0	1	0	1
LB5	1	0	0	1	1	0	1	0	1
LB6	1	0	0	1	1	0	1	0	1
LB7	1	0	0	0	1	0	1	0	1
LAS1	0	0	0	0	1	0	1	0	1
LAS2	0	0	0	0	0	1	0	0	1
LAS3	0	0	1	0	1	0	1	0	1
LAS4	0	0	0	0	0	1	0	0	1
LAS5	0	0	0	0	1	1	0	0	1
LAS6	0	0	1	1	1	0	0	0	1
LAS7	0	1	1	0	1	0	0	0	1

The presence and absence of bands are indicated as 1 for present and 0 for absent. Because microsatellite had more than one allele present, the smallest sized allele is labeled from ‘a’ increasing alphabetically.

Table 5 b: *The presence and absence of each allele in all the four microsatellites*

Code	STR60A	T3-13A	T3-13B	STRUTTA12A	STRUTTA12B
LNO1	1	0	1	0	1
LNO2	1	0	1	0	1
LNO3	1	0	1	0	1
LNO4	1	0	1	0	1
LNO5	1	0	1	0	1
LNO6	1	0	1	1	1
LNO7	1	0	0	0	1
LFM1	1	1	0	0	1
LFM2	1	1	1	0	1
LFM3	1	1	1	1	1
LFM4	1	0	1	1	1
LAM1	1	0	1	1	1
LAM2	1	0	1	1	1
LAM3	1	0	1	1	1
LAM4	1	0	1	1	1
LAM5	1	1	1	1	1
LAM6	1	1	1	1	1
LAM7	1	0	1	0	1

Table 5a and 5b shows the presence and absence of alleles of each individual sampled. The presence and absence of bands are indicated as 1 for present and 0 for absent. Because microsatellite had more than one allele present, the smallest sized allele is labeled from ‘a’ increasing alphabetically.

Microsatellites Stra60 and Stutta12 alleles were most frequent throughout sampled populations, with the exception of some individuals. From the 56 samples only 6 did not have any allele of the microsatellite Stra60 present 2 from LL, 2 from SR and 2 from LAS. A similar pattern is also seen in microsatellite Strutta12B; however only 5 individuals lack this allele 2 from LL and 3 from Sands river; these three brown trout from Sands river and LL2 also lack the presence of any allele from microsatellite Stutta12, a pattern which is not seen in the rest of the trout within the studied population. No data were obtained for Microsatellite SSA197 (Figure 6).

5.4 Statistical analysis

Table 6: *Person correlation for genetic diversity and genetic distance*

	Genetic diversity	**Genetic distance**
Area (km^2)		
Pearson correlation	-0.095	-0.171
P-Value	*0.840*	*0.458*
Altitude (m)		
Pearson correlation	-0.173	0.556
P-Value	*0.710*	*0.009*
Distance from nearest road (km)		
Pearson correlation	-0.714	-0.123
P-Value	0.072	0.596
Distance from nearest car park		
Pearson correlation	-0.431	-0.483
P-Value	*0.334*	*0.027*
Geographical distance (m)	----------	0.093
Pearson correlation		
P-Value		*0.687*

Results obtained from SR and LNO have been removed. Given that SR is a river values for the area variable, distance from nearest road (km) and distance from nearest car park (km) were not obtained. As the road crosses over river also it is not necessary to park as river can be approached by foot. In addition no there were no values for the area variable in LNO as samples were from the out flow of loch LNO.

Genetic diversity has a negative correlation with all variables, but the only significant correlation is with distance from nearest road (Km). A negative correlation is also

observed with genetic diversity and the following variables: Area, difference in distance from nearest road (Km), and difference in distance from nearest car par which is the only significant value. Both Geographical distance and altitude appear to have a positive correlation with Genetic distance, however only the altitude value has significance.

Table 7: *Multiple Regression analysis for genetic diversity and genetic distance*

	Genetic.diversity	**Genetic distance**
Area (km2)	0.182	0.888
Altitude (meters)	0.306	0.064
Distance from nearest road (km)	0.165	0.152
Distance from nearest car park	0.556	0.016
Geographical distance (meters)	**--------**	0.121
R-Sq	54.5%	41.0%

The regression equation is: Genetic diversity = 0.823 - 0.0287 Area (km2) + 0.000907 altitude (metres) - 0.0724 Distance from nearest road (km) - 0.0344 Distance from nearest car park

Again as in table 6, results obtained from SR and LNO have been removed because they were river sites and thus do not have a defined area. Equally, as linear features, distance to road' is also a problematic variable to compute. Given that SR is a river values for the area variable or distance from nearest road (km) as road crosses over river or distance from nearest car park (km) as it is not necessary to park as river can be approached by foot. In addition, there were no values for the area variable in LNO as samples were from outflow.

Results demonstrate genetic diversity shows to have no significant relationship with any of the variables, all p-values are >0.05. In contrast genetic distance appears to have moderate relationship with altitude and a strong relationship with difference in distance from car park.

Table 8*: Multiple Regression analysis for genetic diversity and genetic distance Data which included all populations*

	Genetic diversity	**Genetic distance**
Area (km^2)	**--------**	**--------**
Altitude (m)	0.620	0.127
Distance from nearest road (km)	0.136	0.233
Distance from nearest car park	0.358	0.384
Geographical distance (m)	**--------**	0.031
R^2	53.1%	26.9%

Results from all populations were included; however, results from variable area were removed as SR and LNO did not have values for this variable, as can be seen in table 8 the only significant relationship is genetic distance and geographical distance; however the R-sq value is moderately low. As geographical distance only influences 26.9% of genetic distance.

Table 9: *Spearman correlation for genetic diversity and genetic distance*

	Genetic diversity	Genetic distance
Distance from nearest car park		
Pearson correlation	0.117	--------
P-Value	*0.765*	
Distance from nearest road (km)		
Pearson correlation	0.000	-0.317
P-Value	1.000	0.063
Altitude (m)		--------
Pearson correlation	-0.350	
P-Value	0.356	
Area (km^2)		
Pearson correlation	-0.857	-0.205
P-Value	0.014	0.372
Geographical distance (m)		
Pearson correlation	--------	- 0.261
P-Value	--------	0.131

"-------" represents data that was parametric therefore results for these are found in table 6.As before the two populations SR and LNO have been removed as there are no values for the p area. The only significant correlation with the non-parametric data is genetic diversity and variable area.

6. Discussions

A total of 9 different alleles were found at the 4 microsatellite loci under study. Allele frequency and abundance varied between and within populations and, when genetic diversity and distance were analysed, they showed some evidence for variation with environmental and anthropogenic factors.

6.1 Genetic diversity and genetic distance

Both genetic diversity and genetic distance showed significant relationships with anthropogenic and environmental influences. Genetic distance quantifies the degree of similarity between the groups of individuals (brown trout); (Lowe *et al.,* 2004). The results indicated that genetic distance is influenced by altitude(*r* value = -0.556), distance from the nearest car par (*P*-value= 0.016) and geographical distance (*P*-value = 0.031). Genetic diversity varied between all the populations used for this study and a significant relationship existed between genetic diversity and distance from the nearest road (*r* value = -0.714) and loch area (*r* value= -0.857). Genetic diversity measures and estimate the amount of variation that is found in a population (Lowe *et al.,* 2004). In nature, selection favours those traits that adapt a population to local environmental conditions (Jonsson &J onsson, 2011).Thus genetic diversity is required for populations to evolve in response to environmental changes (Reed and Fankham, 2003). This may be of particular relevance as concerns regarding effects of climate change increase; it has long been recognised that communities at high altitude face a rapidly-changing milieu as temperatures increase (Woodward *et al., 2010).* From an angling perspective and in the current context, this is not insignificant, as genetic diversity is also directly important because it results in angling diversity in terms of size, run timing and behaviour(Harris and Milner, 2006).

6.2 Ecological and geographical influences

6.2.1 Altitude

Although similar studies have indicated that altitude influences the levels of diversity in freshwater habitats (Ostergren and Nilsson, 2011), this study show that altitude has only a weak correlation (r- value= 0.173) with genetic diversity. Normally, high-altitude populations are expected to be more physically isolated, either because of increased probability of physical barriers to gene flow (e.g., impassable waterfalls) and/or due to more pronounced founder effects, assuming that the number of colonists decreased with altitude (Castric *et al*., 2001), due to impossibility of trout to leap over high altitude but be able to migrate down a waterfalls. Loch LAS has the highest altitude and the

lowest genetic diversity; however Sands River has the lowest altitude but does not show the highest genetic diversity (Table 3 in the appendix B, going against the expected pattern. As most rivers (including Sands River, Cunningham, *perscomm*), have an anadromous population of brown trout, they tend to be more genetically diverse. These can often have potentially larger population sizes than in small, dystrophic hill lochs because of the larger river habitat and the potential for anadromous feeding(Ostergren and Nilsson, 2011).A brown trout population which migrates to the sea is expected to have a higher gene flow because of the connectivity through the open sea environment (Bakke, 2011). This can take place through straying (inaccurate homing) of spawning sea trout that do not return to their natal streams, but spawn in other rivers. This introduces novel alleles, leading to the increase of genetic diversity in the receiving population and a lower genetic distance from the donor population (Bakke, 2011). Although , successful straying rates, in terms of gene flow, among most brown trout populations are probably less than 1% (Castle 2006). Sands River (SR) population may act as a donor population (population which strays into nearby lochs via connecting watercourses), as SR does not have the highest genetic diversity. Possible reasons for such low diversity in fish populations may be because gene flow could be restricted as a result of a very accurate homing (Ostergren and Nilsson, 2011). These results may suggest a genetic bottleneck; rapid loss of genetic variation in the derived founder population can occur under the combined effect of genetic bottleneck and genetic drift (Launey *et a*.,2010).

When considering geological features of importance which may influence genetic structures of fish populations, commonly subdivision of the stream into tributaries and the presence of waterfalls (Carlsson *et al.,* 1999) are firstly considered. However, the geological context and nutrient status of the river or loch (table 5; appendix B) may also have a great impact on a fish population genetic structure. Sands River (SR) is a small, oligotrophic (conductivity = ~96 µS) river; based upon Torridonian sandstone (poor in mineral nutrient), limiting food supply and available trout territorie sand production (Cunningham, 2007).

Genetic distance among brown trout populations increased with the increase in altitude (table 6). Genetic distance is the frequency of recombination between two loci (Passarge, 2007). Brown trout at lower altitude are likely be more genetically similar, the low gene flow in high-altitude areas could be attributed to geographic isolation (Pradee *et al.,2011*) by impassable waterfalls; the low gene flow is due to incapacity of brown trout to leap over hill lochs above waterfalls that are inaccessible to sea trout

(Neville *et al.,2006)*or any brown trout population at a low altitude (Pettersson *et al.,*2001). Trout within high altitude there is a greater possibility of homogeneity to occur, population at lower altitude have the genetic benefit of heterogeneity. Due to this possibility, trout may be lower in numbers at high altitudes, and trout's in lower altitudes possibly may have larger population size due to the migratory ability of the species.

6.2.2 Area

It is logistically problematic to assess population size in fish (Heath *et al.,* 2001), therefore habitat size is commonly used to infer population census size and thus diversity (Castric *etal.,* 2001). Thus, surface area of the loch is used as an estimator of population size. A positive correlation between population size and genetic diversity is theoretically expected (Avise, 1994; Amos & Harwood, 1998; Bouzat et al., 1998). There was absence of correlation between area and both genetic diversity and distance (tables 6 and 7) and a similar observation has been observed in another salmonid fish (charr) (Castric *et al*., 2001). The lack of a trend between genetic diversity and habitat size (area) could be the result of factors such as the difficulty of reliably quantifying habitat size; the loch area alone may not accurately reflect the complex ecological interactions that determine the carrying capacity of lacustrine habitats for brown trout (or any other salmolid fish). For instance, the availability of spawning grounds (Blanchfield and Ridgway, 1997) and the relative abundance of other species (Magnan, 1988) could also influence the abundance of Salmonids in different lakes (lochs). Alternatively, the absence of correlation between habitat size and genetic diversity may indicate the persistent effect of founder events. (Castric*et al*., 2001).

With further analysis (table 9), both SR and LNO are rivers so could not be included, within correlations between sample site area and genetic data area. Data from remaining, loch, sites were non-parametric; so were retested using Spearman rank correlation. Results obtained indicate a strong correlation(*r* value= - 0.857 *p* value= 0.014) between area and genetic diversity. The assumption that habitat and effective population size are correlated tested the hypothesis that loch area should be positively correlated with the levels of intra-population genetic diversity (Castric *et al*., 2001; Launey et *al.,* 2010). However, the strong correlation that was obtained was negative (r value= -0.857) apparently suggesting, the smaller the loch area the higher the trout population genetic diversity. This initially seems counterintuitive but a solution to this apparent contradiction may occur when taking into account values obtained for

genetic diversity, in which the smallest loch (Elfs Loch) has the largest value for genetic diversity (0.85). Trout populations tested in this study showed a narrow range of genetic diversity (D = 0.69 to 0.85).

This loch has the smallest area, a relatively high altitude compared with lochs with a lower genetic diversity (e.g. LLM and LL) and a longer distance (2.99 km) to the nearest car park. All these suggest a restricted environment with a small population(supported by the initial data from preliminary capture, mark recapture data, could be as low as 60+ individuals , Kett *pers comm.* 2012) with a potentially sensitivity to environmental changes. This, in turn, implies fluctuation of population size, which may also reduce genetic diversity further (Shrimpton and Heath 2003). Studies (Frankham, 1996; Ostergren and Nilsson, 2011) have shown that, under normal circumstances, isolated brown trout populations have significantly lower genetic diversity and higher genetic distance (F_{ST}) resulting from higher genetic drift in small, isolated populations. Elf's Loch, however, is small, isolated from the ocean, has low connectivity with other watercourses/bodies but still displays high genetic diversity. In the absence of extraordinary natural events, the other explanation for such high genetic diversity may be anthropogenic introduction of brown trout from other lochs by. Elf's Loch has characteristics of a site with a low population so anglers, recognising this may possibly have used this loch as a site of introduction of small brown trout from different lochs. This type of movement of fish will be described in more detail when the "Satellite Movement" model is discussed.

6.2.3 Geographical distance and gene flow

Genetic and geographical distance show only weak and non-significant correlation (tables 6 p- value, 0.687 and table 7, p-value 0.121). Isolation by distance (IBD) describes the tendency of individuals to find mates from nearby populations rather than distant populations (Handley *et al.,* 2007) but these data do not support presence of an IBD effect among sampled trout populations.

The lack of positive correlation between geographical distance and genetic distance within and between brown trout populations has been observed in other studies (Nielsen *et al.,* 1996; Carlsson and Nilsson, 2001). This lack of correlation between

isolated populations also contrast with tagging experiments with anadromous trout, which show even in the case of non-isolated riverine populations straying brown trout, are most likely to ascend a river in close proximity to a natal river and thus bring in closely related genes(Hansen& Mensberg,1998). Hansen &Mensberg (1998) explain that studies which include populations which are effectively reproductively isolated from each other because of physical barriers (i.e, landlocked lochs), waterfalls, impassable dams, may experience genetic drift leading to stochastic changes of allele frequency without the homogenizing force of gene flow. The isolation by distance analysis of associations between the geographical and genetic distances (Hansen *et al*., 1998 Samuiloviene *et al.,* 2009,Bouza*et al.,* 1999) Estoup *et al.,(*2008) indicates that isolation-by-distance acts significantly on brown trout populations. However Hansen &Mensberg (1998) challenge the importance of correlation between genetic and geographical distance, explaining that distances may not necessarily be valid; due to the life history of brown trout; studies have shown that anadromous populations are generally more variable than resident population (Hansen and Mensberg(1998) cited ; Ferguson *et al.,* 1995; Tonteri *et al*.,2007). This outcome is ascribed to gene flow within anadromous populations, effectively rendering each anadromous brown trout (sea trout) population part of a large meta-population. Furthermore, since gene flow may in principle take place among all sea trout population, the genetic structure may differ considerably from that of populations of purely resident and landlocked trout in isolated lochs (and rivers or lakes) (Hansen &Mensberg, 1998). However, this evidence seems to be limited as several studies have shown isolation by distance patterns in anadromous trout population (Bouza *et al* 1999; Fritzner *et al .,*2001sited Moran *et al.,*1995; Hansen and Mensberg, 1998; Bouza *et al* 1999).

Not only that, once site area was excluded from the multiple regression as a variable (because Sands River and Loch na h-Oidhche sites were riverine and could not be accorded a specific area) evidence of isolation by distance is observed in this study, (multiple regression $p< 0.05$ $R^2 = 26.9$) i.e. when all 9 populations were included (table 8). Even though the coefficient of determination was low (R^2=26.9%); the gene flow among basins reflected a positive relationship with geographical distance. This trend was confirmed by the significant correlation observed between geographical and genetic distances, including all population pairs, which suggest a component of isolation by distance in these seven population. Colton and Bower (2002) points out that R^2 can be low, and meaningful relationships may still exist as other factors also influence. Thus this relationship would indicate a limited gene flow between locations (Ostergren and Nilsson, 2011) and strengthens the correlation between altitude and

genetic distance (table 6) suggesting that high altitude trout populations face significant barriers to inter-population gene flow. This observation (correlation) indicates an inter-population genetic differentiation, low gene flow between lochs, thus a discernible IBD pattern, i.e. that increasing geographical distance poses increasing difficulties for trout migrating from one loch to another (Gallucci *et al*.,2010).

Physical obstacles, however, are not the only barrier to gene flow between trout populations. Genetically differentiated salmonid populations can coexist without any physical obstacles interrupting gene flow. Precise homing to natal areas for spawning , combined with low frequency of straying , can result in reproductive isolation maintaining differentiation (Carlssone *t al* 1999). However, even though their homing is very precise, some gene flow usually occurs among populations via straying (Hansen & Mensberg, 1998 and Carlson *et al.,* 1999). As a result, within population genetic diversity seems generally unrelated to geographical distance. This is evidenced by many populations of brown trout showing more genetic difference within populations living in rivers a few kilometres apart in some cases in the same river system compared to populations that are 500 km apart (Moran *et a.,*1995)

Although there is some evidence of IBD effect and the is some evidence to suggest that environmental factors have played a role in determining genetic distance genetic diversity within the studied trout populations these are sufficient indefinite to admit the possibility of further factor; one of these factors is humans in influencing trout population genetic variation.

6.3 Anthropogenic influences

The Gairloch hill lochs and other surrounding water bodies are subject to continue angling pressures of a small but dedicated group of anglers for reasons specific aspects of anthropogenic influence and biogeography were examine to this study.

If anglers are assumed to be relatively “lazy” this is the pattern that may be expected ; The expected relationships would indicate that the genetic distance would decrease with the smallest difference in distance from the nearest car park or road; as distance from the nearest road or car park would decrease, there would be an increase in genetic diversity. This relationship may be an indication that anglers place the fish into

lochs nearest to a car park or road. This may be true when taking into consideration the geography of the area, where the terrain is rough and at high altitude. However this pattern is not observed, suggesting that perhaps anglers are taking the trouble to move fish further, in order to improve the blood or simple create super sized fish .

Genetic diversity correlated with the distance from nearest road (km) (r-value -0.714). A strong correlation and significant relationship was also observed between genetic distance and difference from nearest car park (km)(table 6 and 7). This suggests that anthropogenic influences may affect both genetic diversity and genetic distance of these populations.

These correlation between genetic diversity and distance from nearest road (table 6) and the correlation between genetic distance and difference in distance from the nearest car park (table 7) are both negative; these values therefore suggest that the greater the increase in distance (the further a loch is from the road, there will be a decrease in the genetic diversity, instead of an increase. The negative correlation of the genetic distance indicates that; the further the difference from the nearest car park, the greater the decrease in genetic distance.

A possible explanation for these results implies that this can be an indication that anglers do not place the fish into lochs closest to the road, but into those further away. These results suggest that anthropogenic influences may have a large impact on the genetic diversity and genetic distance of in fish populations. However, it is also possible that anglers are all taking trout from the some loch and placing then into the same isolated loch, therefore homology would occur and not genetic differentiation due to the movement of the same gene stock. There could also be a situation in which there is more than one big loch (figure 7), and small satellite lochs associated with it. With this situation there may be an increase in genetic diversity, as some anglers may place the trout from the nearby bigger loch where as other will bring the gene stock from a distance larger loch, all into the same small loch.

Some may question if the movement of trout from loch to loch may have an impact their genetic integrity, however Reed and Frankham(2003) explain that genetic diversity is required for populations to evolve in response to environmental changes also heterozygosity levels are linked directly to reduced population fitness via inbreeding depression. However a study by Fritzner*et al.,* (2001) indicated that genetic differentiation between rivers which have been stocked was non- significant, whereas non-stocked populations indicated that the genetic differentiation had highly significance. Also, stocking of trout from one river to the other had limited or no genetic impact on the indigenous population**.**

6.4 Satellite movement

Figure 7: Model of the satellite movement. The loch marked as “A” is the presumably the main source from which brown trout may be removed and placed into the surrounding lochs (marked in red (**.....**) and white circles). The Lochs surrounded by the red dotted circles are smaller in size, therefore they will probably, be the greater target of anglers for the placement of trout from the larger loch. The blue dotted line (**.....**) represent the lochs which may also be used as source lochs, which small trout are removed and placed into the small isolated lochs.

This is a recognised but largely unacknowledged (because it is illegal) phenomenon within the Highlands and involves movement of small brown trout from big lochs (where competition for scarce resource precludes rapid growth) into small isolated 'satellite' lochs to create 'super-size' trout(such as those marked in red,figure 7). Small, isolated hill lochs are often trout free – either because of poor colonisation potential or because they do not possess breeding substrata. Stocking small trout into these lochs, where they will have access to all the feeding resources and a minimum or no competition from other fish (and cannot breed), means they increase in size rapidly giving the anglers the opportunity to catch the same fish again and have it photographed as a highly prized 'super-sized ''trophy' fish.

Because the small loch chosen for this activity should, ideally, be trout free, because of the covert nature of the activity and because anglers tend to view such isolated lochs as 'private' and 'known only by them', the more isolated the loch, the greater the chances of anglers placing fish into them. Ironically – this can result in such tiny lochs being stocked (covertly) by several anglers – all convinced they have carried out unique and exclusive translocations (Peter Cunningham pers comm).In some cases trout can be transported significant distances for such translocations if anglers are convinced of the 'superior quality' of a specific population.

Thus, where results indicate that genetic diversity increases as the area of the lochs decrease it may well suggest that these negative correlations are due to satellite movement of brown trout by anglers. Where breeding is possible, this would lead to rapid population expansion and will be likely to retain genetic diversity from multiple founder events, through low genetic drift and higher gene flow (Hinder *et al.,1991* and Launey *at al.,*2010) On the other hand, Carlsson and Nilsson (2001) explain that over small geographic scales, populations may diverge in sympatry or allopatry from a common founding population by random genetic drift, selection, and to a lesser degree, mutation(Hansen and Loeschcke,1996).

The negative correlations obtained from genetic diversity and genetic distance with distance from road and car park are reinforced by the negative correlation with the genetic diversity and area. This also suggests that the phylogeography of hill loch brown trout is influenced by the method of satellite movement.

Weak relationships between genetic distance and geographical distance mentioned earlier may be an indication which can support the heavy impact of the stocking granted by the anglers , as a relationship between geographical and genetic distance

would not be expected amongst populations, which have had trout introduced from different lochs (Fritzner*et al .,*2001) If the significant relationship had a stronger R^2, it would suggest the stocking of brown trout by anglers may not have great impact on the breakage of the natural genetic population structure. This is supported by Fritzner*et al.,* 2001 findings, which indicate that populations do not mate randomly (Wahlund effect).

6.5 Connectivity of the lochs

Studies have shown that Connectivity of lochs indicates a potential influence on both the genetic diversity and genetic distance of fish population. Assessment of connectivity requires biologically realistic classifications of landscape structure (Neville *et al 2006*.,). As landscape characteristics (such as type of coast, accessibility of river mouth, distances between rivers, river length) play a role in shaping directions and rates of migration, and thus the genetic structure of the colonizing populations (Launey *et al., 2007)*.Many of the lochs in this study are connected (via small, occasionally temporary burns) through other bigger lochs or, potentially via anadromous genetic contact. In this case, however, data obtained from Jones (2011, unpublished), which analysed the probability of connectivity affecting the studied populations' genetic structure, indicated that connectivity of the lochs have no significant impact on these populations genetically.

6.6 Limitation of the study

With this study it is important to always recall the number of limited data set, Carlson and Nilson, 2001 point out that, one of the problems with a limited data set can result in the "family effect" for population genetic studies in fish. This can take place if only a few families are included in the study, if this takes places samples may be biased and give incorrect information about the true genetic structure of the studied populations. For this study, samples were collected from different locations within the Lochs and river, Carlsson and nilson, 2001 explain that in order to avoid this taking place, samples should consist of several cohorts. This is a way of avoiding a bias towards a few families and should be representative of the true genetic structure at each location (Carlsson and Nilsson, 2003).

Table 4a indicates microsatellite Str30 had a band range of 66-111; however the range of band size in this microsatellite is 87-111, which can possibly mean that any sample which may have a band size of 86 or below the absence of the target sequence in the template DNA. These miscalculated extra bands (or ghost bands) are a common

problem observed with microsatellite analysis. These usually arise due to misparing slippage of the DNA polymerase during copying of the di-nucleotide repeat units (McPerson and Moller, 2000). This can influence the final results giving an inaccurate reality of the genetic diversity or genetic distance of populations. Also another limitation of the study was the lack of a quantifiable area variable of two of the sampled populations (SR and LNO).

7 conclusions/ further research

Although study limitations may have influenced results, if the data obtained were considered in a larger scale they might have indicated the following suggestions:

The phylogeography of hill loch brown trout has different influences; these include anthropogenic, environmental and geographical factors. The evidence of the gathered data from the study concludes that the phylogeography of hill loch brown trout has geographical influences, as is expected in salmonid fish population due to their life cycle characteristics. The decrease of genetic distance with lochs located in high altitude is probably due to geographical barriers. This correlation would also be expected in genetic diversity, however, other studies have indicated that within a river or loch, brown trout form genetically diverse populations; the geological composing of a loch of river also plays a role on the genetic richness of the brown trout populations which may influence the Phylogeographic structure. The geological composition may also influence the lack of correlation on the connectivity of the lochs as straying individuals possibly would avoid lochs (or any aquatic habitat) in which the conditions were poor and lacked sufficient nutrition.

The obtained data for this study suggests that the genetic structure of hill loch brown trout is also influenced by the movement of trout from loch to loch by anglers; the negative relationship and correlation with genetic distance and distance from nearest car park and also the moderate negative correlation with genetic diversity and distance from nearest road (Km). Furthermore, the negative relationship with area and genetic diversity, suggests that brown trout are deliberately moved by anglers to produce larger fish, therefore it is unlikely that the movement is random.

With the gathered evidence, although the data is limited, further studies will focus on the extent of introgression when populations are mixed. Using a different method of investigation, where the independent variables used in this study are excluded; and the main variables considered would be the genetic similarity between a small isolated loch

and a large loch. Future investigation would focus on a new model of fish movement, which would investigate the hypothesis that fish are taken from large lochs and placed in nearby small isolated lochs that are thought by local anglers to be unpopulated. Thus considering, the genetic similarity of a brown trout in small lochs which surround a large loch (figure 7).

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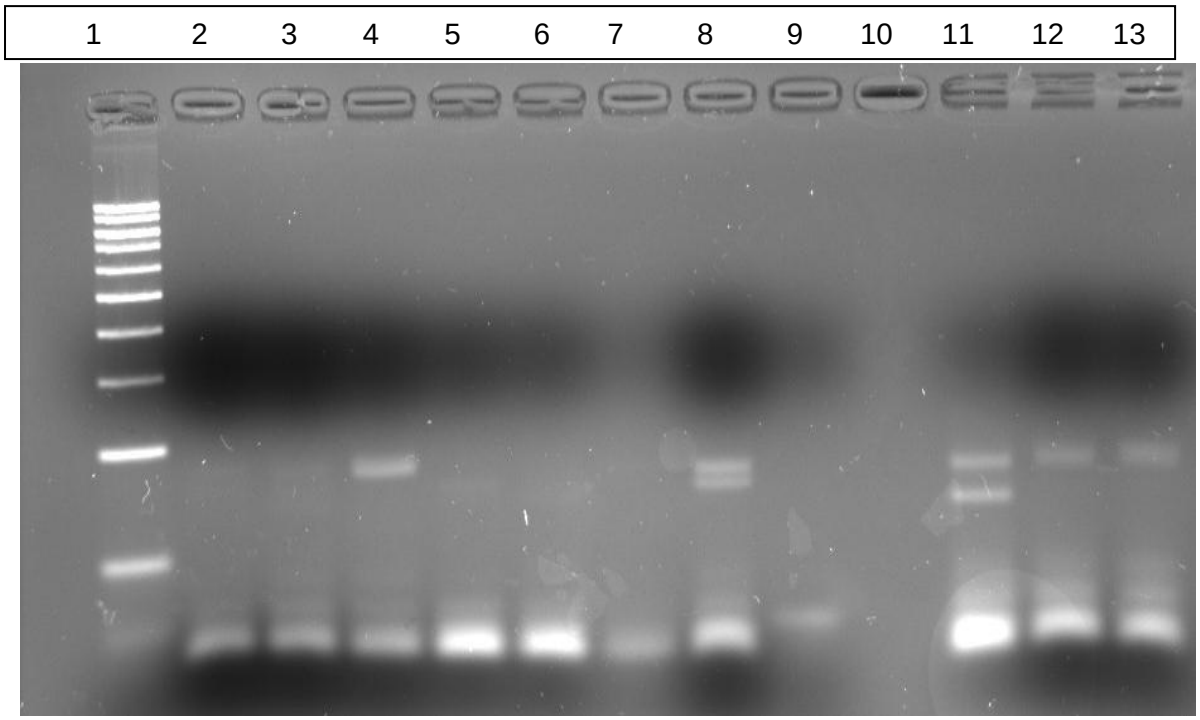
http://www.gub.ac.uk/bb-old/prodohl/TroutConcert/fr_moleculermarkers.htm#MtDNA%20markers

Appendix A

Microsatilite : SSA197



Ima ge 1: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch Laraig and Loch Laraig 'minor' relating to their genetic relationship using Microsatellite SSa197.

Table 1 : the layout of the possiton each product was placent into the gel in image 1

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Ladder	LL 1	LL 2	LL 3	LL 4	LL 5	-------	LL 6	NEG	LLm 1	LLm 2	LLm 3	LLm 4

Lane 1 ladder (HyperLadder™ IV) lane 2 to 6 Loch Laraig samples 1-6 (LL 1-5) lane 8(LL6) ,lane 9 negative control. Lane 10 to13 from Loch Laraig 'minor' samples 1-4 (LLm1-4).

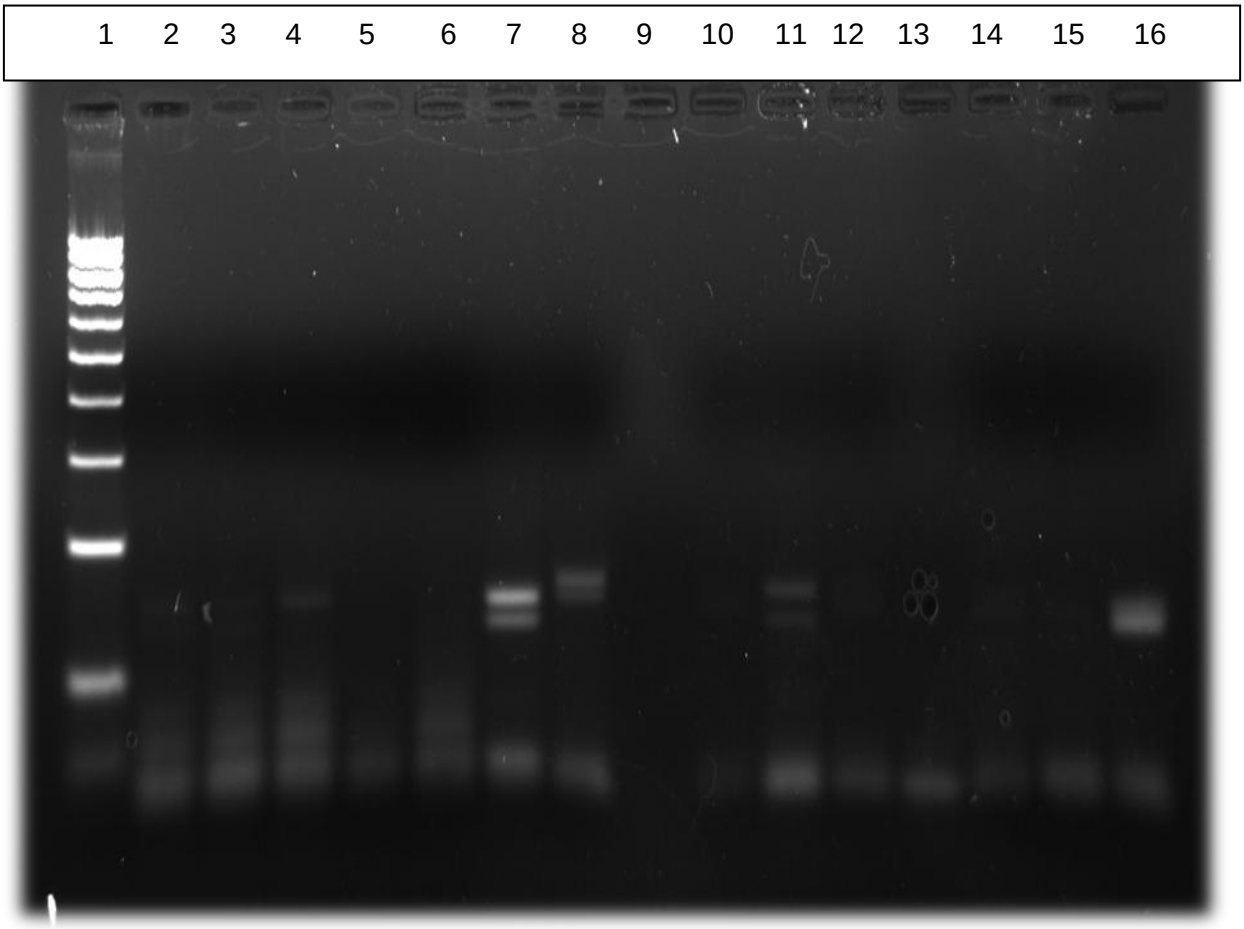


Image2: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch an Aird Shielg and Sands River relating to their genetic relationship using Microsatellite SSa197.

Table2: The layout of the possiton each apliconte was placent into the gel in image 2

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Ladder	LAS1	LAS2	LAS3	LAS4	LAS5	LAS6	LAS7	negive	SR1	SR2	SR3	SR4	SR5	SR6	SR7

Lane 1 ladder (HyperLadder™ IV)) lane 2 to 6 Loch an Aird Shielg samples 1-7 (LAS 1-7), lane 9 negative control. Lane 10 to16 samples from Sands River 1-7 (SR 1-7).

STR60

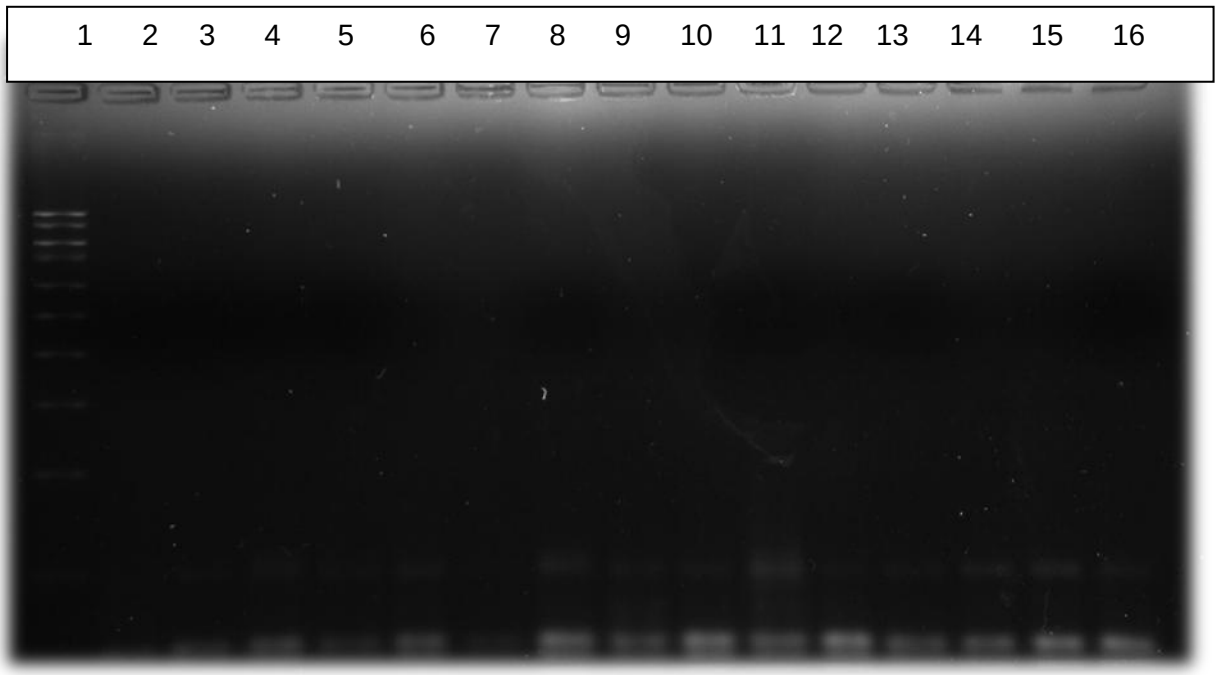


Image 3 Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch Laraig ,Loch Laraig 'minor' and Loch na Feithe Mingaig relating to their genetic relationship using Microsatellite Str60.

Table 3 The layout of the possiton each product was placent into the gel in image 2

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ladder	LL1	LL2	LL3	LL4	LL5	LL6	LLM1	LLM2	LLM3	LLM4	NEG	LFM1	LFM2	LFM3

Lane 1 ladder(HyperLadder™ IV) , lane 2 to 7 Loch Laraig samples 1-6 (LL 1-6); Lane 8 to11 from Loch Laraig 'minor' samples 1-4 (LLm1-4); Lane 12 negative control ; lane13 -16 samples from Loch na Feithe Mingaig(LFM1-4)

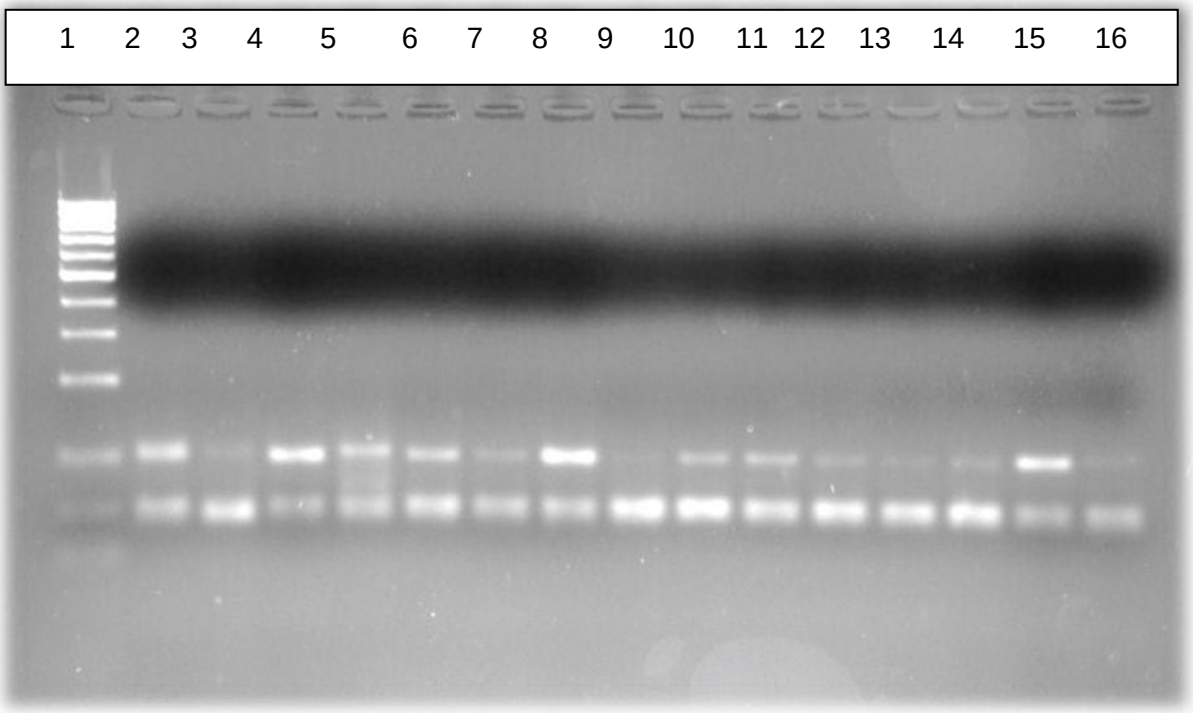


Figure 4 : Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Elf's Loch and Loch nan Buainichean relating to their genetic relationship using Microsatellite Str60.

Table4

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	ELF 1	ELF 2	ELF 3	ELF 4	ELF 5	ELF 6	ELF 7	NEG	LB 1	LB 2	LB 3	LB 4	LB 5	LB 6	LB 7

Lane 1 ladder (HyperLadder™ IV), lane 2 to 8 Elf's Loch 1-7 (ELF 1-7); Lane 9 negative control, lane 10 to16 from Loch nan Buainichean (LB 1-7).

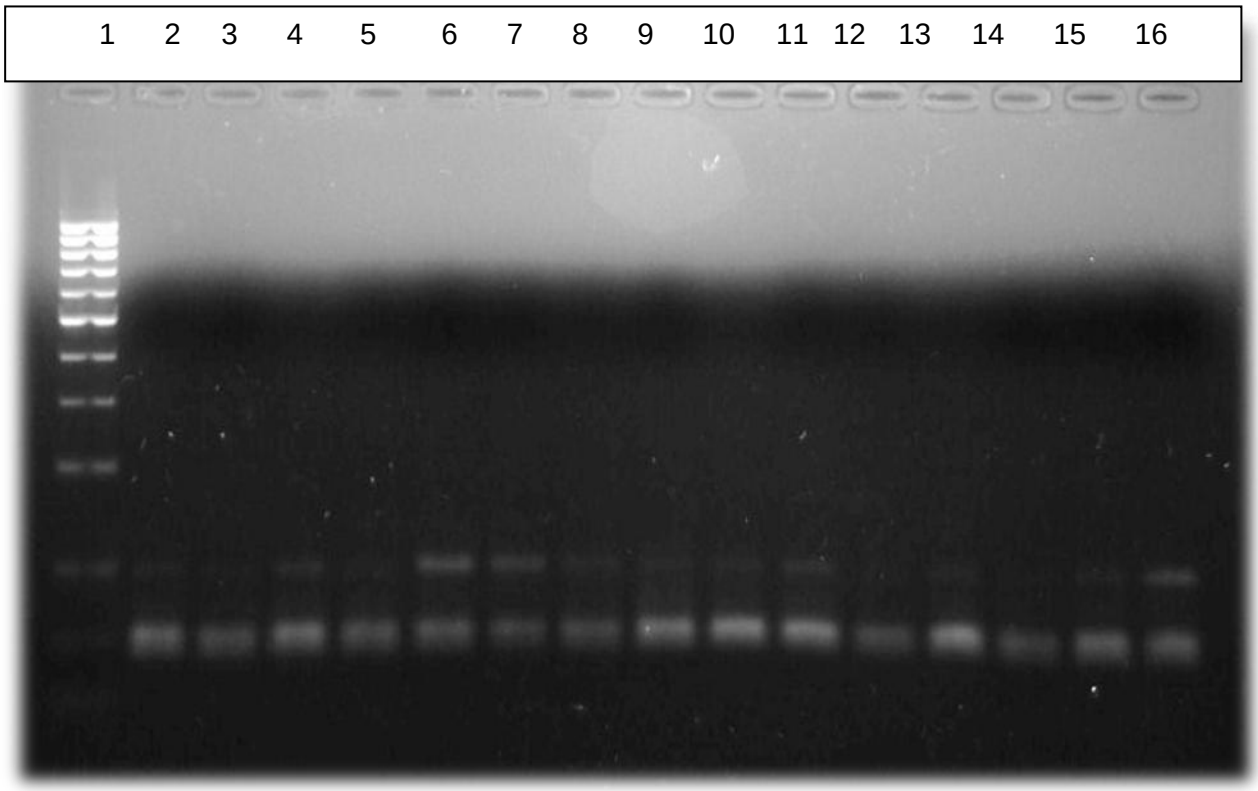


Figure 5 : Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch an Aird Shielg and Sands River relating to their genetic relationship using Microsatellite Str60.

Table 6: **The order in which products from PCR were placed into the gel electrophoresis in image5**

1	2	3	4	5	6	7	8	9	10	11
Ladder	LAS 1	LAS 2	LAS 3	LAS 4	LAS 5	LAS 6	LAS 7	NEG	SR 1	

Lane 1 ladder (HyperLadder™ IV), lane 2 to 8 Elf's Loch 1-7 (LAS 1-7); Lane 9 negative control, lane 10 to16 Sands River (SR 1-7).

T3-13

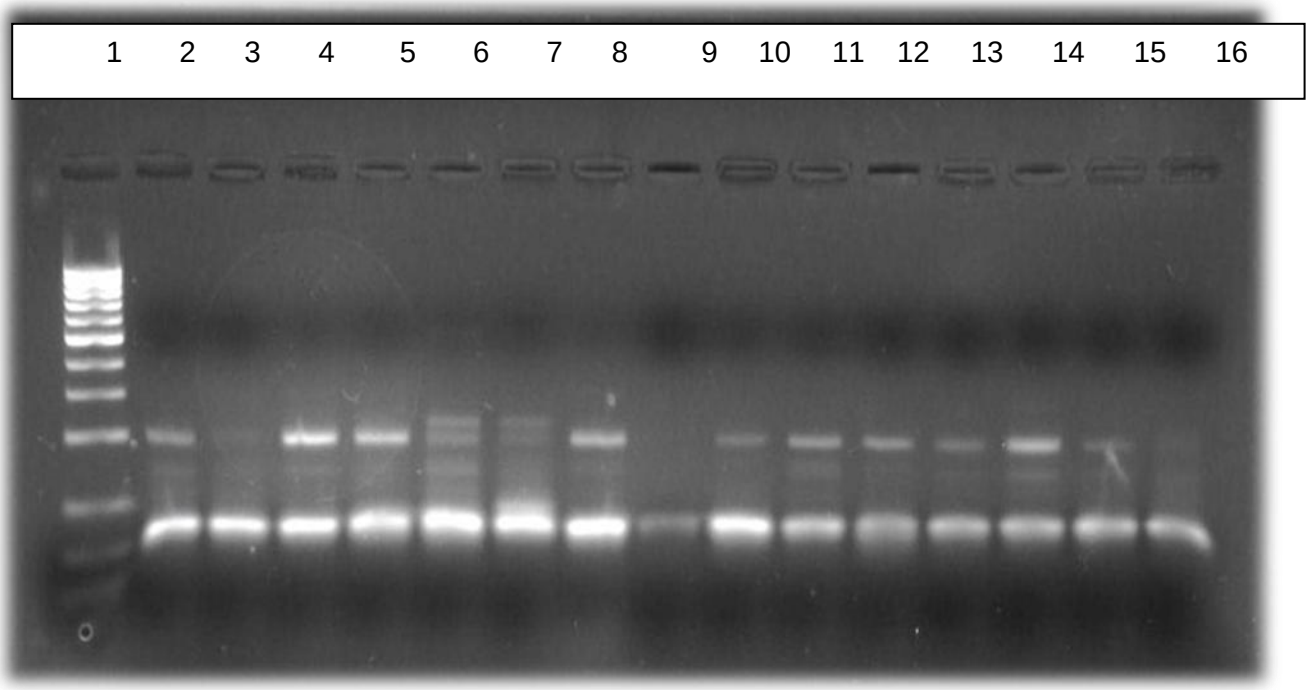
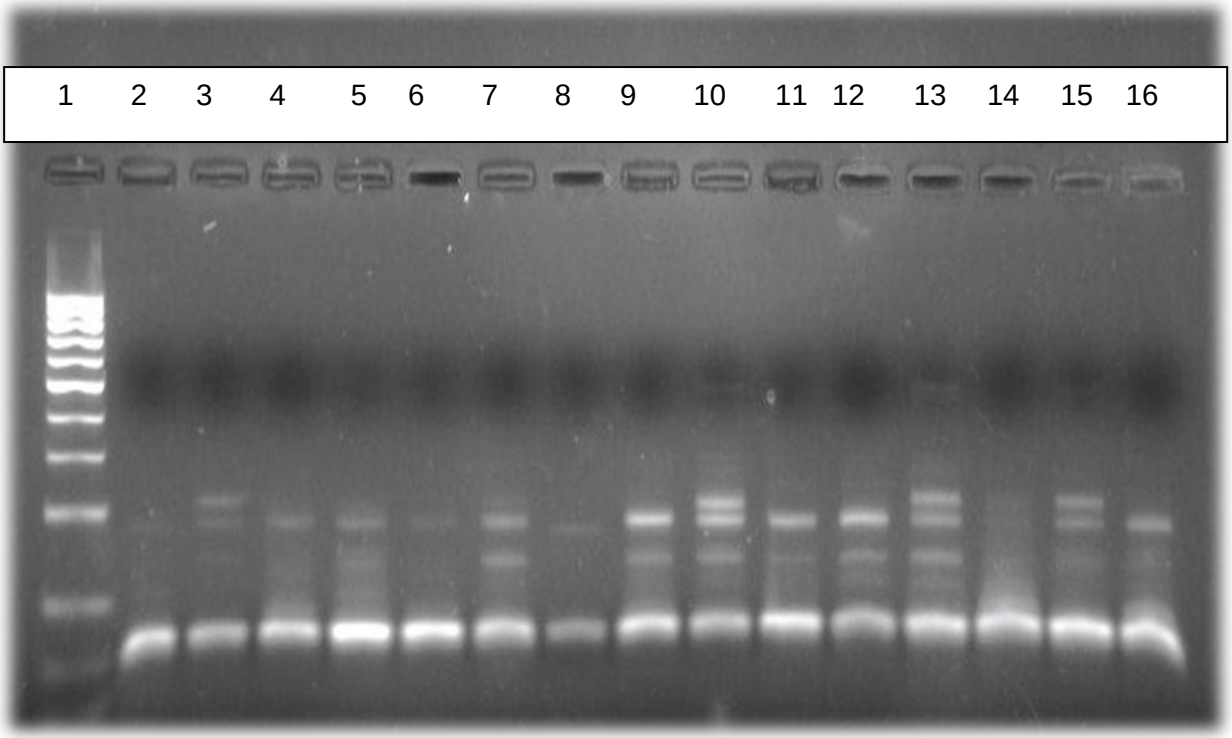


Figure 6: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch Atrigh Mhic Criadh and Loch na h-Oidhche outflow relating to their genetic relationship using Microsatellite T3-13.

Table 6: The order in which products from PCR were placed into the gel from figure 6

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	LAM 1	LAM 2	LAM 3	LAM 4	LAM 5	LAM 6	LAM 7	NEG	LNO 1	LNO 2	LNO 3	LNO 4	LNO 5	LNO 6	LNO 7

Lane 1 ladder(HyperLadder™ IV), lane 2 to 8 Loch Atrigh Mhic Criadh samples 1-7 (LAM 1-7); Lane 9 negative control, lane 10 to16 samples from Loch na h-Oidhche outflow (LNO 1-7).



Figuere 7: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch Laraig , Loch Laraig 'minor' and Loch na Feithe Mingaig outflow relating to their genetic relationship using Microsatellite T3-13.

Table7: The order in which products from PCR was placed into the gel electrophoresis inform figure7

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	LL 1	LL 2	LL 3	LL 4	LL 5	LL 6	LLm 1	LLm 2	LLm 3	LLm 4	NEG	LFM 1	LFM 2	LFM 3	LFM 4

Lane 1:ladder(HyperLadder™ IV), lane 2 to 7 Loch Laraig samples 1-6 (LL 1-6); Lane 8 -11 samples from Loch Laraig 'minor' (LLm 1-4) negative control lane12, lane 13 to16 samples from Feithe Mingaig (LFM 1-4).

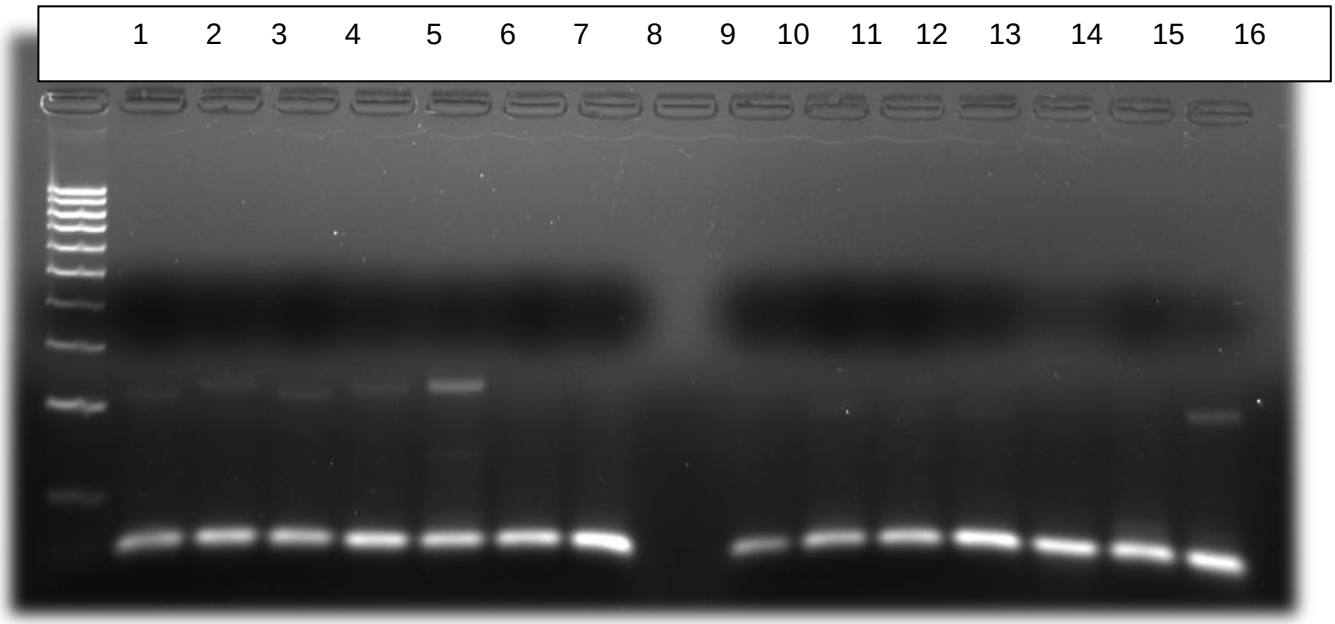


Figure 8: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Sands River and Loch an Aird Shielg outflow relating to their genetic relationship using Microsatellite T3-13.

Table 8: the position in which PCR products were placed into gel electrophoresis from figure8

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	LAS 1	LAS 2	LAS 3	LAS 4	LAS 5	LAS 6	LAS 7		SR 1	SR 2	SR 3	SR 4	SR 5	SR 6	SR 7

Lane 1 Biolader , lane 2 to 7 Loch an Aird Shielg samples 1-7 (LAS 1-7); Lane 9, empty, lane 10 to16 samples from Sands River (SR 1-7).

STRUTTA 12

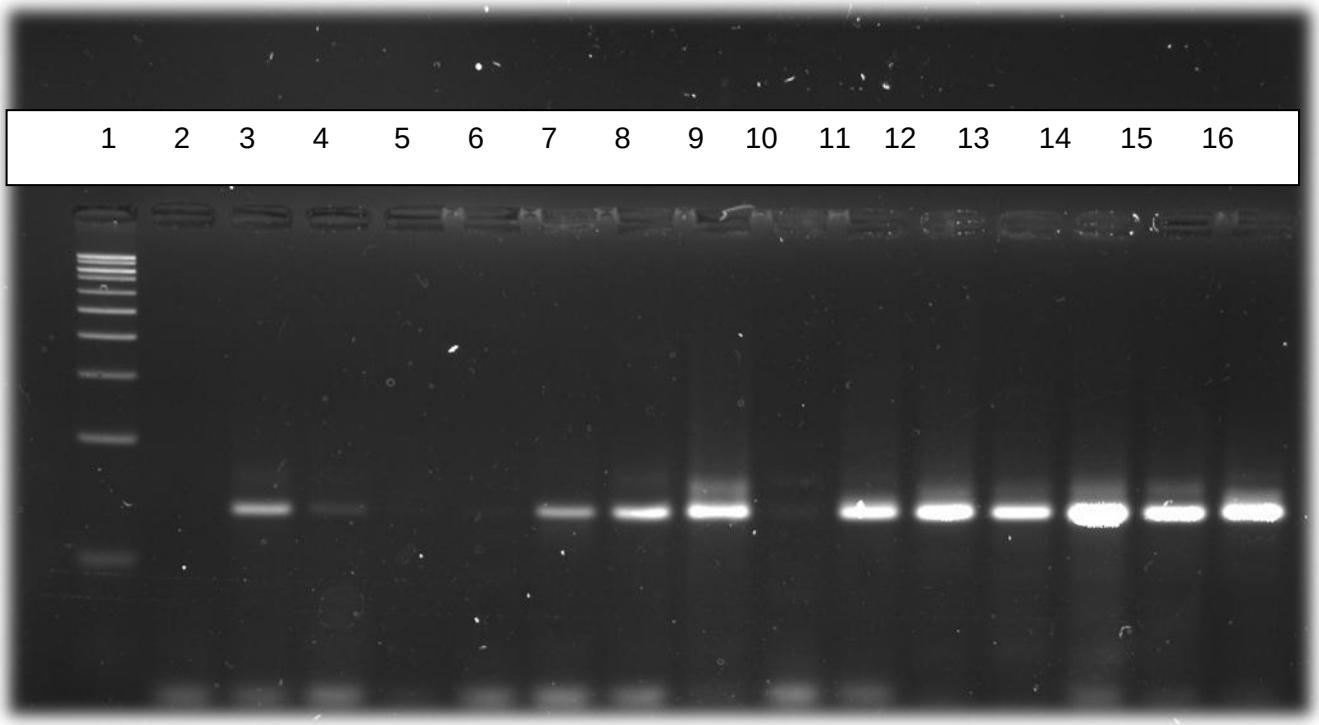
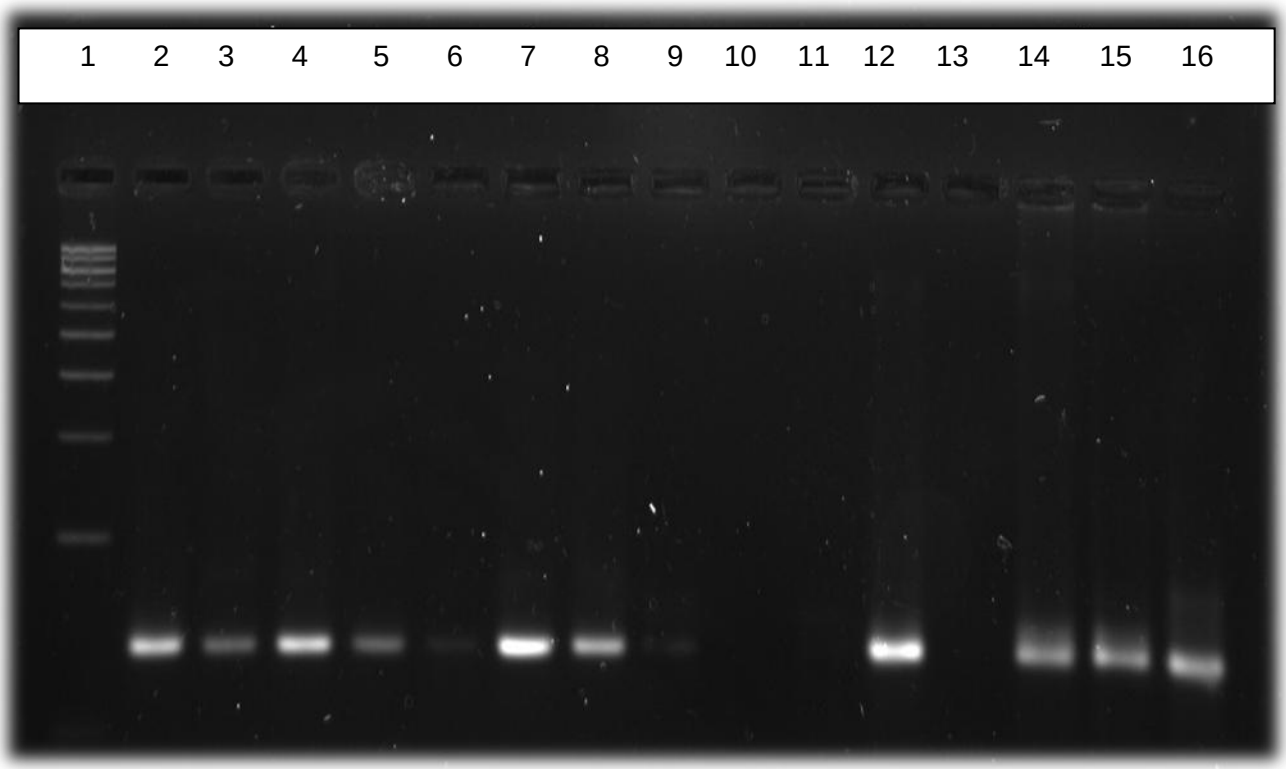


Figure 9: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout Sands River and Loch nan Buainichean outflow relating to their genetic relationship using Microsatellite Strutta 12.

Table9: The position in which PCR products were placed into gel electrophoresis in figure 9

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	SR1	SR2	SR3	SR4	SR5	SR6	SR7	NEG *	LB1	LB2	LB3	LB4	LB5	LB6	LB7

Lane 1 ; ladder(HyperLadder™ IV), lane 2 to 8 Sands River samples 1-7 (SR 1-7);
Lane 9, negative control lane 10 to16 samples 1-7from Loch nan Buainichean (LB1-7).



Figuere10: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch an Aird Shielg and Elf's Loch outflow relating to their genetic relationship using Microsatellite Strutta 12.

Table10: The position in which PCR products were placed into gel electrophoresis in figure 10

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	LAS1	LAS2	LAS3	LAS4	LAS5	LAS6	LAS7	NEG*	ELF1	ELF2	ELF3	ELF4	ELF5	ELF6	ELF7

Lane 1 Biolader , lane 2 to 8 Loch an Aird Shielg samples 1-7 (Las 1-7); Lane 9, negative control, lane 10 to16 samples 1-7 from Elf's Loch (EFL 1-7).

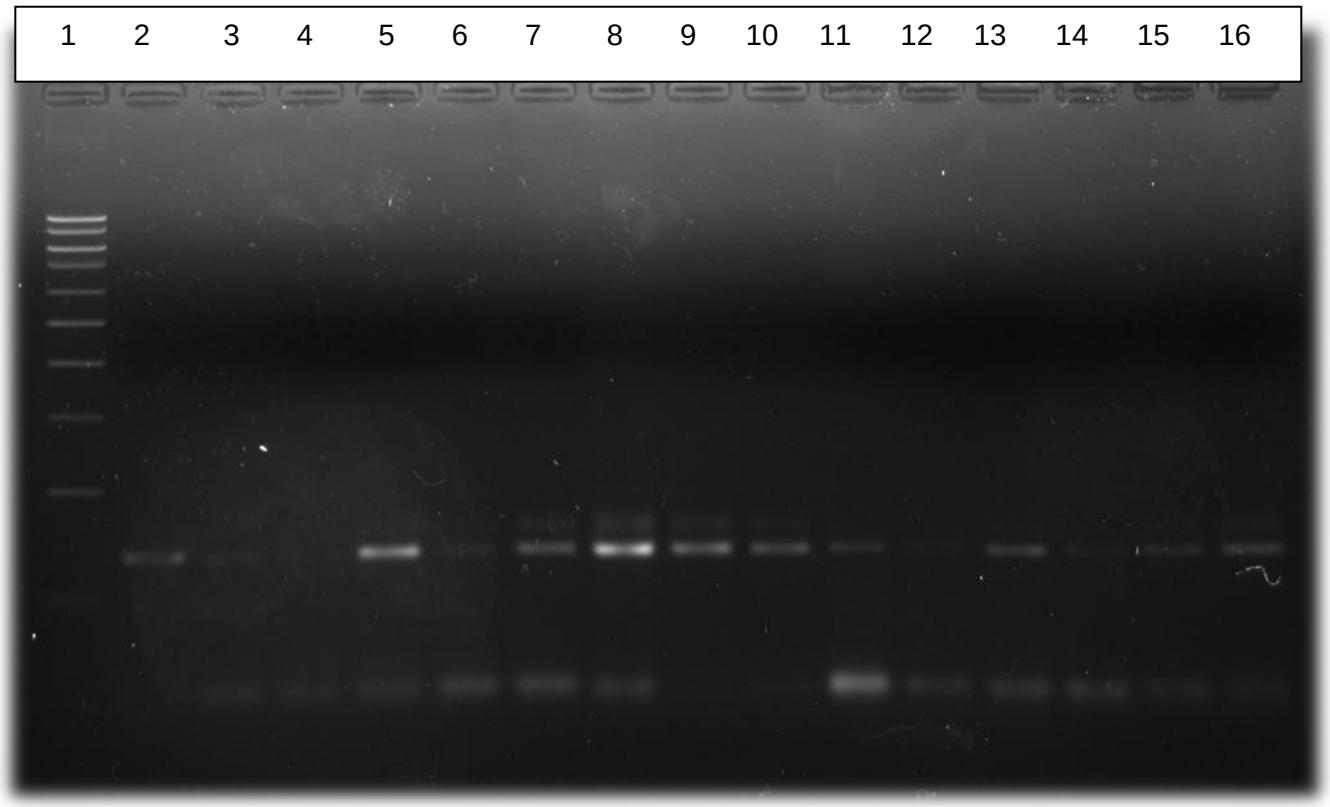


Figure 11: Gel electrophoresis of PCR showing presence or absence of alleles in brow trout in Loch Laraig, Loch Laraig 'minor' and Loch na Feithe Mingaig outflow relating to their genetic relationship using Microsatellite Strutta 12.

Table11: The position in which PCR products were placed into gel electrophoresis in figure 11

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ladder	LL 1	LL2	LL3	LL4	LL5	LL6	LLM1	LLM2	LLM3	LLM4	Negative	LFM1	LFM2	LFM3	LFM4

Lane 1 ; ladder(HyperLadder™ IV), lane 2 to 7 Loch Laraig samples 1-6 (LL 1-6); lanes 8-11, samples 1-4 from Loch Laraig 'minor' (LLM 1-4) Lane 12, negative control, lane 13 to16; samples 1-4 from Loch na Feithe Mingaig (LFM 1-4).