THE UNIVERSITY OF HULL

Spatial and temporal genetic variation in small and declining populations of Atlantic salmon (*Salmo salar*)

being a Thesis submitted for the Degree of Doctor of Philosophy in the University of Hull

by

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Dedicated to Mum and Dad

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Summary

Atlantic salmon is protected under national and European legislation but most populations are declining. The research investigated population size, habitat quality, population structure and stability over time using Atlantic salmon parr in the River Frome (a chalk stream in Dorset, England) surveyed at 15 sites, in summer and autumn over three consecutive years. The hypotheses were:

- Abundance of juvenile Atlantic salmon in a chalk stream is related to habitat quality.
- There is significant genetic differentiation between Atlantic salmon within one river, despite small geographic distance between sites and no barriers to migration.
- Temporal stability of spatial population structure of Atlantic salmon occurs within a river.

Habitat quality, assessed using HABSCORE, varied between sites and over time. Habitat quality was not a predictor of juvenile density but presence of adult brown trout reduced juvenile numbers. DNA for molecular analysis was extracted from fin-clips (removed non-lethally) and genetic variation and distribution of genetic variation was assessed using 5 microsatellites. Relatedness tests indicated that some 0+ parr within a site were closely related. It was inferred that 1+ parr had moved from their natal site, as they could not be assigned to a site of origin. Moderate heterozygosity was detected at each sample site. Low, but significant, genetic differentiation over all sites was detected in summer and autumn for three consecutive years (F_{ST} 0.023 to 0.047). Isolation by distance was detected at two out of the six sample times, despite small distances between sites. This was strong evidence for structuring within the population. Using a new statistical test, no temporal stability of spatial population structure was detected in the River Frome, but significant temporal stability was detected using published data for Atlantic salmon in a Canadian river.

Chapter 1

Introduction

1.1 Introduction

1.1.1 Atlantic salmon biology and distribution

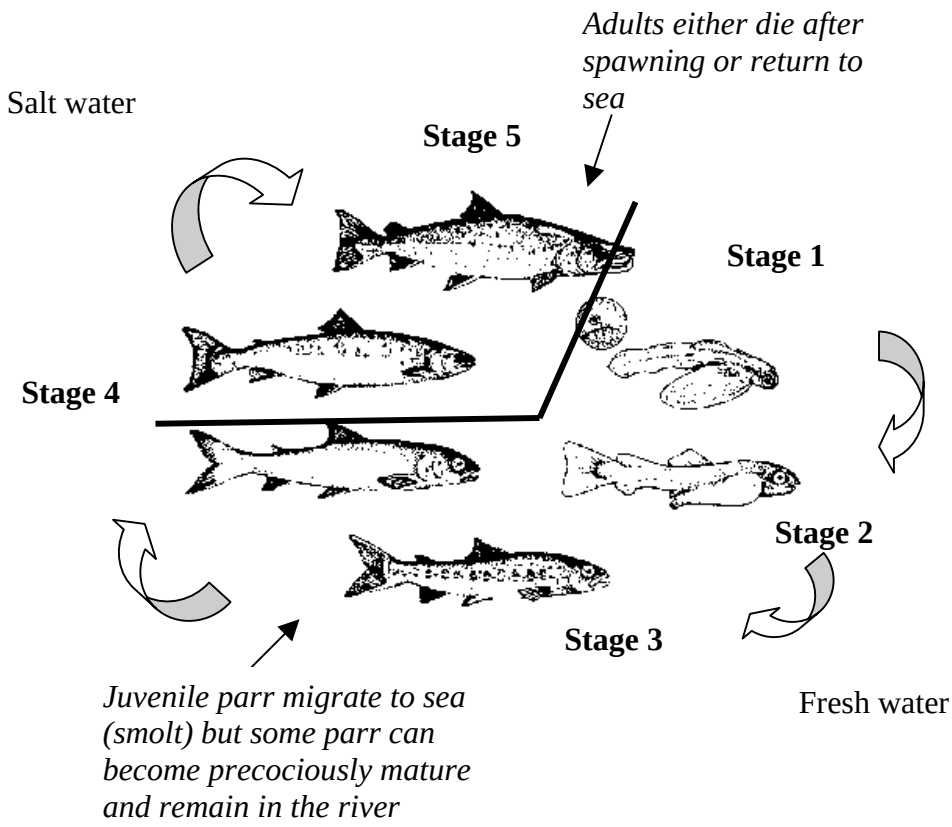
Atlantic salmon (*Salmo salar* L. 1758) is part of the family Salmonidae, which also contains the genera *Oncorhynchus* (Pacific salmon), *Salvelinus* (Charr), *Hucho, Brachymystax* and *Thymallus* (grayling). It is estimated that the salmonids diverged from a common ancestor approximately 25 to 100 million years ago (Allendorf and Thorgaard 1984). Classical salmonid phylogeny, constructed using morphological, karyological and ontogenic techniques (Mills 1989) placed the genus *Salmo* most closely to the genus *Oncorhynchus*. Atlantic salmon is most closely related to brown trout (*Salmo trutta* L 1758). More recent studies of taxonomy using molecular markers such as allozymes, mitochondrial (McVeigh and Davidson 1991), ribosomal (Phillips *et al.* 1992) and growth hormone DNA sequences (Oakley and Phillips 1999) indicated that that *S. salar* (but not *S. trutta*) may be closer to *Salvelinus* than to *Oncorhynchus*.

1.1.2 Life history overview

The Atlantic salmon life history influences its global distribution and population structure. The majority of Atlantic salmon populations have a life history of anadromous semelparity. This means that spawning occurs in rivers, the juveniles go out to sea and mature into adults and the adults feed at sea and return to rivers to spawn (Figure 1.1). It is accepted that adult Atlantic salmon home to the natal river to spawn (Mills 2000), thus each river is likely to be distinct from neighbouring populations. Almost all adults die after spawning, but some (normally females) can survive. Fish that survive spawning and return in subsequent years are iteroparous and spawning years can be detected from scale growth-ring pattern. In a review of empirical studies, Fleming (1998) found between 0.7% and 43% of Atlantic salmon populations were iteroparous. Reproductive success of adults is dependent on size (Garant *et al.* 2001) because larger females produce more eggs and larger males are more able to defend territory (Fleming 1998). The advantages of large breeding size are balanced by increased risk of mortality at sea as each extra year in the ocean incurs a 50-90% increased risk of mortality (Fleming 1998).

Not all Atlantic salmon go to sea to compete their lifecycle. Landlocked forms exist in some Scandinavian (Berg 1985 and Tessier *et al.* 1997) and Canadian lakes (Potvin and Bernatchez 2001), where adults migrate to a lake to feed. Landlocked populations have lower genetic variation compared with anadromous populations (Primmer *et al.* 2000) but higher genetic differentiation between sites (Tessier *et al.* 1997). Atlantic salmon from rivers on both sides of the Atlantic (with the exception of Baltic rivers and landlocked populations) migrate to the feeding grounds on the west coast of Greenland (Mills 2000). Most data on the distribution of adults at sea are from the location of commercial catches. Distribution may be affected by the location of prey such as crustaceans, pelagic fish and squid (Hansen and Quinn 1998). Salmon from the Baltic rivers do not migrate into the Atlantic Ocean but remain in the Southern Baltic Sea to feed (Hansen and Quinn 1998). Adults feed at sea for between one and six years then return to rivers. Grilse (one-sea winter) are around 50 cm long, whereas multi-sea winter fish can be at sea for up to 5 years and can be over 1 m long (Garant *et al.* 2001; Welton *et al.* 1999).

Salmon navigation mechanisms are not precisely understood. It is possible that salmon migrate towards land using magnetic orientation and then use recognition of physical and chemical characteristics to precisely locate the natal river (Quinn 1990). The earliest homing experiments were from 1653, when a ribbon was tied in the tail of smolts and the number of the marked fish returning as adults was observed (Mills 1989). In 1905, smolts on the River Tay were marked with ribbons and 1.7% of these were recaptured in the Tay as adults, though some were found in other rivers (Mills 1989) and in 1952 migrating smolts on two branches of a river were tagged and 80% of the original tagged fish returned to their natal branch (Mills 1989). Most of the missing fish will be due to mortality although some could have migrated to other rivers. Spawning occurs in winter and time of spawning can vary by up to four months but differs between rivers, depending on temperature (Fleming 1998). Within river, however, the spawning time is short, with most salmon spawning within a two-week period. Females construct nests in ‘redds’ in gravel in areas of moderate water velocity and depth (Fleming *et al.* 1996). Adult Atlantic salmon do not feed in river and expend about 60% of their lifetime energy budget in spawning (Fleming 1998). After spawning, fish loose condition and are called kelts. Most kelts die a couple of weeks after spawning from disease (Mills 1989).



Not to scale. Picture from Mills (1989).

1. Alevin Atlantic salmon hatch in river. The juveniles are called 'alevin' from hatching to end of dependence on the yolk sac as the primary source of nutrition.

2. Fry Stage from independence of the yolk sac until dispersal from the redd (redd: gravel area where eggs are deposited).

3. Parr Stage from dispersal from the redd, to migration as a smolt. Some parr (mostly males) become precociously mature and can breed with adults females.

4. Smolt Fish migrating out of the river towards the sea.

5. Salmon All fish at the end of the first winter in the sea. Fish return from the sea after 1-5 years. Semelparous adults die after spawning, but some adults survive (iteroparous), migrate to sea and return to spawn again.

Figure 1.1 Life cycle of Atlantic salmon.

Figure 1.2 Atlantic salmon parr

1.1.3 Atlantic salmon juveniles

When Atlantic salmon juveniles hatch from the eggs they remain at the redd site until initial passive dispersal at about two weeks (Mjolnerod *et al.* 1999). At the first free-moving stage, juveniles are called 'fry' and after this stage they are called 'parr' (Figure 1.2). Parr remain in the river for between one and five years (Mills 1989), and parr of more than one cohort can be present at a site (Beacham and Dempson 1998; Fontaine *et al.* 1997). Different growth rates were detected between juvenile Atlantic salmon within a site (Webb *et al.* 2001) and between sites (Heggberget and Lund 1986). Differences may be genetically controlled and under selection (Wilson *et al.* 2003) or may be influenced by environmental factors, for example, Riddell *et al.* (1981) found that juveniles have slower growth in colder, northerly rivers where there is less food. Some parr (mostly males) can become precociously mature and have the ability to spawn by 'sneak mating' with adult salmon. At less than 20 cm in length, precocious parr are much smaller than adults so do not directly compete with adults and can avoid detection by remaining quiescent and by adopting cryptic colouration (Fleming 1998). Morán *et al.* (1996) observed precocious mating at high frequency in a semi-natural system and in some populations 100% of parr are precocious (Fleming 1998). Precocious parr can migrate to sea and return to spawn again as full-sized adults (Mills 1989).

Experiments show that juveniles can move quite a long distance from the natal site: using artificial nests in a natural stream, Webb *et al.* (2001) detected dispersal of up to 1 km, mainly downstream, by 17 weeks after hatching. Work on tagged juveniles

in a semi-natural system in Scotland indicated that 80% established a home range within 8 days (Armstrong *et al.* 1999).

Before migrating to sea, juvenile Atlantic salmon undergo physiological changes necessary for survival in salt water, in a process called smoltation. Juveniles must reach a critical size (around 10 cm) before being able to smolt and timing of smoltation depends on latitude (Thorpe *et al.* 1998). Riley *et al.* (2002) used passive integrated transponder tagging of 86 Atlantic salmon juveniles in the River Itchen to investigate migration. Possible environmental cues for migration, such as flow rate or temperature, were measured. Migration occurred between October and March and all fish had left the river by 29th April. Smolts tended to move in darkness, possibly to avoid predators, however, no relationship was detected between migration and flow rate or temperature. There are some data for smolt movement in estuaries (Moore *et al.* 1998) but there are limited data on movement and survival at sea until fish are captured as adults on the feeding grounds off Greenland (Friedland 1998).

1.1.4 Atlantic salmon natural range

In North America, Atlantic salmon occurs in rivers from the north coast of Quebec, Canada, south to the Connecticut River, USA. In Europe, Atlantic salmon is found in rivers from the Ural Mountains and all countries to the west including Northwest Russia, Norway, Ireland, Iceland and Greenland. The southerly limit in Europe is the rivers on the border between Portugal and Spain. In the UK, Atlantic salmon is found in rivers in Northern Ireland, Scotland, Northern England, Wales and the West and Southwest Coasts of England. There is fewer Atlantic salmon in rivers on the east coast of England due to loss of populations (Figure 1.3).

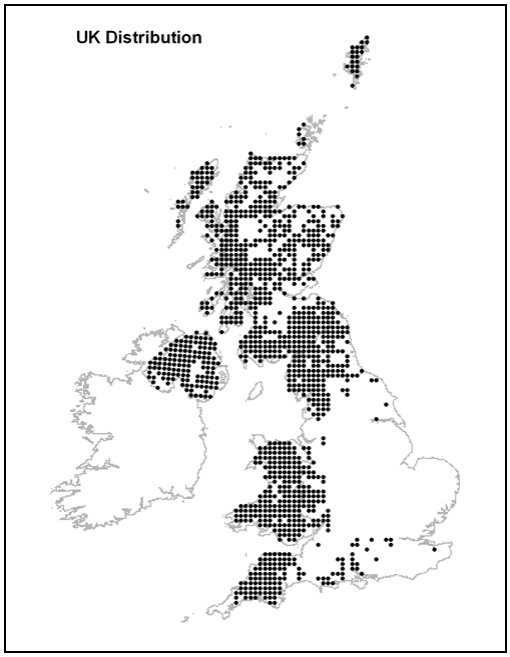


Figure 1.3 Distribution of Atlantic salmon in the UK
Image from Joint Nature Conservancy Council.

1.2 Atlantic salmon importance and decline

1.2.1 Decline and re-introduction

Wild Atlantic salmon have been lost from 294 rivers worldwide (World Wildlife Fund 2001). In Europe, Atlantic salmon disappeared from rivers in Germany, Switzerland, Netherlands, Belgium and the Czech Republic by 1960. Parrish *et al.* (1998) found generally that populations were relatively stable in northerly regions, have declined in temperate latitudes and have been lost at the southerly limits. Commercial fisheries and river angling catch data indicate how serious the decline had been. Since 1970 the commercial catch at sea has declined by 80% (World Wildlife Fund 2001). This severe decline was partly due to tighter fishing regulations imposed to prevent further loss, but reconstruction models indicate that commercial catch of grilse declined by 46% and commercial catch of two sea-winter salmon declined by 65% (Mills 2000). In rivers, the decline of multi-sea winter salmon is of great concern. For example, in the River Eden, U.K., spring fish (mostly multi-sea winter) declined from 80% of the rod catch between 1941 and 1972 to around 10% of the total from 1987 to 2002 (data from Environment Agency, Penrith). Many

threats to wild Atlantic salmon have now been recognised and stringent regulations to protect remaining stocks have been imposed to prevent exploitation at sea and in rivers (see Section 1.3). In certain rivers, mitigation actions such as improvement of water quality have led to the recovery of salmon and salmon have been re-introduced to some rivers where the stock was lost completely. For example, Atlantic salmon were completely eradicated from major European rivers, such as the Rhine in Germany and the Thames, UK, due to industrial pollution. Atlantic salmon disappeared from the Thames in 1821 and by the late 1970s it was suggested that the water quality had been sufficiently improved to attempt to reintroduce salmon. The Thames Salmon Rehabilitation Scheme (run by the Environment Agency) was launched in 1979. However, it is not certain if any of the fish stocked as juveniles spawn successfully, therefore the river has to be stocked each year, at a cost of £6 million (to 2006).

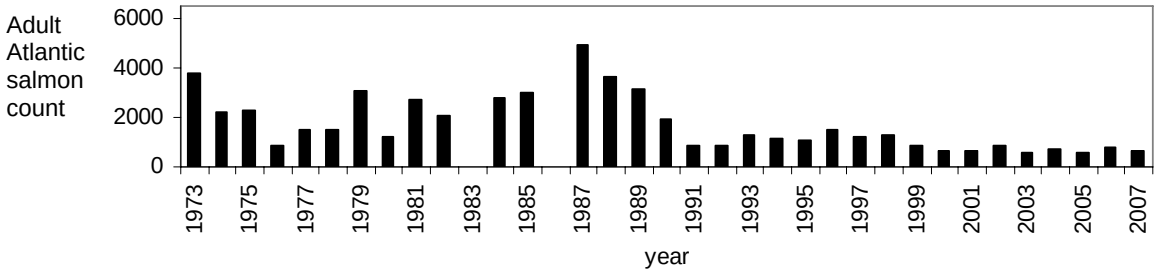
1.2.2 Atlantic salmon population decline in the River Frome and nearby rivers

From the 14th Century, adult salmon were intercepted by a stone weir located at the tidal limit at Wareham, (for location see site map Figure 2.1) and records indicate that the population size has declined in the past due to human activities. Salmon were nearly obliterated by 1850, and use of a weir was prohibited in 1861 (Solomon 2000) which allowed the population to recover. Since 1973, the number of adult Atlantic salmon ascending the River Frome has been measured using a fish counter on the river at East Stoke (Grid reference SY870867). Between 1973 and the end of the 1980s, there were fluctuations between years (Figure 1.4) but the average number was around 2500 each year, with over 4000 adults in 1997 and 1988. Before 1990, there were years of low adult return, for example in 1976 (this was correlated with low flow) but the population recovered to over 3000 within three years. Since 1991, the number of adults had reduced to less than 1000 per annum and there is no sign of recovery. (In years 1983 and 1996, adult Atlantic salmon ascended the River Frome, but accurate data from the fish counter were not available for these years).

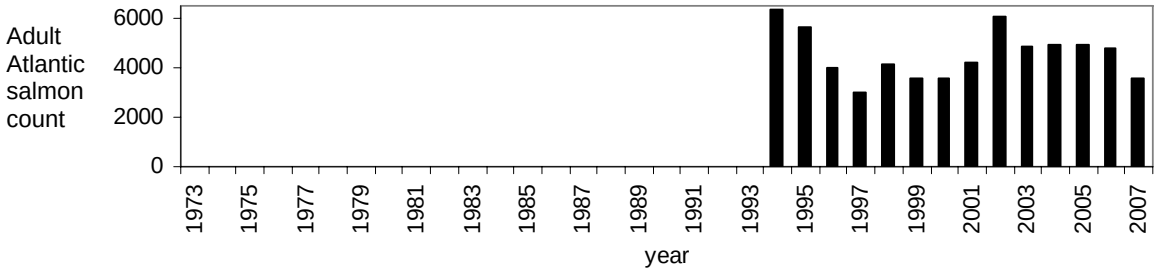
Although Atlantic salmon has declined in the River Frome, the population is larger than in many other rivers. The nearest rivers to the Frome for which there are data, are the rivers Test and Itchen to the east in Hampshire, and to the west, the River

Tamar. The Test and Itchen are chalk streams and thus have similar habitat to the Frome, whilst the Tamar is a granite river. There are differences in population size between years in the Tamar, Test and Itchen (Figure 1.4). The number of Atlantic salmon, averaged over 10 years, in the Frome was much higher than the Test and the Itchen, despite the Test being almost twice as large, measured by discharge (Table 1.1). The Tamar population is over 3.5 times larger than the Frome and this may reflect the Tamar having roughly 3.5 times higher discharge than the Frome.

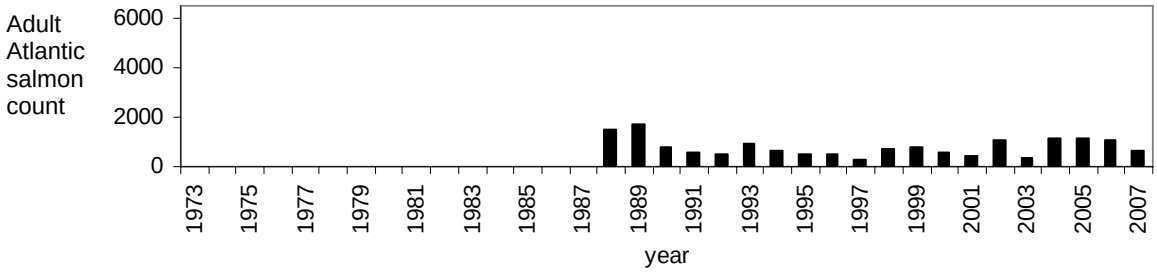
a) River Frome



b) River Tamar



c) River Test



d) River Itchen

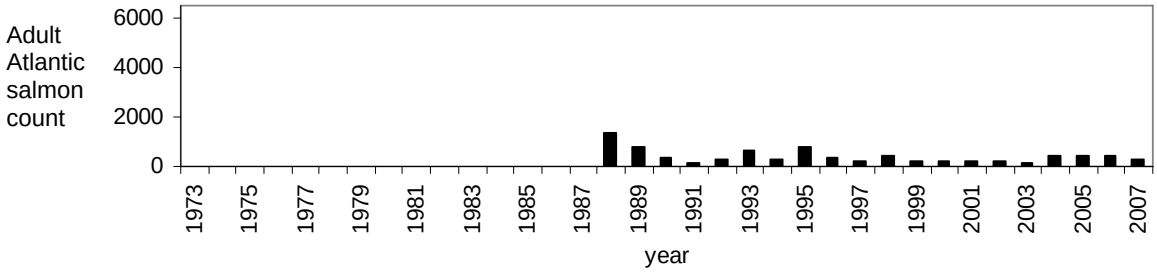


Figure 1.4 Adult Atlantic salmon fish counter data for a) River Frome (Welton 1999; Environment Agency 2007), b) River Tamar, c) River Test d) River Itchen (Environment Agency 2007), Southern England, UK.

Table 1.1 Adult Atlantic salmon counter data for the River Tamar (Environment Agency, 2003), River Frome (Welton *et. al.* 1999), River Test and River Itchen, UK (Environment Agency, 2003) and discharge m^3s^{-1} (www.ceh.ac.uk/data/nrfa/uk_gauging_station_network.html).

River	*Average Atlantic salmon adult count (time period)*	*Discharge*
Tamar	4500 (1994-2002)	22.61 m^{3s-1}
Frome	1165 (1991-2002)	6.43 m^{3s-1}
Test	710 (1991-2002)	11.02 m^{3s-1}
Itchen	386 (1991-2002)	5.37 m^{3s-1}

1.2.3 Importance and legislation

Atlantic salmon has high protection status at international, national and river levels, to mitigate population decline and to conserve the remaining populations (Potter *et al.* 2003). Since 1992, Atlantic salmon in fresh water only (excludes estuaries and the sea), has been protected by law under Annexe II of the European Habitats Directive (http://europa.eu.int/comm/environment/nature, 92/43/EEC). In the UK, Atlantic salmon is now a Priority species under the 2007 Biodiversity Action Plan (www.ukbap.org.uk), which sets out criteria that populations need to meet to receive recognition and the targets that will help to maintain and enhance current populations. As well as the requirement to achieve favourable conservation status across all rivers, there is an additional obligation to meeting the conservation objectives of Special Areas of Conservation. For Atlantic salmon in the UK, the Special Areas of Conservation were selected as rivers with large populations across the geographical range of the species, taking into account variation in the ecological and hydrological characteristics of salmon rivers in the UK, and in the life-cycle strategies adopted by the salmon within them (www.jncc.gov.uk/page-1374). There are 18 Special Areas of Conservation and 4 additional Sites of Special Scientific Interest (SSSIs) designated for Atlantic salmon in England and the target is to achieve favourable status for Atlantic salmon in SSSIs by 2010.

1.3 Threats and mitigation

Threats to Atlantic salmon include commercial exploitation at sea, aquaculture, obstacles to up-river migration such as dams and weirs (which prevent adults from reaching spawning grounds) and destruction of habitat through pollution. Threats posed by over-fishing are now well-recognised but the threats posed by barriers to migration and aquaculture have yet to be effectively dealt with in all rivers.

1.3.1 Commercial fishing at sea

Although Atlantic salmon was observed off the West Coast of Greenland in 1935 (Reddin and Friedland 1999), it was not until the late 1950s that commercial exploitation began here (Mills 1989). Initially it was assumed that fish captured were of Greenland origin, however, there is only one river in Greenland with a run of Atlantic salmon. By the 1960s it became clear that the Greenland fishery was exploiting a mix of adults from both the United States and Canada, and European stocks. Tagging fish in their river of origin, and recapture of these fish in Greenland feeding areas provided evidence for this migration (Reddin and Friedland 1999).

The main problem of fishing adult Atlantic salmon at sea is that this reduces the number of adults returning to the natal rivers to spawn. If all the source populations (within river) were large and not at risk then it would be possible to set sustainable targets for fishing at sea. However, as adults from different rivers mix on the feeding rounds off Greenland, some of these adults may be from rivers with very low population sizes (100 or less fish). Fish from rivers with low population size are exploited at sea at the same rate as fish from rivers with high population size. It has now been recognised that mixed-stock fisheries are particularly damaging for anadromous species such as Atlantic salmon where the exploited stock is composed of populations from different rivers and mixed-stock fishing has been implicated in regional decline (Chen and Blair Holtby 2002). In response to the crash in Atlantic salmon at sea, first noticed in the 1970s, the North American Salmon Conservation Organisation (NASCO) pressurised governments to reduce or halt commercial fishing. Strict regulations were introduced in 1983 by the ‘Convention for the Conservation of Salmon in the North Atlantic Ocean’ enforced by NASCO. Catch

limits were set to allow the escape of a sufficient number of potential spawners to ensure future recruitment to maintain the stock (Dodson *et al.* 1998). In 1988 all commercial fishing for Atlantic salmon in Canadian coastal waters was halted.

1.3.2 The risks of aquaculture to wild Atlantic salmon

The consumer demand for Atlantic salmon as a relatively cheap food has lead to development of a farmed salmon industry which is substantial in both numbers of fish and economic impact. It is estimated that of all the Atlantic salmon in the world, over 94% are aquaculture fish (Gross 1998). Farming was developed in Norway in the 1960s and is now a multi-million pound industry in Europe and Canada (Cross 2000). Fish farms pose both direct threats (such as pollution and disease), and longer-term indirect threats to wild salmon (such as hybridisation). Salmon farms sited in estuaries can form a physical barrier to wild adult and juvenile salmon migration (Hindar *et al.* 1991). Aquaculture causes pollution to the local environment from the antibiotics, pigments, vitamins, pesticides and large amounts of fish waste that are released into the water. This pollution can affect the behaviour of wild salmon (Armstrong *et al.* 1998). Hatchery fish harbour parasites and disease, such as infectious salmon anaemia, which can be transmitted to wild fish (Bakke and Harris 1998).

Aquaculture salmon can escape from farm cages when the cages are damaged in storms or are attacked by seals. Farmed fish can rapidly disperse, for example, escaped farmed salmon from a river in N. Ireland were detected in 10 rivers in Scotland and N. Wales within 16 days of escape (Milner and Evans 2003). Escaped farmed fish threaten local populations by competition for resources and hybridisation. This is a problem because, although farmed and wild Atlantic salmon are the same species, there are behavioural and genetic differences between them. Farmed fish tend to be bigger and more aggressive (Fleming and Jonsson 1994 and Gross, 1998) out-competing the wild fish for food. Escaped adult farmed salmon can mate with wild fish and although reproductive success of farmed Atlantic salmon has been estimated at 16% of the native population (Fleming *et al.* 2000), this can still have a large impact because a) the farmed fish have very low genetic variation and b)

offspring may not learn the behavioural traits to return to the same river to breed, causing the population to die out over time.

Farmed fish normally have low genetic variation (Verspoor 1998) because they are bred from a small number of stock lines. If farmed fish with low genetic variation do breed with wild salmon then this can have the detrimental effect of reducing genetic variability of wild salmon (Cross 2000). Introduction of genes from farmed salmon can alter life history traits of wild salmon, such as return rates and number of years spent in river or at sea, if these traits are heritable (Crozier 1997 and Crozier 1998). Farmed salmon can also escape at the juvenile stage and if these escaped juveniles survive, they may breed in future and this was detected as allele frequency change in a wild salmon population (Clifford *et al.* 1998 and Crozier 2000).

1.3.3 Decline caused by barriers to migration

The construction of hydroelectric dams in the 1960s and 1970s caused the complete loss of Atlantic salmon in some rivers in America, Canada, Spain, Denmark, Finland, France and Sweden (Parrish *et al.* 1998). Dams prevent upstream migration by adults and juveniles can get cut up in the turbine blades (Mills 1989). Despite evidence of the detrimental effect of dams, for economic reasons there can be resistance to removing dams or constructing passes. For example, in Oregon, USA, construction of fish passes on 14 dams to aid recovery of Pacific salmon, would have cost $1 billion and was ruled out by the US Government (Mann and Plummer 2000).

1.3.4 Decline caused by pollution in rivers

Many rivers are affected by chemical and particulate pollution from agriculture, urbanisation and industry, and low flow rates caused by dams and water abstraction can exacerbate pollution problems. Particulate pollution reduces oxygen availability and siltation (particles of less than 2mm) causes mortality at the egg stage (Hilton *et al.* 2001 and Soulsby *et al.* 2001). Silt can be removed by cleaning the riverbed with high-pressure hoses (Hendry *et al.* 2003). Chemical pollution can alter salmon behaviour (Mackenzie 1999) and can form a barrier to adult trout migration and thus disrupt the pattern of population structuring (Welters and Bond, submitted). Acid

pollution affects Atlantic salmon in Northern Europe and North America. For example, in Norway, Atlantic salmon were lost from 25 rivers due to lethal acidity (Parrish *et al.* 1998).

1.3.5 Atlantic salmon decline in rivers caused by deliberate introduction of non-local salmon

Deliberate introduction or stocking of Atlantic salmon was carried out historically for sport fishing and to augment existing stocks and often records of the origin of stocked fish are imprecise. Introduction includes the movement of populations classified as the same species but considered to be genetically distinct (Rhymer and Simberloff 1996). Introduction can have direct negative consequences for small populations by displacement of native stock (Weiss and Schmutz 1999), competition for resources, alteration of natural predator-prey relationships and introduction of disease and parasites. Introduced fish may have different behavioural and life history traits, for example, a lower adult return rate has been detected in stocked fish (Crozier *et al.* 1997 and Verspoor and Garcia de Leaniz 1997). Stocked fish can have low genetic variability, for example, heterozygosity and number of alleles of stocked Atlantic salmon in a Canadian river were 12-26% lower than wild fish (Verspoor 1998). It is not always possible to determine if loss of variation was immediate or more recent (Reilly *et al.* 1999). Current stock transfer to English and Welsh rivers is strictly regulated by the Environment Agency (under the Salmon and Freshwater Fisheries Act 1975), and in North America, NASCO has strict guidelines on movement of fish.

1.3.6 Threats to Atlantic salmon from hybridisation with brown trout

Atlantic salmon and brown trout can occur naturally together, but do not normally breed together due to mate selection and spawning at different times (Heggberget *et al.* 1988). Hybridisation occurs if these barriers break down (Campton 1988) for example by precocious parr mating with heterospecific adults (Olsen *et al.* 2000 and Garcia-Vazquez *et al.* 2002). Hybridisation will have greater detrimental impact on small Atlantic salmon populations in contact with more abundant brown trout

populations (Rhymer and Simberloff 1996), although the impact of hybridisation will depend on the viability of hybrids and the extent of genetic introgression (Allendorf *et al.* 2001).

Natural Atlantic salmon/ brown trout hybrids were identified in British rivers at a rate of 0.4% by Payne *et al.* (1972) and similar rates have been detected in other countries (1.2% by Hurrell and Price, 1991 and 1.8% by Beland and Roberts, 1981). A hybridisation rate of 2.3% was detected in Spanish rivers (de Leaniz and Verspoor 1989) due to the presence of precocious parr (all 1+ and older juveniles were precocious). Where brown trout were introduced outside their natural range a hybridisation rate of 0.9% was detected (Verspoor and Jordan 1989). The abundance of hybrids may be underestimated because they are difficult to distinguish morphologically (Verspoor and Hammar 1991), however, high hybridisation rates (35-41%) were detected in Swedish rivers which had been stocked and contained a large percentage of Atlantic salmon precocious parr (Jansson and Ost 1997) and in a natural stream, Garcia-Vazquez *et al.* (2003) detected 67% survival of hybrids into the second year. Unless historic allele frequency data are available it can be difficult to identify hybrids using molecular markers.

1.4 Population structure and genetic diversity

Population structure in species such as migratory fish, is influenced by

- geographical distribution
- historical distribution and dispersal patterns
- geographical distance or physical barriers between populations
- habitat structure
- degree of dispersal or migration between sites i.e. amount of gene flow among populations
- behaviour such as mate selection
- historic genetic factors such as a genetic bottleneck
- genetic drift
- mutation

Some of these factors lead to increased genetic differentiation between populations and some serve to reduce genetic differentiation (after Frankham *et al.* 2002).

Populations with limited gene flow tend to diverge over time due to genetic drift. Genetic drift is the change in allele frequency over time due to stochastic transmission of alleles to the next generation. In small, isolated populations, genetic drift is considered to be the most important factor causing loss of genetic diversity (Lacy 1987 and Whitlock 2000). Natural selection will promote population divergence (Steinger *et al.* 2002), but gene flow will act to oppose this (Lenormand 2002). Migration of individuals between populations counteracts the effects of genetic drift (Lenormand 2002) and is generally greater than introduction of new alleles by mutation (Frankham *et al.* 2002).

Isolation, due to physical barriers, can also be expected to influence genetic variation. More isolated sites are expected to have lower genetic variation, all other factors (such as population size) being equal (Haenfling and Brandl 1998). Physical barriers and geographical distance between sites will reduce migration and therefore will tend to increase genetic differentiation between sites. Geographical distance between sites can lead to genetic differentiation known as isolation by distance,

where sites further apart tend to have higher genetic differentiation than neighbouring sites.

In extreme cases isolation between groups can lead to speciation i.e. complete differentiation between two groups, such that they are designated as separate species. In physically isolated populations, reduced gene flow coupled with natural selection can eventually lead to allopatric speciation (Utter 1981). Even in populations that are not completely physically isolated, genetic differentiation can still occur between groups due to reproductive isolation. Ultimately reproductive isolation will lead to sympatric speciation (Utter 1981). Divergence may occur rapidly, for example Hendry *et al.* (2000) reported reproductive isolation between two groups of sockeye salmon after only 13 generations.

Historical genetic factors will influence the current population structure. For example, when a species (re-) colonises a habitat, often only a few individuals found the new population. The population will grow in size over time, but genetic variation may remain low. This is known as the 'founder effect'. Founder effects can occur when a small number of individuals are bred to reintroduce populations into the wild. Higher differentiation between populations would be expected if current population sizes are low or if only a small number of individuals initially colonised each site. Low genetic variation in a population may be also be due to a past genetic bottleneck. The severity of a bottleneck effect is proportional to the smallest population size experienced and the number of generations over which the bottleneck occurred and populations that have passed through a severe bottleneck can show reduced ability to respond to evolutionary change (Amos and Balmford 2001).

Genetic diversity can be estimated using molecular markers. Using standard techniques it is possible to estimate genetic diversity in different populations (or even different species) and compare the level of diversity between different groups and to factors such as population size and fitness. Genetic diversity is often estimated as percentage heterozygosity. Individuals are homozygous if two identical alleles are present at a locus and are heterozygous if the alleles are different. New mutations introduce variation and new alleles can also be introduced by migration. The amount of genetic variation in a population is a therefore a balance between gain of alleles

from mutation and migration and loss of alleles by natural selection and genetic drift. The level of genetic diversity is important in small and declining populations, because low genetic diversity can be related to population decline. Population structure can be estimated from the distribution of genetic diversity among the population.

It is recognised that structuring below the species level occurs in Atlantic salmon, but to legislate for protection, the subgroups must be defined. Evolutionarily Significant Units (ESUs) (Fraser and Bernatchez 2001) were developed to give official status to subgroups and the ESU can be treated as a 'management unit' (Moritz 1994). ESUs are subject to debate because there is currently no standard amount of significant genetic differentiation required to delimit an ESU, either for conservation-regulation purposes or to define speciation (Dodson *et al.* 1998).

1.4.1 Genetic diversity in small and declining populations

Low genetic diversity can be correlated with low fitness and reduced fitness can in turn lead to a reduction in population size. Small, isolated populations with low genetic variation and inbreeding are more sensitive to stochastic factors than larger populations, resulting in further loss of genetic variation, leading to a reduced ability to adapt to evolutionary change which leads to further decline. This is known as an extinction vortex (Frankham *et al.* 2002). Fitness is a measure of number of surviving offspring in the next generation and can be inferred from sperm mobility, birth weight and by resistance to disease, predation and environmental stress. Significant correlation between fitness (or a component of fitness) and genetic diversity was detected in 34 studies of wild populations (Reed and Frankham 2003). The correlation between low genetic variation and reduced fitness in these populations could have been caused by few low quality individuals, however, using data for sea mammals and birds, Amos and Balmford (2001) concluded that the effect was spread throughout the population.

A reduction in fitness due to inbreeding is called inbreeding depression (Frankham, 2002). In small populations, inbreeding can contribute to population decline through the effects of low genetic variation. Many studies showed correlation between

reduced fitness and low genetic diversity in natural populations, but low genetic diversity is not always associated with reduced fitness (Frankham *et al.* 2002). Detection of reduced fitness with neutral markers may be due to a physical linkage between the marker and the fitness gene (Tsitrone *et al.* 2001).

Slate *et al.* (2000) found that heterozygosity was positively associated with lifetime breeding success in a wild population of red deer (*Cervus elaphus* L. 1758*)* on the Island of Rhum, Scotland. (Keller and Waller 2002) detected inbreeding depression in 13 bird species and 15 animal species and Crnokrak and Roff (1999) detected biologically significant inbreeding depression in 20 wild animal species and 15 plant species. Inbreeding effects in the wild are expected to be more severe if resources are limited and environmental conditions are unstable. A study of Granville fritillary (*Melitaea cinxia* L. 1758*)* butterflies indicated that the extinction risk increased with decreasing heterozygosity, even after accounting for ecological factors (Saccheri *et al.* 1988).

Loss of heterozygosity can lead to 'mutational meltdown' in populations with small effective population size (<100) and high inbreeding (Keller and Waller 2002) and some recessive homozygotes can be lethal (Hansson and Westerberg 2002). However, in populations with extreme inbreeding, loss of deleterious recessive alleles (purging) is sometimes used as reason to explain that that, despite low heterozygosity, the population is still viable (Wang 2000). At specific genetic loci, heterozygotes can be fitter than either homozygote, this is over-dominance.

Adaptation can be disrupted by gene flow, as introduced genes, which are not adapted to the local habitat, lead to a reduction in fitness called outbreeding depression. Outbreeding depression is difficult to quantify in the wild, unless data were available previous to the breakdown of spatial or temporal barriers. Reduction in fitness is often not immediately apparent. There is evidence that Atlantic salmon can actually avoid breeding with close relatives, increasing the heterozygosity of the offspring (Landry *et al.* 2001). In contrast to out-breeding depression, gene flow between isolated populations, each with low genetic variation, can sometimes result in an increase in fitness known as heterosis. This is because introduction of new alleles increases genetic variation and can increase fitness.

1.4.2 Estimation of genetic variability and population structure

Population structure is the way that genetic diversity is held within-individuals, between individuals and between populations and can be estimated using molecular markers. Many molecular methods are available to estimate the distribution of genetic variation. The ideal marker for this research would be variable and heterozygous, inherited in a Mendelian fashion, neutral with regards to natural selection and co-dominant.

The marker needs to be sufficiently variable to be able to discriminate between populations that may have potentially small levels of differentiation. The marker must be inherited with no male or female bias and also without linkage because this would be a deviation from expected Hardy-Weinberg ratios and would contravene the expectations of population statistics. Natural selection acts against the phenotype, so strictly speaking at the DNA level, natural selection can only act against coding sections of the DNA, i.e. genes. Non-coding markers cannot be directly selected against, however, non-coding sections may act as though they are under selection if they are linked to a gene.

For population genetics it is preferable to use non-coding markers (generally assumed to be neutral) to infer population structure rather than selection. Dominance is a term applied strictly speaking to genes: alleles have a dominance hierarchy and co-dominance indicates that both alleles contribute to the phenotype. For non-coding DNA the locus can have many alleles and of course these do not have a dominance hierarchy because they are neutral. On a gel, a homozygous individual will have one band and a heterozygous individual will have two bands. The ability to score two alleles per locus for an individual (e.g. for microsatellites) provides greater statistical power because within-individual variation can be estimated.

Microsatellites fulfil more of these criteria than many of the markers available, and in addition a number of microsatellite primers have already been developed specifically for Atlantic salmon, cutting down the time to set up the screening system and also possibly allowing for comparison of data across studies. RFLP, AFLP and RAPD were ruled out for use in this research because of the problems of

reproducibility and because they are not co-dominant (therefore can not use within-individual level of variance). Analysis of variation at allozyme loci has been extensively used in earlier studies to investigate global population structure of Atlantic salmon (Stahl 1998), European structure (Bourke *et al.* 1997) and between-river structure (McElligott and Cross 1991, Galvin *et al.* 1994 and Jordan *et al.* 1992). The low variability of allozymes means that they not useful for small scale genetic differentiation. For example Bourke *et al.* (1997) tested 32 allozyme loci stains in Atlantic salmon and found that only three were variable across Europe. Minisatellites have previously been used to investigate population structure in Atlantic salmon at the within-river level (Galvin *et al.* 1995, Galvin *et al.* 1996), but were not used for this research because of the difficulty in PCR amplification of larger fragments.

The most important feature of microsatellites for analysis population structure analysis is that they are assumed to be neutral with regards to natural selection. Microsatellites are highly variable and are therefore sensitive to small changes and are replicable. Microsatellites are inherited in a Mendelian fashion and are co-dominant, thus microsatellite data can be used to analyse population structure using standard population statistics packages (Chambers and MacAvoy 2000).

Microsatellites have a high mutation rate, thus in large populations, over a long time period, it is possible that new alleles detected in the population result from mutation and not migration. In a randomly mating population if the allele frequencies change between parents and offspring deviation from Hardy-Weinberg occurs but selection and drift are more likely than mutation to occur in one generation. One generation of random mating restores Hardy-Weinberg ratios. For neutral markers such as microsatellites, the number of alleles and allele frequency results from the interaction between mutation and genetic drift. The expected frequencies of homozygotes and heterozygotes in a randomly mating population are given by the Hardy-Weinberg equilibrium equation $p^2 + 2pq + q^2 = 1$ (Hartl and Clark 1989). Homozygotes are p^2 or q^2 and heterozygotes are 2pq. Hardy-Weinberg equilibrium would be expected in large-randomly mating populations, however deviations from Hardy-Weinberg occur due to genetic drift, migration, overlapping generations, assortative mating, unequal

male: female ratio, population subdivision, inbreeding and selection. Mutation and recombination can also affect allele frequencies detected. Statistical tests, known as F statistics were developed in to estimate genetic differentiation and this is discussed in detail in results Chapter 4 (section 4.1.8).

1.5 Outline of the research

The broad context of the research was driven by the following questions:

- Do Atlantic salmon breed at specific sites within the river, what influences this and is it stable over time?
- What within-river factors could contribute to observed population decline?
- Are there any conservation management suggestions that could be made to help reverse the situation?

The behaviour of adult Atlantic salmon on their return to river to breed is an important aspect of biology and can influence population structure. If there is population structuring, this inbreeding would cause the effective population size to be reduced, leading to reduced genetic variability, which could lead to reduced fitness. As discussed in section 1.4.1 these are all factors associated with small populations and can lead to further population decline. On the other hand, division of a population into subpopulations should not always be viewed in a negative framework: sections of the population may be adapted to the site and thus require protection to maintain the diversity of the entire system.

Salmon rivers vary from granite substrate to chalk substrate (Elliott *et al.* 1998), but even within one river there may be differences in habitat quality between sites and this could influence adult selection of spawning site. Most studies on Atlantic salmon are carried out in large rivers with large distances between spawning sites and potential differences in habitat quality between sites (see Chapter 4, sections 4.1.3 and 4.1.4 for a literature review of previous studies). Thus, in these rivers it would not be surprising to detect genetic differentiation between sites and even increased genetic differentiation with increasing spatial distance between sites (isolation by distance). However, what is potentially more interesting is to investigate a system

where there is limited habitat structure (e.g. no barriers to migration such as waterfalls) and small differences in habitat quality between sites, such as that found in chalk rivers (see Chapter 3, section 3.1.1 for a summary of the habitat characteristics of chalk rivers). It is possible in a situation such as this, that no population structuring would be detected, i.e. the fish returned randomly to spawn.

1.5.1 Selection of the study river

The River Frome, Dorset, UK was used in this research for a number of reasons:

- It is a chalk stream and thus has a different habitat structure from 'classic' salmon rivers on granite (see above).
- The river had a relatively healthy population of Atlantic salmon, allowing a good chance of there being some fish from which to take samples and also allowing the Environment Agency to permit sampling of juveniles (but not adults, see below).
- It has a known historic population size; this is important for comparing number of juveniles with number of adults and inferring past changes (see below).
- The river has not been stocked with Atlantic salmon from outside the system thus any population structure should be intact (reported by local anglers, the Environment Agency and CEH fish biologists).
- There are no known barriers to migration, either physical or chemical.
- There is good site access.

The research river was close to the lab, which meant it was easier to get local access permissions to the river bank and sampling could be carried out at a number of sites in one day. It was possible to the same sites in three years to take temporal replicates of the spatial samples. This was essential to investigate the distribution of genetic variation over time. Another consideration in using the River Frome was the population size of the adults, because, although this work was concerned with small and declining populations, it was important to have some fish to work with for statistical reliability. The Frome currently has an adult census size of around 1000 adults (Figure 1.4).

There were strict sampling restrictions on Atlantic salmon in the River Frome set by the Environment Agency, to protect the population. The Environment Agency did not allow sampling, radio-tracking or anything that could impede adults in any way (even though this is permitted on other rivers). Thus all the samples in this research were from juveniles. Although tracking and direct monitoring of Atlantic salmon was not permitted, the molecular analysis used in this research was a powerful tool to investigate both current genetic variation and population structure and to infer past genetic variation. Thorough long-term records of census size are very uncommon and so one of the deciding factors for working on the River Frome was the availability of adult return counts, dating back to 1973. These data were important to investigate potential genetic bottlenecks to census size and to estimate stock-recruitment (Chapter 3).

There were no barriers to migration in this system and this was important because the aim of this research was to investigate the potential for genetic structuring in the absence of obvious physical structuring of the habitat. The River Frome is rare compared with many other rivers in that it has never been stocked with fish from outside the system. Disruption of population structuring has been detected in other systems where fish have been introduced (Ayllon 2006, Vasemägi 2004).

1.5.2 Novel aspects of the research

Previous studies have estimated genetic variation between (Stahl 1983, Fontaine et al. 1997, O'Connell et al. 1995, King et al. 2001, Galvin et al. 1996, McElligott and Cross 1991, Stahl 1998, McConnell et al. 1997, Verspoor et al. 1999, McConnell et al. 1995, Verspoor et al. 2002, Nielsen et al. 1996, Bourke et al. 1997) and within rivers (Verspoor *et al*. 1991, Jordan *et al*. 1992, Garant *et al*. 2000, Beacham and Dempson 1998, Galvin *et al*. 1994, McElligott and Cross 1991 and Spidle 2001), but no detailed study of Atlantic salmon in a chalk stream has previously been made. The research aimed to investigate population structuring over a small geographical area, within one chalk stream. No previous study had specifically investigated the relationship between spatial and temporal genetic variation. Atlantic salmon parr were sampled at 15 sites (for map of sample sites see Figure 2.1) in summer and autumn for three consecutive years. Sampling with very small geographic distance

between sites would allow isolation by distance to be estimated in this system, where there were no obvious barriers to migration between sites. Although isolation by distance had been investigated in other systems, this had not previously been carried out in a chalk stream, where the habitat was likely to be relatively homogenous. Repeat sampling of the same sites in summer and autumn would allow juvenile migration between sites to be inferred. Many of the methods used in this research had previously been used in similar systems and thus the aim was not to develop new sampling or genetic methods, but rather to use established techniques with the emphasis on testing a different system. However, a novel statistic to estimate spatio-temporal genetic variation (the *Q*-test) was developed, which has a different rationale than existing tests. This is discussed in detail in Chapter 5.

1.6 Hypotheses and aims

The decline of Atlantic salmon in the River Frome is of conservation concern and work is needed to understand aspects of salmon biology, habitat and genetic diversity to reverse the decline. The research set out to test three hypotheses, each with specific aims and objectives:

Hypothesis 1: Abundance of juvenile Atlantic salmon in a chalk stream is related to habitat quality.
This was tested in Chapter 3.

> **The main aim** was to estimate habitat quality at 15 sites in the River Frome, to estimate abundance of Atlantic salmon juveniles at each site and to determine if habitat quality was a predictor of abundance.
>
> **The second aim** was to test the assumptions that there was no dispersal between sites between summer and autumn and that most parr were able to smolt after one year.
>
> **The third aim** was to investigate the relationship between predators and juvenile abundance and between adult population size, and juvenile abundance.

Hypothesis 2: There is significant genetic differentiation between Atlantic salmon within one river, despite small geographic distance between sites and no barriers to migration. This was tested in Chapter 4.

The main aim was to estimate genetic differentiation between sites and determine if isolation by distance occurred.

The second aim was to estimate the level of genetic variation, because low variation can be related to population decline.

The third aim was to determine if older parr had moved from the original site.

Hypothesis 3: Temporal stability of spatial population structure of Atlantic salmon occurs within a river.

This was tested in Chapter 5.

The **main aim** was to estimate temporal genetic differentiation and to determine if temporal stability of spatial population structure occurred.

The **second aim** was to estimate relatedness between individuals within a site. It was not expected that a high incidence of relatedness would be detected, however, it was important to test for this as relatedness would affect the conclusions made about spatial population structuring.

Chapter 2

Methods

2.1 Introduction

The sampling and genetic methods used in this research followed established protocols. For example, DNA extraction and estimation of genetic variability and population structure using microsatellites, were techniques that had been used before and were modified where necessary for this research. There are a plethora of statistical techniques available to estimate genetic variation and population structure and many statistics packages have been developed. For each test it was important to select an appropriate statistics package that used the most appropriate estimator (see section 2.3). Some of the statistical tests were modified for this research and a novel test, the *Q*-test, was devised specifically to investigate spatio-temporal population structure.

2.1.1 Sampling

The aim of the sampling was to investigate the distribution of Atlantic salmon juveniles in the River Frome and to determine parr abundance at each site. A small fin clip was removed from each fish for molecular analysis, at the same time as the sampling. This also served to mark the fish to estimate the recapture rate at the same site in the autumn survey. Habitat quality was assessed at each site, to investigate the relationship between habitat quality and juvenile abundance.

2.1.2 Sample site location

A large number of sites were sampled within one river, with small distances between sites. The distance between the furthest upstream and furthest downstream sites was approximately 50 km and the shortest distance between any two sites was 0.5 km. For practicality, sites were used that could be easily accessed from the bank.

Fifteen sites were selected on the River Frome, Dorset, UK, which covered the entire range of habitat where adult Atlantic salmon were known or expected to spawn. Spawning sites on the River Frome were obtained from redd counts and from angler's reports. Distribution maps of redd counts for 1980/1981 and 1982/1983 (carried out by Wessex Water Authority) indicated that suitable spawning sites

occurred over the entire river between West Holme, (site 1) 5 km upstream from the tidal limit at Wareham, to Muckleford Bridge (site 15), 5 km upstream of Dorchester (Solomon 2000). In addition to sites on the main channel, it was known that Atlantic salmon spawned in a tributary, the Tadnoll Brook, therefore this site was included (site 9-TN) (Figure 2.1).

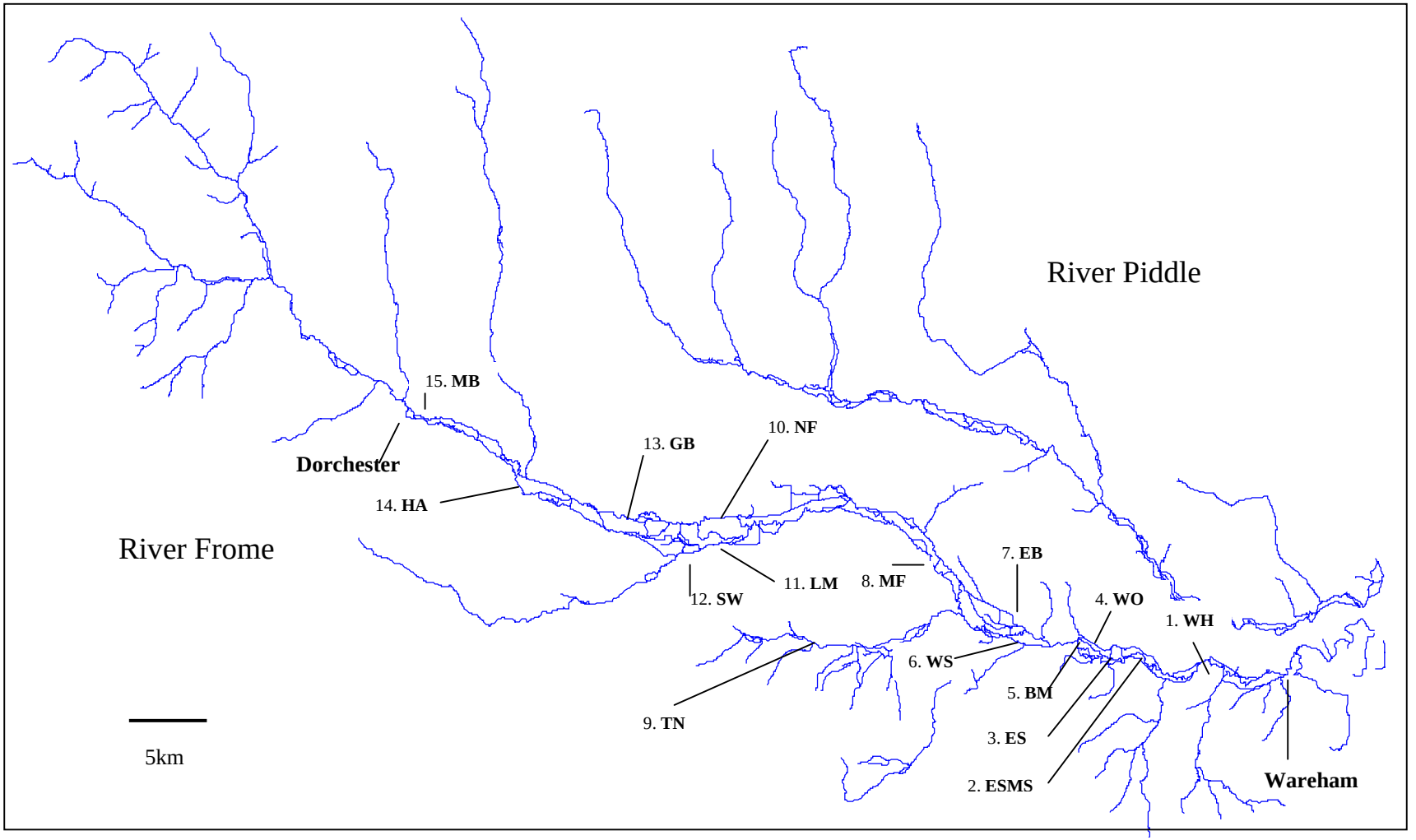


Figure 2.1 Location of sampling sites on the River Frome, Dorset.

1. WH West Holme
2. ESMS East Stoke Millstream
3. ES East Stoke
4. WO Wool Stream
5. BM Bindon Millstream
6. WS Waterbarn Stream
7. EB East Burton
8. MF Moreton Ford
9. TN Tadnoll
10. NF Norris Farm
11. LM Lewell Mill
12. SW South Winterbourne
13. GB Greys Bridge
14. HA Whitfield Hatches
15. MB Muckleford Bridge

Sites were plotted onto the river network using a map based on digital spatial data licensed from the Centre for Ecology and Hydrology (Moore *et al.* 1994).

2.1.3 Electric fishing

Electric fishing is a highly efficient method for studying fish and was first proposed in a patent application in 1863 (Beaumont *et al.* 2000). Electric fishing works by over-riding the voluntary muscles causing the fish to swim towards the anode (Cowx 1990). The fish can then be removed from the water using a net. There are concerns that electric fishing may alter behaviour, cause stress, physical injuries and ultimately mortality to fish (Cowx 1990; Cowx and Lamarque 1990). To assess the potential stress and injury effects of electric fishing juvenile Atlantic salmon, the Fish Department at CEH Dorset carried out experiments using farmed rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) in an artificial channel. Significantly increased plasma cortisol concentration, a measure of stress, was detected in the fish, but no damage to the spine or other bony tissues occurred and mortality rate was 0.2% (Beaumont *et al.* 2000). The voltage and the choice of pulsed or direct current influence the extent of injuries. Direct current has previously been demonstrated to cause fewer injuries, however, using pulsed current conserves the battery life of the electric backpack. Electric fishing was carried out by trained members of the Fish Biology Department of CEH Dorset, due to safety considerations for the operators and to optimise the speed of the fishing to minimise stress to the fish. Experienced fish biologists were able to differentiate between brown trout and Atlantic salmon juveniles which can appear similar at the parr stage.

Electric fishing is not known to cause behavioural changes promoting parr to move from the original site. For exampled, Dunham *et al.* (2002) repeated an electric fishing of brook trout (*Salvelinus fontinalis*, Mitchill 1814) over 4-5 days and found no statistically significant movement .In this study there was at least 3 months between each sample, which should be more than sufficient for any short-term effects to have ended.

Electric fishing was carried out at a frequency of 100 Hz, pulsed current, using a handheld anode 280 mm in diameter. Fishing started at the downstream end of the site and moved upstream for 100 m. Each site was fished twice. Abundance was estimated by number of fish captured multiplied by river width. Captured fish were

held in river water until completion of the survey and were then sedated in 2-phenoxyethanol. A 0.5 cm pelvic fin clip was removed from each individual and preserved in 95% ethanol. Scales were removed from any suspected 1+ parr to confirm the age. Age was determined by counting the number of growth rings using a microscope. After processing, all fish were returned alive to the same section of river.

Sites were electric fished in the summer and autumn of three consecutive years. The earliest sampling time permitted by the Environment Agency was June. In the autumn sampling, the pelvic fins of juveniles captured were examined, and individuals with previous clips were noted, but in the autumn, a fin clip sample was only taken from juveniles that were *not* previously marked. This prevented the same individual being sampled in both summer and autumn. Any 1+ parr or older were not included in the abundance estimates, because the older parr may have moved between sites. Abundance was determined per 100 m^2 (per unit area in this case river width x length).

2.1.4 Predators

Predators of juvenile Atlantic salmon, eel (*Anguilla anguilla* L 1758*.)*, pike (*Esox lucius* L. 1758*)* and adult brown trout, were noted at the time of electric fishing at each site. This was in daylight and under non-flood conditions.

2.1.5 Habitat measurements

HABSCORE takes into account habitat use by different age parr, models were derived using stepwise multiple regression for parr of different size and age categories (Armstrong *et al.* 2003). HABSCORE V habitat assessment was designed for use under normal summer flow conditions.

Measurements for HABSCORE v5 (Barnard and Wyatt 1995) were made at each site and all details were recorded immediately after sampling.
a) catchment record form- general site description

- site location

- position of the site in the catchment - distance from source, distance from tidal limit, plus link number, altitude and gradient. The link number is the total number of first order streams upstream of the site. First order streams are all sources of the river.

b) fish form

- river and catchment name
- number of salmon of different age classes
- accessibility of site to adults
- potential negative impacts: potential migration barriers, acid water, pollution, river engineering, habitat modification or stocking.

c) site habitat record form

- riverbank vegetation (riparian) shading of the site: total percentage area of the water surface overhung by vegetation to nearest 10% (categories into deciduous trees and shrubs, coniferous trees and herbaceous vegetation)
- submerged vegetation percentage cover including mosses and algae
- percentage of area covered by different substrate types: concrete, silt, sand, gravel, cobbles, boulders, bedrock
- substrate embeddedness, typified by silt or sand accretion round boulders, cobbles and gravel.
- site dimensions - length, width and water depth (shallow was less than 30 cm, deep was over 30 cm)
- flow types: slack or still, cascade, turbulent – white water, riffle or plunge pool, horizontal eddies (glide).

HABSCORE data were analysed by the Fish Ecology team at CEH Dorset.

2.2 Molecular analysis

2.2.1 DNA extraction

To extract DNA from samples, the cells are disrupted to release the DNA and unwanted cell constituents are then removed. For this research, a method to extract DNA rapidly and obtain DNA of sufficient quality to analyse microsatellite variation using polymerase chain reaction (PCR) was required. DNA extraction methods vary in terms of purity of product achieved, cost, length and complexity of the process and the use of toxic chemicals (reviewed by Milligan, 1998). The simplest extraction methods, such as boiling, NaOH and Chelex, do not have a DNA purification step but are suitable for certain applications. Isolation methods such as phenol/chloroform, SDS (sodium dodecyl sulphate) and CTAB (hexacetyltrimethyl ammonium bromide) can be used to obtain purified DNA. Boiling and high alkalinity (pH>9) disrupt cell membranes and release DNA into solution, but DNA may become degraded by metal ions released from the cells (Milligan, 1998).

Chelating resins such as Chelex (www.bio-rad.com) have been used to extract DNA of sufficient quality for forensic PCR applications (Walsh *et al.* 1991). Chelex resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions that act as chelating groups (Walsh *et al.* 1991). Chelex removes metal ions from solution and thus prevents DNA degradation. Walsh *et al.* (1991) found that Chelex was at least as efficient as proteinase K and phenol-chloroform extraction. Although the Chelex method does not yield purified DNA, this is not required for micorsatellite analysis. The main advantage of Chelex extraction for large sample sizes is that it is cheaper than commercial DNA extraction kits (for example, www.promega.com and www.qiagen.com), which although they produce high quality DNA of a consistent yield, are very expensive. In addition, the Chelex method has few steps therefore reduced the danger of mixing samples by crossover between tubes and is very quick to carry out.

A concern with Chelex-extracted DNA is that due to the high alkalinity of the chelating resin (pH 10-11) that the samples may degrade over time (Walsh *et al.* 1991). If the extracted DNA were intended to be stored for a long time (for example,

over 5 years) then perhaps a method with a purification step would be preferred and thus the DNA could be dehydrated and stored for very long time periods. This was not necessary in this study however.

DNA was extracted following the Beacham and Dempson (1998) method, using high temperature to disrupt the cells and Chelex resin to chelate metal ions. Extractions following this method were found to be degraded therefore a number of modifications were made. The initial method required a 15 minute autoclave step, this is likely to cause degradation of DNA, and instead, samples were digested overnight at 37°C. To further stabilise the DNA extract, samples were digested in a buffer.

One fin clip was digested in 300 µl of Chelex Buffer A (5% Chelex-100, Biorad, 100 mM NaCl, 50 mM Tris, 1% Triton, 10 mM EDTA in autoclaved distilled water) in a 1.5 ml Eppendorf. Chelex beads were suspended in the buffer using a magnetic stirrer and a repeater pipette was used to add Chelex to the Eppendorf tubes to ensure even distribution of beads. 3 mg proteinase K and 0.1 mg RNAse was added and samples were digested overnight at 37°C with rotation. After digestion, samples were spun for 10 minutes at 13000 revs. The supernatant was removed and transferred to a new tube. 300 µl of Chelex Buffer B (5% Chelex-100, Biorad, 10 mM Tris and 1 mM EDTA in autoclaved distilled water) was added and extracts were stored at minus 20 °C. To prevent any possibility of contamination, no DNA extraction for any other species was carried out when the salmon DNA was extracted. DNA was extracted from 2099 salmon parr fin clips. DNA was stored at –20 °C. The DNA extracted was of sufficient quality for microsatellite allele amplification without further purification steps. There was no evidence for sample contamination.

2.2.2 Molecular markers

Molecular makers are used to screen for genetic variation and if sufficiently variable, can be used to discriminate between individuals. Markers vary in practical application (time and cost to set up, time and cost to run and reliability of replication)

and quality of data generated, making some more suitable than others for this research.

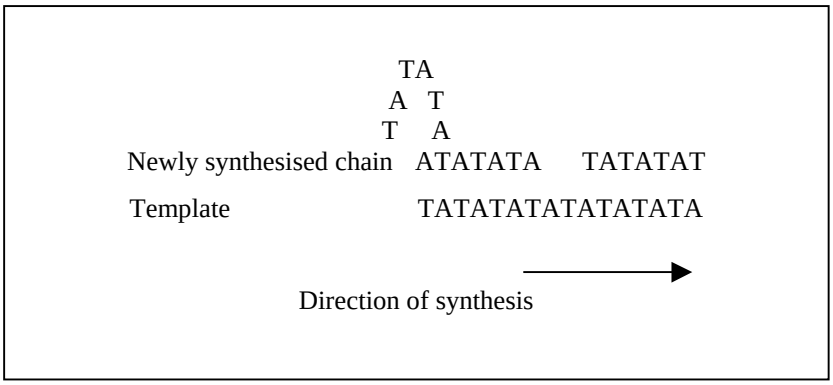
Allozymes were of the first molecular methods for estimating genetic variation. Allozymes are different forms of a particular enzyme and differences in the mobility of allozymes across starch or polyacrylamide gels are scored to estimate genetic variation. An advantage of allozymes is that no prior knowledge of the species is required because a standard set of reactions can be used, but a strong disadvantage is that often the study organism needs to be killed to carry out the analysis, which is obviously undesirable in small and declining populations. Further disadvantages of allozymes are that they are likely to be under natural selection and genetic variability is generally low. In Atlantic salmon, evidence for selection at the Mep-2 locus (NADP- dependent malic enzyme-2 locus) has been found (Verspoor and Jordan 1989, Verspoor, Fraser *et al.* 1991 and Jordan, Verspoor *et al.* 1997). Allozymes are generally less variable than microsatellites and are therefore not suited to small-scale studies where it is expected that genetic differentiation will be low.

2.2.3 Microsatellites

Microsatellites are tandemly repeated arrays of up to six nucleotides (Freimer and Slatkin 1996). The majority of microsatellites are dinucleotides (e.g. ATATAT) (Li *et al.* 2002). Trinucleotides (e.g. GTCGTCGTC) and tetranucleotides (e.g. CTCACTCACTCA) are less common. Compound (e.g. $(CA)_n(CTCA)_n$) and interrupted repeats (e.g. $(TC)_{10}N_n(TC)_3$) also occur (Jarne and Lagoda 1996).

The existence of microsatellites in eukaryotes has been known since the 1970s (Bruford *et al.* 1998). Microsatellites are five times more abundant in mammalian genomes than plants (Lagercrantz *et al.* 1993) and have been found in the genomes of every organism analysed so far (Li *et al.* 2002). Microsatellites occur throughout the genome but are more common in non-coding areas of the chromosome (Jarne and Lagoda 1996). It is thought that mutation of microsatellites occurs by mis-pairing of the DNA strands caused by slippage of the strands during replication (Levinson and Gutman 1987) (Figure 2.2).

Repeats are added when the newly synthesised DNA chain loops out



Repeat units are removed if the template loops out

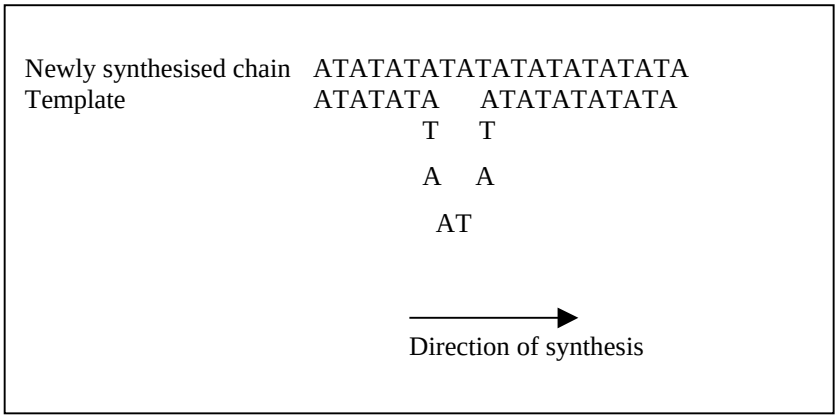


Figure 2.2 Microsatellite mutation by slipped strand mispairing.

As mutations add and remove units from the microsatellite a series of different alleles accrue in the population (Harding *et al.* 1992 and Gordenin *et al.* 1997). Microsatellites appear to have an upper size limit and rarely exceed a few tens of times the repeat unit (Tautz and Renz 1984). Long alleles tend to mutate to shorter alleles preventing infinite expansion (Ellegren 2000) and this has been experimentally observed in *Drosophila* (Whittaker *et al.* 2003).

Microsatellites have a high mutation rate and this is important when using microsatellites to infer population structure because new alleles detected in the population are assumed to have come from migration but may occur due to mutation. Weber *et al.* (2002) suggested that tetranucleotides have a higher mutation rate than di and tri nucleotide loci, although Chakraborty *et al.* (1997) suggested the opposite.

Characteristics that make some loci more mutable are not clearly understood (Gardner *et al.* 2000).

The number of repeats (Primmer *et al.* 1996), the structure of the repeat array (Estoup *et al.* 1995) or heterozygote instability (Amos *et al.* 1996) may influence mutation rates. The mutation rate is influenced by the size of the microsatellite as larger microsatellites will mutate more rapidly (Ellegren 2000). The mutation rate of a microsatellite locus can be estimated directly by counting mutations in pedigrees, from linkage data or from comparison of observed and theoretical mutation values (Jarne and Lagoda 1996). Rates of microsatellite mutation are several orders of magnitude higher than other parts of the DNA and values between 10^{-5} to 10^{-2} mutations per meiosis have been observed (Jarne and Lagoda 1996).

2.2.4 Microsatellite primer selection

To estimate genetic variation, DNA extracted from each sample is screened using primers, which are short (e.g. 10-20 bp) sections of DNA, designed to be complementary to a region upstream and a region downstream of the microsatellite on the different DNA strands. The selected microsatellite region is amplified using the polymerase chain reaction (PCR). There are several steps in the selection of microsatellite primers: firstly a literature search was carried out to find all the primers available and then the search was narrowed using specific criteria (below), secondly, a test set of approximately 20 primers was selected and thirdly a screening set of 6 primers was selected.

Before screening, criteria such as published variability, allele size and previous use in the target species were used to narrow the search. Primers designed in the target species are most likely to be useful. Primers from different species may fail to amplify due to mutations at the primer binding site or the product may be monomorphic (Chambers and MacAvoy 2000). Ascertainment bias may occur where a microsatellite in the target species tends to be longer than that in related species

using the same primer because longer microsatellites are selected when primers are being designed (Crawford *et al.* 1998). This is not a problem if cross-species comparisons are not being made, thus does not affect this study. Microsatellites developed in one species can often be used to investigate genetic variability in related species, however, mutation in the primer binding site may cause failure of some cross-species amplification (Chambers and MacAvoy 2000). Higher polymorphism and larger alleles are generally found in the species in which the primers were characterised (Crawford *et al.* 1998).

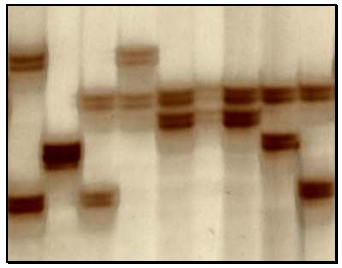
A moderate degree of heterozygosity was required because the analysis must be sensitive to differences on a small spatial and temporal scale. If primers have been previously used in population studies this should indicate that the primer provided a good quality product. Small microsatellites, under 300 bp, were preferred because if DNA is degraded, larger alleles will not amplify and therefore 'false' homozygotes may arise.

Microsatellites have different numbers of nucleotides in the repeat unit. Dinucleotides are more prone to slippage during PCR, which results in 'stutter' a ladder of bands. Presence of stutter bands does not necessarily prevent the primer from being used as the alleles can generally be scored unless the largest band, scored as the allele size, cannot be distinguished or if bands from two alleles overlap. Due to the potential stuttering of dinucleotides, it is preferable to use tri or tetra nucleotides to screen a population.

Seventeen previously-published primers from Atlantic salmon and related species were selected and tested initially. The criteria were for primers to have reproducible, clear results which were polymorphic with preferred product size of less than 300 bp. Thirteen primers cloned from Atlantic salmon were selected for testing (Table 2.1) Primers Ssa 4, Ssa 14, Ssa 171, Ssa 197, Ssa 202 and Ssa 289 have been used by other researchers for population studies and resulted in polymorphic products with product sizes generally below 300 bp. (Primer Ssa 85 could have been used but was not selected in this study, in order to avoid confusion with Ssosl 85). Primers Ssosl 85, Ssosl 417, Ssosl 438, Ssosl 439, Ssosl 444 were selected because these had been

used previously for Atlantic salmon. Primers F43, 20.19 and D30 (Sanchez *et al.* 1996) were also selected for testing. Two primers cloned from brown trout, μ60 and μ73 and two primers cloned from *Oncorhynchus,* Ogo1a and F_{GTI} (Table 2.2), were also selected for testing.

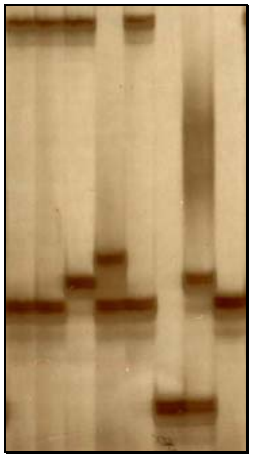
Some primers were rejected from the test set and were not used for screening: primers Ssa 4, Ssa 14, Ssosl 438, Ssosl 439, Ssosl 444, F43, 20.19 and D30 were not used to screen the samples because PCR conditions resulting in clear bands could not be optimised. Loci μ60 and μ73 from brown trout were rejected due to a high amount of stutter and the loci cloned from *Oncorhynchus* (Ogo1a and $F_{GTI)}$ were rejected because the products were monomorphic. Primers Ssa 202, Ssa 171, Ssa 197, Ssosl 85, Ssa 289 and Ssosl 417 were selected to screen the samples. See Table 2.3 for annealing temperatures and primer sequences. PCR was repeated for all samples that either did not amplify on the first reaction or where the product was difficult to score. The primers selected gave rise to products which were replicable, and easy to score on silver stained gels, except for primer Ssosl 417 which was rejected because not enough samples could be scored per population. See Figure 2.3 for pictures of silver-stained gels.



Locus Ssa 171

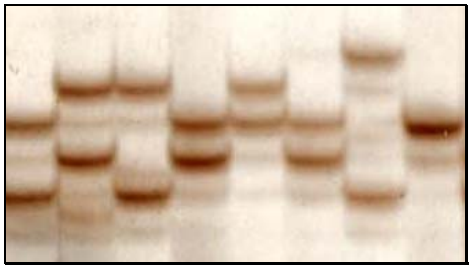
Microsatellite structure

$(TGTA)_{14}(TG)_7$



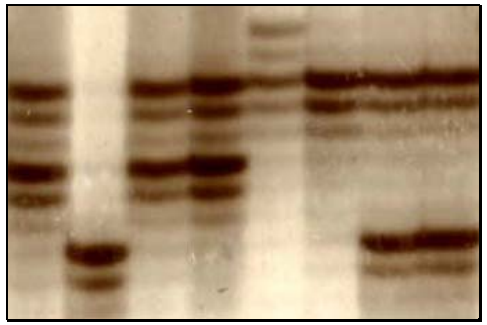
Locus Ssa 197

Microsatellite structure $(GT)_5C(TG)_4TC(TG)_3A(GTGA)_{15.}$



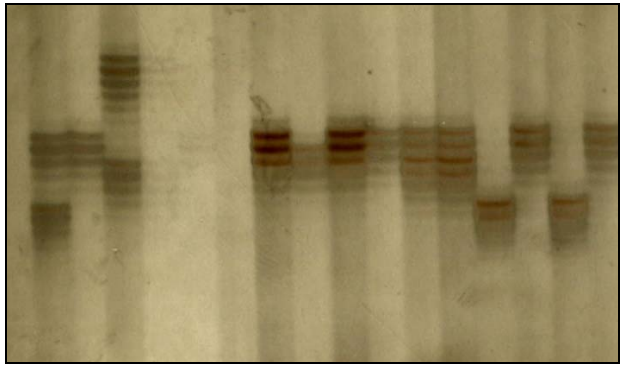
Locus Ssa 202

Microsatellite structure $(CA)_3(CTCA)_7$

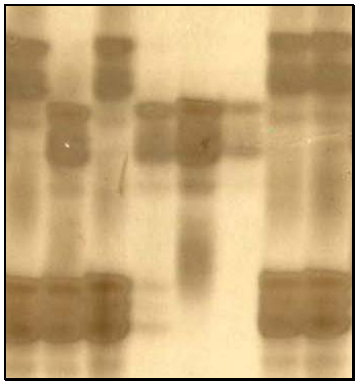


Locus Ssosl 85

Microsatellite structure $(GT)_{22}$



Locus Sssol 417 Microsatellite structure (TG)



Locus Ssa 289

Microsatellite structure $(GT)_{12}$

Figure 2.3 Silver stained gels at six microsatellite loci.

Table 2.1 Microsatellite primers cloned from *Salmo salar*.

	Locus cloned by	*Repeat type*	*AT ℃*	*N_A*	*size bp*	*Ho*	*Published use for Atlantic salmon population structure*
1	Ssa 4 McConnell et al. 1995	$(GT)_{39}$	65	30	112-190	0.6-0.89	McConnell et al. 1995
2	Ssa 14 McConnell et al. 1995	$(TC)_{10}N_{15}(TC)_3N_2(AC)_{12}(TC)_3N_5(CA)_4$	57	3	138-145	0.3-0.5	Beacham and Dempson 1998, McConnell et al. 1995
3	Ssa 171 O'Reilly et al. 1996	$(TGTA)_{14}(TG)_7$	58	29	233-267	0.6-0.9	Fontaine et al. 1997, Tessier et al. 1997, Tessier and Bernatchez 1999
4	Ssa 197 O'Reilly et al. 1996	$(GT)_5C(TG)_4TC(TG)_3A(GTGA)_{15}$	58	21	150-200	0.4-0.8	Beacham and Dempson 1998, Fontaine et al. 1997 Tessier et al. 1997, Tessier and Bernatchez 1999
5	Ssa 202 O'Reilly et al. 1996	$(CA)_3(CTCA)_7$	58	18	270-320	0.4-0.8	Beacham and Dempson 1998, Fontaine et al. 1997, Nielsen et al. 1999
6	Ssa 289 McConnell et al. 1995	$(GT)_{12}$	46	6	110-119	0.3-0.9	Beacham and Dempson 1998
7	D30 Sanchez et al. 1996	(AG/TC)n	53	5	226-242	0-0.5	Sanchez et al. 1996
8	F43 Sanchez et al. 1996	$(AC/TG)_n$	60	9	103-127	0.5	Sanchez et al. 1996
9	20.19 Sanchez et al. 1996	$(AC/TG)_n$	62	4	96-102	0.7	Sanchez et al. 1996
10	Ssosl 85 Slettan et al. 1996	(GT)	55	8	194	0.7-0.96	Fontaine et al. 1997 Garant et al. 2000
11	Ssosl 417 Slettan et al. 1996	(TG)	53	7	187	/	None*
12	Ssosl 438 Slettan et al. 1996	$(AC)_{26}AT(AC)_6$	50	8	140	/	None*
13	Ssosl 439 Slettan et al. 1996	$(AC)_{30}$	56	7	154	/	None*

AT- annealing temperature, N_A- number of alleles, Ho- observed heterozygosity.

* no previous use of this primer in Atlantic salmon was found in a literature search at the time, however, later studies may have used this primer.

Table 2.2 Microsatellite primers cloned from *Salmo trutta* and *Oncorhynchus* sp.

	Locus	*Repeat type*	*AT ℃*	N_A	*size bp*	*Ho*	*Published use for Atlantic salmon population structure*
1	μ60 S. trutta Estoup et al. 1993	$(CT)_{13}ACCA(CT)_3$	58	6	97-111	0.49	None*
2	μ73 S. trutta Estoup et al. 1993	$(GT)_{13}TTATCT(GT)_3$	58	6	140-158	0.63	None*
3	Ogo1a Oncorhynchus Gorbuscha (Walbaum, 1792) Olsen et al. 1998	$(GTCT)_{26}$	59	21	183-323	0.94	None*
4	F_{GTI} Oncorhynchus mykiss (Walbaum, 1792) Sakamoto et al. 1994	GT	53	7	15	/	None*

AT- annealing temperature, N_A- number of alleles, Ho- observed heterozygosity.

* no previous use of this primer in Atlantic salmon was found in a literature search at the time, however, later studies may have used this primer.

Table 2.3 Primer sequences and PCR conditions.

Locus	*Primer sequence*	*TMAC*	*formamide*	*AT[a]/AT[b] ℃*	*published AT ℃*	*ramp*
Ssa 202 O'Reilly et al. 1996	Fwd-5'-CTTGGAATATCTAGAATATGGC-3' Rev-5'-TTCATGTGTTAATGTTGCGTG-3'	60mM	2.5%	46/50	58	0.5
Ssa 171 O'Reilly et al. 1996	Fwd-5'-TTATTATCCAAAGGGGTCAAAA-3' Rev-5'-GAGGTCGCTGGGGTTTACTAT-3'	60mM	2.5%	46/50	58	0.5
Ssa 197 O'Reilly et al. 1996	Fwd-5'-GGGTTGAGTAGGGAGGCTTG-3' Rev-5'-TGGCAGGGATTTGACATAAC-3’	30mM	1.25%	50/54	58	none
Ssosl 85 Slettan et al. 1995	Fwd-5´-TGTGGATTTTTGTATTATGTTA-3’ Rev-5´-ATACATTTCCTCCTCATTCAGT-3’	None	None	52/56	55	none
Ssa 289 McConnell et al. 1995	Fwd-5'-CTTTACAAATAGACAGACT Rev-5´-TCATACAGTCACTATCATC	60mM	2.5%	44/48	46	none
Ssosl 417* Slettan et al. 1995	Fwd-5´-TTGTTCAGTGTATATGTGTCCCAT-3´ Rev-5´-GATCTTCACTGCCACCTTATGACC-3´	None	1.25%	58/56	53	none

AT- annealing temperature, TMAC and formamide are additives to improve PCR, ramp is a step to slow PCR cycle to promote polymerisation and increase product. *Ssosl 417 was rejected from screening set. *Ssosl 417 rejected from screening set after not enough samples could be reliably amplified.

2.2.5 Polymerase chain reaction (PCR)

PCR is an in vitro version of natural DNA replication, but only small sections of the DNA are amplified (Frankham *et al.* 2002). In the PCR, extracted DNA from the individual of interest is used as a template and DNA *taq* polymerase is used to create copies of the original strand. Magnesium chloride and nucleotides (A, T, C and G) are added to the reaction. Repeated temperature cycles are used to denature the DNA, to allow the primers to bind (annealing) and to replicate the DNA. Between 25 and 35 cycles are needed to produce sufficient DNA to detect using silver staining, following separation of fragments by polyacrylamide electrophoresis, or automated sequencing. The PCR is automated using a thermal cycler.

Published conditions for PCR are generally stringent (this means that the reaction is carried out at high temperature and low $MgCl_2$ concentration which results in highly specific products. If the conditions are too stringent however, there will be no product at all). PCR conditions were determined experimentally. A standard volume of 1 µl of DNA extract in a 1 in 5 dilution was used as a template in the reaction mix. $MgCl_2$ concentration was optimised for each primer separately. Low magnesium concentration was required for specific binding. TMAC (Tetra Methyl Ammonium Chloride) and formamide were added to some reactions to increase specificity of the primer binding. PCR amplifications were performed in 10 µl reaction volumes using 0.2 µl DNA extract, 1.0 µl Bioline NH_4 buffer, 1.0 or 1.5 mM $MgCl_2$, 1.0 mM dNTP, 2 pmol forward and 2 pmol reverse primer and 0.01 µl Bioline Taq DNA polymerase.

A higher annealing temperature was used for five cycles and the annealing temperature was then reduced by 2 °C for a further 28 cycles. The reasoning behind this is that the higher temperature should promote specific primer binding. After 5 cycles the temperature can be reduced because the accurate copies form the template for further amplification cycles. Using a ramp step of 0.5 added to the second 72 °C step was tested. This increases the elongation time available for the product to be synthesised and thus increased product yield. It was found that lower Annealing Temperatures (AT^a/AT^b) than published were generally useful.

PCR reactions were carried out in 96 well plates on a Hybaid 96 well OmnE PCR machine. The following PCR profile was used: 2 min at 95°C x1, 1 min at 94 °C, 30s at AT^a, 40 s at 72 °C x5, 1 min at 90 °C, 1 min at AT^b, 50 s at 72 °C x28. To minimise the danger of sample contamination, PCR was carried out in a different laboratory to the DNA extraction, using pipettes which were only used for PCR and never for DNA extraction.

2.2.6 Microsatellite detection

Microsatellites can be detected with an automated sequencer after labelling with fluorescent dyes, or by size differentiation on a polyacrylamide gel and detection using silver stain. An advantage of an automated sequencer is that it is often possible to multiplex loci, reducing costs and improving run time. However, there was no automated sequencer in the lab at the time of the molecular analysis and it would have been too expensive to process the samples elsewhere.

Silver staining was first used to detect proteins on polyacrylamide gels and has since been optimised for DNA detection (Sprecher *et al.* 1996). Silver staining allows sizing and scoring of alleles at equivalent accuracy to that obtained from an automated sequencer and in addition, silver staining is much cheaper to set up and run. Silver staining has been used to detect alleles on polyacrylamide gels in a wide range species for example in bovines (Comincini *et al.* 1995) and conifers (Rahman *et al.* 2000). Some multiplexing on silver stained gels is possible if the loci are not very variable.

PCR products were separated to single base resolution on 6% denaturing polyacrylamide gels run in 1.5% TBE buffer. For products of around 100 bp, the gel was run at 70 W for approximately 60 minutes, for products between 100 and 200 bp the gel was run for a total of 90 minutes. Products were visualised using SILVER SEQUENCE™ Staining Reagents (www.promega.com). 2 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficol in H_2O) was added to 10 µl PCR product and 3 µl of this was loaded onto the polyacrylamide gel. Samples were denatured at 95 °C for 2 minutes before loading to enhance band clarity. On

completion of gel running, the glass plate with the gel was placed immediately into 2 L of 10% acetic acid in distilled water for 40 minutes to remove urea from the gel which would interfere with staining. All steps of silver staining were performed with the gel on a gently rocking platform to ensure even treatment of the plate. The gel was then washed twice for 10 minutes using distilled water to remove all traces of acid, before being placed in staining solution for 30 minutes. The staining solution was 2 g silver nitrate ($AgNO_3$), 37% formaldehyde (H_2CO) in 2 L distilled water. Formaldehyde was included to increase sensitivity and contrast of the stain.

After staining, the gel was washed very briefly (2 seconds) in distilled water to remove excess stain, and then placed in developer solution (37% formaldehyde (H_2CO), 10 mg/ml sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$), 60 g sodium carbonate (Na_2CO_3) in 2 L distilled water). The development procedure works by an abrupt change in pH causing the formation of insoluble silver salts. DNA initiates precipitation of silver and will thus become visible. Inclusion of sodium thiosulphate reduces non-specific background staining. The developer solution was chilled to 4 °C to slow the development reaction. The developer solution was carried out on a more rapid rocking to allow even development. After development, the reaction was stopped by placing the plate into 10% acetic acid for two minutes to reduce the pH. Plates were washed in distilled water and left to dry. Water quality was vital to obtaining high quality silver staining therefore all water for staining was obtained from a USF ELGA Option 7 purifier that has a 0.2 μM particulate filter. Formaldehyde is a respiratory sensitisor thus all steps were carried out in a fume cupboard**.** Silver nitrate was precipitated with $NaCl_2$ before disposal. After drying overnight, gels were scanned (using a dedicated computer in the laboratory). After scoring, gels were removed from glass plates by soaking overnight in 5% NaOH. The silver staining method was labour-intensive and it was difficult to judge the length of development, especially if there were differences in product concentration between lanes.

2.3 Statistical analysis

2.3.1 Estimation of genetic diversity- observed and expected heterozygosity

Genetic diversity is an important factor in the genetic make-up of a population and low genetic diversity has been correlated with reduced fitness (see Introduction, section 1.4). Genetic variation was estimated at each site and at each locus and over all loci for each sample time. Estimation of expected heterozygosity permits comparison of diversity between sites. Observed heterozygosity was calculated at each locus and over all loci, for each site and expected heterozygosity was derived for each locus and over all loci, for each site using the program GENEPOP 3.4 (Raymond and Rousset 1981). GENEPOP is a population genetics software package for haploid or diploid data that computes exact tests or their unbiased estimation for Hardy-Weinberg equilibrium, population differentiation and genotypic disequilibrium. The programme is available from http://wbiomed.curtin.edu.au/genepop. This test was used in Chapter 4.

2.3.2 Estimation of genetic variation - allelic richness

Observed heterozygosity is the standard measure of genetic variation, but this is not standardised for sample size. Sample size differed between sites (see Appendix Table 1 a-f for details) and thus a standardised estimator of genetic diversity was needed. Allelic richness, standardised per individual, was used to compare genetic variability between sites and also to compare the results from this study to data from salmon in other rivers.

The observed number of alleles in a sample is highly dependent on sample size therefore an allelic 'richness', corrected for differences in sample size, was devised by El Mousadik and Petit 1996. The allelic richness statistic describes how variable a subpopulation is compared to the total (equation 2.1). N is the number of individuals sampled and n is the number of individuals in the (sub) population being analysed. The expected number of alleles in a sub-sample of 2n genes is estimated, given that 2N genes have been sampled $(N \geq n)$:

Equation 2.1 Allelic richness Rs

$$Rs = \sum_{i-l}^{n} \left[1 - \frac{\binom{2N-Ni}{2n}}{\binom{2N}{2n}} \right]$$

where Ni is the number of alleles of type i among the 2N genes. Each term under the sum corresponds to the probability of sampling allele i at least once in a sample of size 2n. If allele i is so common that it is certain to be sampled -when 2n > (2N-Ni)- the ratio is undefined but the probability of sampling the allele is set to 1. The program FSTAT v2.9.3 (Goudet 2001) used the formula in equation 6.1 for allelic richness (Rs) for all samples, the same sub-sample size n is kept, but N is altered to the number of individuals genotyped at the locus. These results are in Chapter 4.

2.3.3 Checks for null alleles, stutter and mis-scoring

It is important that data derived from microsatellite PCR are accurate. Two potential causes of inaccuracy are difficulty in scoring dinucleotide microsatellites, due to stuttering, and the presence of false homozygotes. Dinucleotide microsatellites are prone to stutter bands, which can cause problems in allele scoring. Stutter bands appear as a ladder of extra (normally fainter) bands from the main allele band and are caused by polymerase slippage during replication. If the stutter bands of the two alleles overlap then it is not possible to score the allele size accurately, and this primer would be rejected. Sufficient Atlantic salmon microsatellite primers exist, that, if stutter bands appeared to be a problem, dinucleotide loci could be avoided.

False homozygotes from either scoring errors or null alleles can potentially bias the data. When scoring the microsatellite gels it is possible to score heterozygotes with confidence: a heterozygote is an individual that has two different-sized alleles at a particular locus, which appear as two bands on the gel. A true homozygote individual will have two alleles at a locus which are the same size, are identical by descent and which appear on a gel as one band. However, some of the homozygotes may be false, that is only one band is present on the gel. Of course, true and false homozygotes can

not be distinguished visually but analysis of the data can detect potential false homozygotes.

Causes of false homozygotes

Null alleles are caused by non-amplification in the PCR due to poor quality DNA (fragmented DNA), a mutation in the primer-binding site (Pemberton *et al.* 1995) or large allele dropout the PCR – this is when only the smaller allele amplifies in an individual (Wattier *et al.* 1988). A mutation in the primer binding site that prevents primer binding, causes the allele to not be amplified. If this affects one allele, this causes a false homozygote and if both alleles are affected, this results in no product being amplified during PCR for that individual at that locus.

Reducing the likelihood of false homozygotes

Some of the potential problems of false homozygotes can be pre-empted at the primer selection stage. The incidence of these problems will vary between loci and also between populations and published studies can be checked to find out if these problems have been mentioned for particular loci. Null alleles are more likely to occur if the primers used were designed in different species to the one that they were cloned from, however in this study, all primers used were cloned from Atlantic salmon. Drop out of large alleles can be minimised by selecting microsatellite loci which have small products and also by avoiding loci with large variance in allele size and therefore loci were selected with relatively small allele size (under 350 base pairs).

The program Micro-Checker (available from www.microchecker.hull.ac.uk) was used to check the data for scoring errors and then to check for stutter and null alleles. The program takes Excel or GENEPOP-style input files. The following settings were made for microsatellite locus checking: Ssa 202, Ssa 197 and Ssa 171 were set as di-tetra complexes and Ssa 289 and Ssosl 85 were set as dinucleotides. Scoring errors were checked, such as alleles outside the expected size. Any scoring/ data entry errors were corrected by reference to the original data and the analysis was performed with the confidence limit set at 95%.

- Large allele dropout was indicated by an excess of shorter-allele homozygotes.

- Stutter bands appear as a ladder of extra (normally fainter) bands from the main allele band and large alleles have a tendency to stutter more. The program checked for an excess of heterozygotes with one repeat unit difference and also checks if this excess is more apparent in the large allele size range.

Null allele detection

A high incidence of null alleles was not expected but it is important to test for this, because false homozygotes could have a large impact on estimation of genetic variability and population structure. In systems where parental information is available, non-amplifying alleles can be tested by comparing allele scores for parents and offspring, but in this study no genetic information was available from the adult generation.

Micro-checker makes no assumption about microsatellite mutation mechanism. The observed frequency of homozygote classes was compared to simulated (expected) heterozygote frequencies (using Monte Carlo randomisation). If homozygote excess was detected then some or all of the deviation from Hardy-Weinberg may be due to null alleles rather than true homozygotes. The program attempts to differentiate between null alleles from biological processes that may have caused deviation from Hardy-Weinberg by comparing deviations across loci. Allele classes with many alleles are expected to have excess homozygotes due to the segregation of common alleles with null alleles. Rarer allele classes are not generally expected to show homozygote excess because the probability of segregating with a null allele is dependent on allele frequency. The program calculates a cut-off frequency for which allelic classes are analysed to show a homozygote excess. When most allelic classes show a homozygote excess, then the program indicates a potential null allele (Micro-Checker help file). The estimated null allele frequency for each locus is compared to the null allele frequency obtained by the program and to Chakraborty *et al.* (1992) and Brookfield (1996) estimates. The results are in Chapter 4.

2.3.4 Linkage

Linkage equilibrium occurs if alleles at locus A segregate independently from alleles at locus B. Linkage disequilibrium occurs if this is not the case. Tests for linkage disequilibrium measure the statistical association of alleles in forming gametes or the linkage of genes on chromosomes. Microsatellite markers are assumed to be neutral with regards to natural selection, but it is possible that a microsatellite may be tightly linked to a gene and thus acts as if it were under selection (Slatkin 1995). The incidence of linkage low because most microsatellites are in non-coding regions (Li *et al.* 2002) but it is important to test for linkage because it causes pseudo-replication (effectively reduces the number of independent loci used). Microsatellites used in this study were tested for linkage the program Linkdos, available from genepop.curtin.edu.au/linkdos.html or via the web version of Genepop. The results of the linkage tests are in Chapter 4.

2.3.5 Statistics to estimate population structure- Wright's F statistics

F-statistics were developed by Sewall Wright in 1921, from work on Shorthorn cattle to relate the pedigree inbreeding to desirable characteristics. Wright defined a fixation index *f* (later designated *F*), which is a correlation between the presence or absence of an allele in uniting gametes compared with the expected correlation if the pair of gametes had been drawn randomly from the population. Wright also designated F as the inbreeding coefficient (the expected correlation between uniting gametes) (Raybould *et al.* 2002).

The principle of fixation indices can be applied to natural populations. Wright defined the 'F statistics' for natural populations, calling samples from discrete sites 'subpopulations'. The genetic variation of the population is characterised at three levels: within individual, between 'subpopulations' and total (he following is as described in Hartl and Clark, 1989). The heterozygosity of individual (*I*) in subpopulation (*S*) (H_I) can be measured using any marker from which single locus genotypes can be inferred. H_I can be interpreted as the probability of heterozygosity of any one gene in an individual. H_I is the observed heterozygosity averaged over subpopulations. The expected heterozygosity of an individual in a randomly mating

subpopulation is termed (H_S). The expected heterozygosity in randomly mating total population is termed (H_T), i.e. the heterozygosity if there was no structuring and all subpopulations were pooled together and mated randomly.

The inbreeding coefficients or *F* statistics for the three levels (within 'subpopulations', between 'subpopulations' and total) can be estimated using the following:

a) The inbreeding coefficient F_{IS} measures the reduction in heterozygosity of an individual due to non-random mating within the subpopulation. $F_{IS} = H_S - H_I / H_S$.

b) The coefficient F_{ST} measures population subdivision by estimating the reduction in the expected heterozygosity of a subpopulation compared with the expected heterozygosity in the whole population. $F_{ST} = H_T - H_S / H_T$. An F_{ST} of 0 means no differentiation (i.e. allele frequencies are the same among subpopulations) and F_{ST} of 1 indicates complete differentiation (i.e. each subpopulation is fixed for a different allele).

c) The total inbreeding coefficient F_{IT} measures the reduction of heterozygosity of an individual relative to the total population.

2.3.6 Nei's G_{ST}

Wright's F statistics were devised for correlation of gametes at a single locus with two alleles, with all alleles except the commonest being pooled (Hartl and Clark 1989). Nei (1973) devised the statistic G_{ST} for multi-allelic and multi-loci systems using the average heterozygosity of all the genes in an individual. G_{ST} is formulated thus:

$$G_{ST} = 1 - \frac{\overline{H}_S}{H_T}$$

$\overline{H}_S$ is the average over sub-populations of the proportion of heterozygotes in a subpopulation assuming random mating and H_T is the expected proportion of heterozygotes if the subpopulations are pooled and mated randomly. $\overline{H}_S$ and H_T are averaged over loci to give the multi-locus G_{ST}. In this form, G_{ST} is an estimator of F_{ST} (Weir and Cockerham 1984).

2.3.7 Weir and Cockerham's θ

The co-ancestry measure θ is concerned with the correlation of alleles in individuals in subpopulations compared with the correlation among alleles within individuals in the whole population, whereas *F*-statistics consider correlations among gametes. For example, co-ancestry is a correlation between a randomly selected allele from individual A and a randomly selected allele from individual B, in contrast to *F*-statistics that are concerned with correlations between randomly selected gametes from A and B (Weir and Cockerham 1984). If there is no bias during meiosis θ can be considered to be equivalent to F_{ST} and thus estimators of θ can be considered to be estimators of F_{ST}.

Weir and Cockerham (1984) used an analysis of variance approach to derive an unbiased estimator of θ. The Weir and Cockerham parameters *f*, *F* and θ are equivalent to F_{IS}, F_{IT} and F_{ST} respectively, and hence estimators of *f*, *F* and θ can be considered estimators of F_{IS}, F_{IT} and F_{ST}. The variance in allele frequency is partitioned as follows (as described in Hartl and Clark, 1989). Using as an example two alleles and three levels of variance: alleles A and a, with frequencies p and q= 1-p, are assigned x = 1 for allele A and x = 0 for allele a.

The expectation of x is the allele frequency p and the total variance = p(1-p).

The total variance of x is $p(1-p) = \sigma_T^2 = \sigma_a^2 + \sigma_b^2 + \sigma_w^2$

and can be partitioned as follows $x_{ijk} = p + a_k + b_{ik} + w_{ijk}$

x_{ijk} is the value (0 or 1) of the *i*th allele in the *j*th individual in the *k*th subpopulation.

The hierarchical F statistics are related to the variance components in the following way:

$\alpha^2_w = (1 - F_{IT})\alpha^2$, this is w_{ikj}, the component for alleles *w*ithin individuals

$\alpha^2_b = (F_{IT} - F_{ST})\alpha^2$, this is $b_{ik,}$ the component *b*etween individuals within subpopulations

$\alpha^2_a = F_{ST}\alpha^2$, this is $a_{k,}$ the component for *a*mong subpopulations.

The *F* statistics in terms of variance components are:

F_{IT} *(F)* $= \alpha^2_a + \alpha^2_b / \alpha^2_a$

F_{ST} *(θ)* $= \alpha^2_a / \alpha^2_T$

F_{IS} *(f)* $= \alpha^2_{b/} \alpha^2_b + \alpha^2_w$

The program FSTAT v 2.9.3.2 (Goudet 2001) was used to estimate and test gene diversities and fixation indices. (In this program a 'sample' is equivalent to a site, not an individual). FSTAT is available from www2.unil.ch/popgen/softwares/fstat.htm FSTAT uses Nei's (1987) estimators of gene diversities and differentiation and uses Weir and Cockerham (1984) estimators of F (F_{IT}), θ (F_{ST}) and f (F_{IS}) per allele, per locus and for all loci, using the Infinite Alleles Model (IAM). The F statistics estimated at each sample time are in Chapter 4.

2.3.8 Detection of significant F_{IS}

If fish mated randomly within a site then F_{IS} will be zero, however, values significantly different from zero can occur. Significant *negative* F_{IS} indicates higher variance at the lower level (i.e. within individuals), which could be caused by dissortative mating.

If significant *positive* F_{IS} is detected within a site, it is possible that more than one population existed at that site i.e that populations were pooled when sampled. If two populations are pooled, which separately are in Hardy-Weinberg equilibrium, the pooled population will have an allele frequency of the average of the two populations and will therefore not be in Hardy-Weinberg equilibrium. This is known as a Wahlund effect (Hartl 1988). It was extremely unlikely that the populations were smaller than the sample site, because sampling was carried out on a very small scale.

Sites with significant positive F_{IS} contravene the assumptions of Hardy-Weinberg equilibrium, i.e. no inbreeding within a site. If F_{IS} is significant, the assumptions that allow estimation of gene flow from F_{ST} are violated. Sites with a large F_{IS} value with low allele number could indicate a small number of families (see Chapter 5 for discussion of sampling families).

2.3.9 Microsatellite mutation models and estimators of population structure

Different statistical approaches to estimate population structure have been proposed, based on different microsatellite mutation models. The two main microsatellite mutation models are the Infinite Alleles Model (IAM) and the Stepwise Mutation Model (SMM). F_{ST} is based on the Infinite Alleles Model (IAM) and R_{ST} on the SMM. In the absence of knowledge about the microsatellite mutation model in a system, then F_{ST} is the conservative statistic to apply.

Microsatellite mutation models:

- The Infinite Allele Model (IAM) was developed by Kimura and Crow in 1964 and states that mutation will lead to new allelic states which are always different to existing alleles and may involve any number of repeat units (O'Connell and Wright 1997). Each mutation creates a new allele at rate *u.* The K-allele model, developed by Crow and Kimura in 1970 assumes that K allelic states are possible and mutation at a given allele occurs with the probability *u/k*-1 to any other of the *k*-1 alleles.
- The Stepwise Mutation Model (SMM), developed by Kimura and Weiss (1964), states that mutation occurs by gain or loss of a single repeat unit, thus some mutations will generate alleles identical in state to existing alleles (O'Connell and Wright 1997). Expansion or contraction is considered to be equally likely.
- The Two Phase Model (TPM) (Di Rienzo *et al.* 1994) is a modification of the Stepwise Mutation Model to incorporate two mutation mechanisms. The TPM states that microsatellites mutate most often by stepwise mechanism but can also mutate in larger chunks and to alleles which are already present in the population.

Although the microsatellite mutation models are conceptually relatively straightforward, it is often difficult to assess which model is most likely in natural populations. Microsatellite mutation has been directly investigated in studies using large sample sizes, but evidence for a particular mutation mechanism is not straightforward. There is some evidence for the stepwise mutation model (SMM):

Weber *et al.* (2002) studied microsatellites in human populations and concluded that most mutations led to new alleles which differed from the parental alleles by a few repeat units and Valdes *et al.* (1993) compared human microsatellite data to a model and concluded that the stepwise model gave the best fit to the observed data. Whittaker *et al.* (2003) analysed over 100,000 parent-offspring transmissions of AC microsatellites in humans and found that most mutations consisted of steps of one repeat unit: 65% were one repeat step, 23% were two repeat steps and 12% were three or more steps. (Gardner *et al.* 2000) found a high proportion of multi-step mutations in a lizard species (*Egernia stokesii,* Gray, 1845*).* Shriver *et al.* (1993) found that tri- and tetra- nucleotide repeats were more similar to the SMM than were dinucleotide repeats.

In a natural population such as in this study, the aim was not to investigate the microsatellite mutation model but to be aware of how mutation model could affects the estimation of population structure. If stepwise mutation does occur, then measures of population differentiation based on allele identity (e.g. F_{ST}) will be biased. Measures based on allele size, for example R_{ST} (assuming SMM), may better reflect differentiation but these measures have high sampling variance (Hardy *et al.* 2003). R_{ST} is based on size differences, and even if most mutations are stepwise but some are larger, then R_{ST} will no longer be accurate. Thus, F_{ST} is the most conservative statistic to apply. Despite the potential drawbacks of microsatellites highlighted, they still have major advantages for estimation of population structure on a small spatial scale. It is possible that, in the future (when sequencing becomes relatively cheaper with faster throughput), molecular ecology studies will employ sequencing to overcome any problems associated with null alleles, homoplasy and locus repetition.

2.3.10 Analysis of molecular variance using AMOVA

AMOVA is a useful way to partition variance between factors, in this case time and space (between years and between sites). Although genetic differentiation over time could be estimated separately, in this case, it was the relationship between-years and between-sites that was of interest. The aim was to partition the variance across all the sites, over the three years. The program ARLEQUIN v2.0 (Schneider *et al.* 2000)

was used to partition variance between sites and between years and the program to do this is available at http://anthro.unige.ch/arlequin. The option 'genotypic data with unknown gametic phase' was selected for the input data. *No* assumption of stepwise mutation mechanism was made. The hierarchical analysis of variance for the data set was 'genotypic data, several groups of populations including within-individual level'. The hierarchal AMOVA output equates to the samples thus: 'Among groups' is the between years level, 'Among populations within groups' is genetic differentiation between sites, equivalent to F_{ST} and 'Among individuals within populations' is equivalent to F_{IS}.

2.3.11 Detection of past genetic bottleneck

Populations which are currently large in census size and be relatively genetically variable, may still have underdone a past genetic bottleneck which may affect population viability (see Introduction, Chapter 1, section 1.4). The aim was to determine if a genetic bottleneck had occurred in the River Frome. The program *BOTTLENECK* v1.2.02 (Piry *et al.* 1996) was used to determine if a population bottleneck had occurred. The program computes expected heterozygosity distribution expected from the observed number of alleles, given the sample size and assuming equilibrium for each locus and for each population. The infinite alleles model was used because heterozygote excess (due to a bottleneck) has only been demonstrated for loci evolving under this model. *BOTTLENECK* takes GENEPOP and GENETIX style input files and is available from
www.ensam.inra.fr/URLB/bottleneck/pub.html. The results are in Chapter 4.

2.3.12 Relatedness estimation

In this research, juvenile samples only were used, as no samples were permitted to be taken from adult salmon. It was possible that there was a high degree of relatedness between individuals at each sample site, due to possible limited dispersal of individuals after hatching. The juvenile samples were used to estimate population structure and from this, infer adult behaviour. If the samples were not random per site, this would not meet the expectations of the F statistics. The aim was to determine the level of relatedness between all samples within each site to see a) how

much dispersal or multiple mating had occurred and b) if the samples per site were reliable i.e. could meet the assumptions required by the population structure estimates. The program *Relatedness* v5.0 (Goodnight and Queller 1999) was used to investigate the level of relatedness. *Relatedness* calculates genetic relatedness within and between demographically-defined groups of individuals using the Queller and Goodnight (1989) regression measure of relatedness. Relatedness is estimated using equation (1), for allele l, at locus k for individual x. P_x is the frequency within the current x individual of the allele l at locus k. P_y is the value of the same allele in all the individuals to which individual x's relatedness will be measured. Available from www.gsoftnet.us/GSoft.html

Equation (1) $$R = \frac{\sum_x \sum_k \sum_l \left(P_y - P^*\right)}{\sum_x \sum_k \sum_l \left(P_x - P^*\right)}$$

Relatedness outputs total relatedness per site and between-individual relatedness per site. Relatedness was estimated for the summer samples only because it was assumed that if juveniles were associated with kin then the pattern would be stronger in the summer. Individuals were not weighted. P_x was defined as all individuals and P_y was defined as P_x. The results are in Chapter 5.

2.3.13 Isolation by distance

Testing for a relationship between geographical distance between sites and genetic differentiation between sites can provide interesting information about the biology and structure of the system (see Introduction Chapter, section 1.4). The aim was to determine if isolation by distance occurred in the River Frome. Geographic distance between sites was estimated using geographic information system (GIS) software to calculate distance along the river network between sites. The river network was based on digital spatial data (Moore *et al.* 1994). Geographic distance between sites was then correlated with genetic differentiation between sites (pairwise F_{ST}) for summer and autumn for the three sample years and the significance of the correlation was tested using a Mantel test. The results are in Chapter 4.

2.3.14 Assignment of 1+ parr to site of origin

Atlantic salmon parr can move between sites in the river and it is possible that older parr have moved from their natal site. Most parr in the River Frome were 0+, however there were some older parr (age 1+). The aim was to see if older parr had moved from the natal site in order to a) increase the understanding of juvenile behaviour within river and b) decide if older parr should be excluded from samples when investigating population structure. 1+ parr were easily recognisable when sampling because they were almost twice as big (15-20 cm) as 0+ parr (<10 cm). The age of suspected older juveniles was confirmed by scale reading under the microscope. The aim was to assign the 1+ parr to a probable site of origin on the basis of genetic similarity to the 0+ juveniles. (A more direct method would have been pit-tagging of juveniles and monitoring their movement by radio-tacking, but no tagging was permitted in this river). Differences in allele frequency over time may be expected due to genetic drift and, in order control for this possibility, the potential populations of origin which were the same cohort year as the 1+ parr were used. For example, 1+ parr caught in 2000 were assigned to 0+ parr caught in 1999. The summer samples were used as the reference population as these parr are less likely to have migrated from the site of origin, compared to the samples taken in the autumn. The results are in Chapter 4.

The program GeneClass (Cornuet *et al.* 1999) was used to assign older Atlantic salmon parr to likely site of origin. GeneClass is a program for assignation and exclusion based on molecular markers. Multilocus assignment is the product of the assignment likelihoods for each locus. When an allele in the individual is absent from the population sample, the estimate of the corresponding allele frequency is zero, thus eliminating the population. However, the allele may be rare and therefore not represented in the sample. This problem can be overcome by replacing null frequencies by a small constant frequency (0.01). The set of reference populations may not include the true population of origin of an individual, thus a confidence measure was obtained. Multilocus genotypes were simulated by randomly taking alleles according to population frequencies. The program is available from www.ensam.inra.fr/CBGP.

Chapter 3

Ecology of Atlantic salmon in the River Frome

3.1 Introduction

3.1.1 Chalk stream habitat

Chalk rivers are found in the south and east of England from Dorset to East Yorkshire. Conservation of chalk rivers is important at a European level and chalk streams are recognised as a Priority Habitat under the UK Biodiversity Action Plan (www.ukbap.org.uk). Habitat is defined as the range of physical, biological and chemical factors that affect an animal (Armstrong *et al.* 2003). Abiotic factors include water temperature, oxygen availability, water depth, turbulence and velocity, catchment and river bed geology. Biotic factors include predators, riverbank vegetation and in-river vegetation that provide cover for juveniles. Atlantic salmon require high quality water (Hendry *et al.* 2003), i.e. well-oxygenated, with natural nutrient content and temperature, buffered to prevent pH fluctuations, with limited chemical contaminants and low natural silt content. Salmon juveniles occur in water with mean velocity of 5-40 cm s^{-1}, have a water depth preference of <25 cm, but can occur in water 5-100 cm deep, and are associated with substrate size range of 16-256 mm (literature survey by Armstrong *et al.* 2003). Atlantic salmon live in rivers with a water temperature of nearly 0 °C at the northerly limit but can survive in water up to 25 °C in the southern limit, such as in Spanish rivers (Elliott *et al.* 1998).

The preference is for colder water, because less oxygen is available as the water warms up. Chalk streams, such as the River Frome and the Hampshire Avon on the south coast of England, provide excellent habitat for Atlantic salmon with relatively stable flow rates and water temperature throughout the year. Chalk streams are ground water fed as well as taking run-off from the surrounding land, which makes a very stable environment throughout the year, with high water quality as the ground water input provides a buffer from pollution. In southern England, the water emerges from chalk aquifers at about 11 °C throughout the year, thus the stream temperature is generally between 5 and 17 °C (Berry 1992).

3.1.2 Juvenile abundance, habitat quality and prediction of abundance

Fifteen sites were sampled to estimate juvenile abundance and to look at differences in abundance between sites. It was expected that parr would be detected at moderate or high abundance in the River Frome, because the river had been identified as a high-quality river for salmon.

The spatial distribution, size and number of suitable habitats affect the dynamics and long-term persistence of populations (Rieman and Dunham 2000; Armstrong *et al.* 2003). Habitat indices can be used to compare habitat between sites and between rivers and also to predict the abundance and distribution of juvenile salmonids in rivers. Habitat variables were measured using the HABSCORE index, a system for measuring and evaluating catchment features and site-specific habitat features, originally developed by the National Rivers Authority, Welsh Region (Milner and Wyatt 1991), based on data from Welsh streams. HABSCORE is based on empirical statistical models that correlate observations of juvenile salmonid abundance with physical and chemical variables (measuring salmon abundance and environmental variables and then fitting the best relationship to describe the data). HABSCORE is not intended as a general indicator of river quality because environmental quality was not defined independently of salmonid abundance. HABSCORE V, used in this study, was developed by Barnard and Wyatt (1995) based on data collected from over 600 sites in England and Wales. Habitat Quality Score (HQS) was calculated for each sample site by recording biotic and abiotic factors (Methods 2.1.5). A drawback of HABSCORE is that it was developed for upland rivers so is not necessarily suitable for chalk streams. The combined data were used to predict the expected abundance of juveniles at each site, to determine if habitat quality was a predictor of abundance. The aim was also to identify any high quality sites that would require protection or low quality sites that could benefit from management, to best focus any conservation effort.

3.1.3 Dispersal from site and age of parr

Atlantic salmon juveniles remain at the site where they were born for a certain number of months after hatching (Chapter 1, section 1.1.3 for an outline of juvenile

biology). Parr dispersal in the River Frome was investigated by comparing relative abundance per site between summer and autumn. Site fidelity was estimated by mark-recapture (a small fin-clip was removed from each fish to identify any individuals that had been previously sampled in the summer). It was also expected that lower abundance would occur in autumn if the parr had dispersed from the natal site and it was expected that dispersal from site would occur by autumn. In some rivers, parr can remain in the river for up to five years before they are large enough to smolt (Chapter 1 section 1.1.3). It was expected that the favourable conditions in a chalk stream would allow most juveniles to smolt after only one year and that therefore there would be few parr found in the river which were over one year old.

3.1.4 Predators of Atlantic salmon parr

Eel, pike and adult brown trout are predators of Atlantic salmon parr (Mills 1989), that are occur in the River Frome. Avian predators were not thought to be high in the River Frome (Hilton *et al.* 2001). Predators were counted at the time of sampling.

3.1.5 Adult numbers

Management of small and declining populations tends to concentrate on the adult population size and ways to increase or protect this. However, the size and viability of the juvenile population (the stock), which is recruited to the next generation (adults), is also critical for the success of the population. This relationship is described in stock-recruitment models. In commercially exploited species, stock-recruitment models have been used to understand and manage exploited fish species, to ensure the stock is not over-fished and to maintain economic viability. The models are used to set a limit on the minimum age (size) for capture, to ensure sufficient number of breeders to maintain the population. Stock-recruitment models have been developed for commercially-fished species such as cod *(Gadus morhua,* L. 1758*)* (Cook 2000) and mackerel (*Scomber japonicus,* Houttuyn 1782) (Tanaka 2003). The biology of the system is different for Atlantic salmon (there is no fishing pressure on juvenile salmon) but the principles of the models can still be applied. A model was developed by Hilton *et al.* (2001), specifically for Atlantic salmon in chalk streams (see discussion).

3.1.6 Hypothesis and aims

Hypothesis

Abundance of juvenile Atlantic salmon in a chalk stream is related to habitat quality.

The main aim was to estimate habitat quality at 15 sites in the River Frome, to estimate abundance of Atlantic salmon juveniles at each site and to determine if habitat quality was a predictor of abundance.

The second aim was to test the assumptions that there was no dispersal between sites between summer and autumn and that most parr were able to smolt after one year.

The third aim was to investigate the relationship between predators and juvenile abundance and between adult population size, and juvenile abundance.

3.2 Results

3.2.1 Juvenile distribution

Up to 76 Atlantic salmon juveniles were obtained per site (100 m^2) using a standardised sampling technique and over 2000 samples were obtained sites over the three years. Atlantic salmon juveniles were present at all 15 sites, in summer and autumn over three consecutive years, with the exception of three sites. Atlantic salmon were distributed from the lowest site downstream near the tidal limit at West Holme (site 1-WH), upstream all the way to Dorchester (site 15-MB), although at 1-WH no salmon juveniles were captured in September 1998 or in October 2000. No fish were caught at site 4-WO in July 1998 and no fish were caught at site 15-MB in September 1998 or in July 1999. For details of parr per site for all sample times see Appendix Table 1 a-f. No obvious injury or mortality to Atlantic salmon juveniles resulted from the electric fishing survey.

3.2.2 Observed abundance of Atlantic salmon juveniles in the River Frome

For sites where at least one juvenile was caught, the lowest abundance was 0.11 parr 100 m^2 in September 1998 and the highest was 49.69 parr 100 m^2 in July 2000. Most of the sites had relatively low densities, with 81% of samples having densities of less than 10 parr 100 m^2 (Appendix Table 1 a-f, for data for all sites and all sample times). Averaged over three years, there were differences in mean abundance between sites (Figure 3.1). Highest mean abundance was detected in the middle of the river (sites 5-BM and 7-EB). Lower mean abundance was detected at upstream and downstream sites and also at site 9-TN, which was on a tributary to the main channel. There was significant correlation between the mean summer and mean autumn abundance per site, which indicated that the juveniles had not dispersed by the autumn.

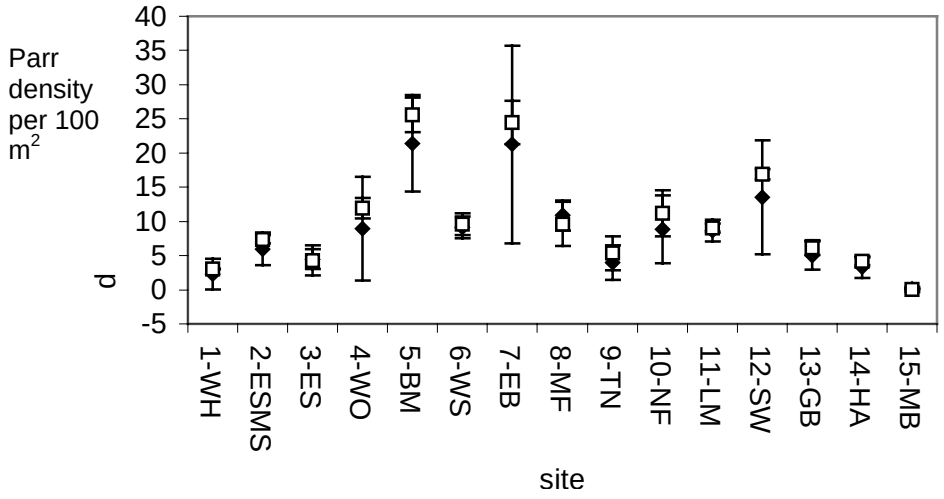
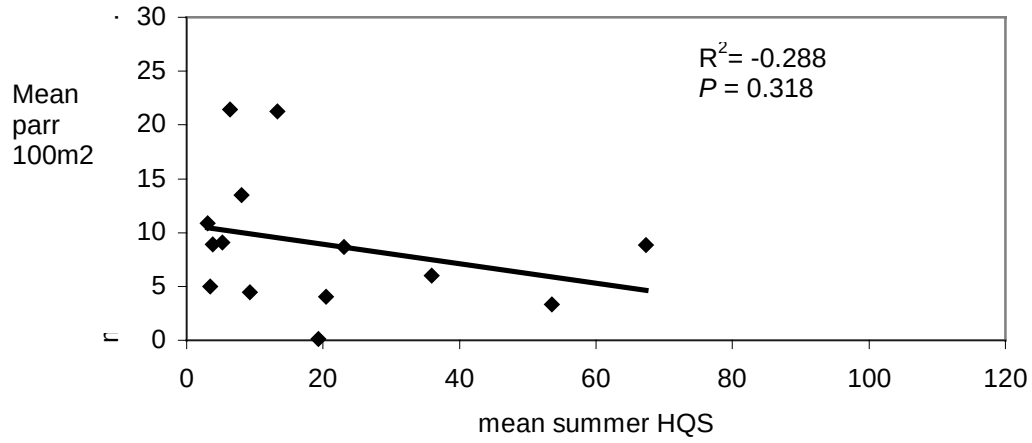


Figure 3.1 Mean (over three consecutive years) Atlantic salmon 0+ parr abundance per site in summer (diamonds) and autumn (squares) in the River Frome.

3.2.3 Habitat characteristics and observed abundance

A large range of habitat quality scores (HQS) were generated. Most HQS were below 20 for both summer and autumn, but very high HQS were detected, with a maximum of 67 in summer and 109 in autumn. HQS is a score of habitat quality specifically for juvenile salmonids thus it was expected that high HQS would be correlated with high observed juvenile abundance, but this was not found (Figure 3.2 and Appendix Table 1 a-f). Both high and lower parr abundance occurred at sites with HQS below 20 and the highest HQS for both summer and autumn had relatively low parr abundance. There was no relationship between HQS and the distance of the site upstream.

a) summer



b) autumn

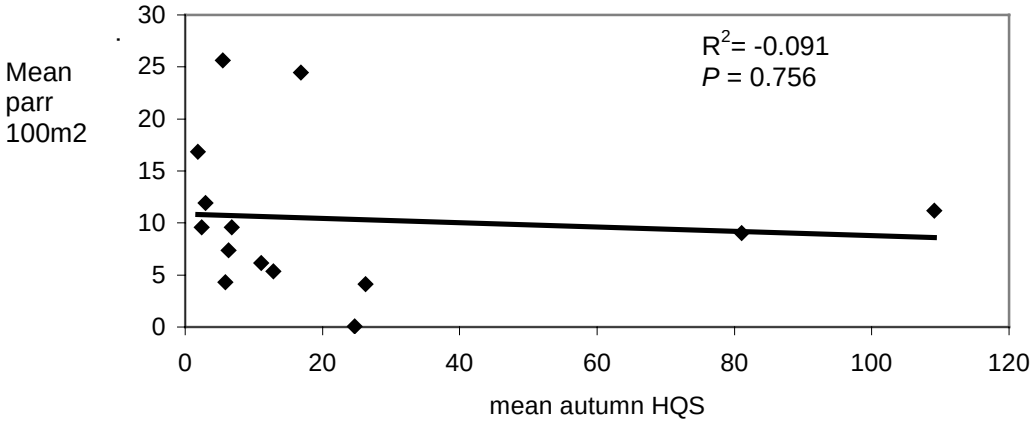


Figure 3.2 Mean (over three consecutive years) parr 100 m^2 and habitat quality score (HQS) from HABSCORE for a) summer and b) autumn.

3.2.4 Habitat characteristics and expected abundance

There were differences between observed parr abundance and abundance predicted from HABSCORE. To visualise the degree of difference, the percentage of observed to predicted was calculated (mean over 3 years per site for summer and autumn) (Figure 3.3). A value of 100% indicated that the observed parr abundance at a site was the same as the predicted abundance and a value greater than 100% indicated

that the observed number was greater than the predicted number. Most of the sites had greater observed number of parr than expected. In the summer, 9 out of 15 sites had higher observed parr abundance than that predicted by HABSCORE and in the autumn, two-thirds of sites had higher observed parr abundance than that predicted. Where the observed was higher than predicted, then this was often much higher, up to mean of over 1000%. There was no trend of higher observed than predicted abundance with site order upstream/ downstream and there was no significant correlation between mean summer and autumn values.

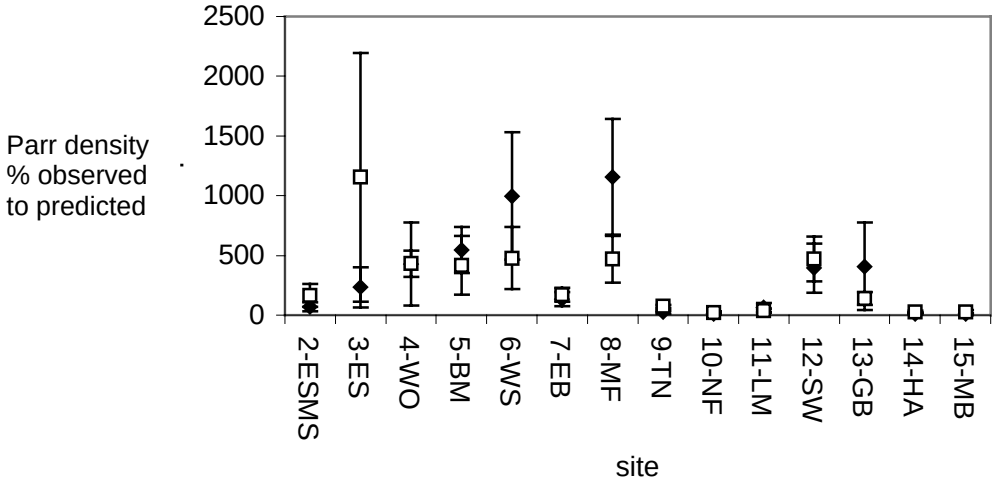


Figure 3.3 Relationship between observed and predicted 0+ parr abundance per site, mean over three years for summer (blue diamonds) and autumn (pink squares), in the River Frome.

3.2.5 Mark-recapture

Site fidelity of parr was estimated by removal of a fin-clip from each parr and noting if previously-clipped parr were found at the same site in Autumn (fish at adjacent sites were clipped on alternate sides). Recapture rates varied between sites and were between zero (site 1, WH) and 90% (site 2, ESMS) recapture. There was no consistent pattern of higher fidelity at specific sites over the three sample years, and nor was there a particular year that had a consistently higher recapture rates. For example, there were no recaptures at site 1, WH, in year 2 but in year 1 the recapture rate was 57 % and in year 3 it was 24% (Appendix Table 1 g).

3.2.6 Affect of predators on 0+ parr abundance

Pike, eel and adult brown trout were counted at time of sampling. No adult pike were found at any of the sites and no relationship was found between the number of eel and salmon parr abundance at any time (data not shown). Brown trout were relatively common at the sampling sites and up to 25 adult brown trout per site were detected. Juvenile Atlantic salmon were negatively correlated with adult trout number in July 1999 (Figure 3.4) only.

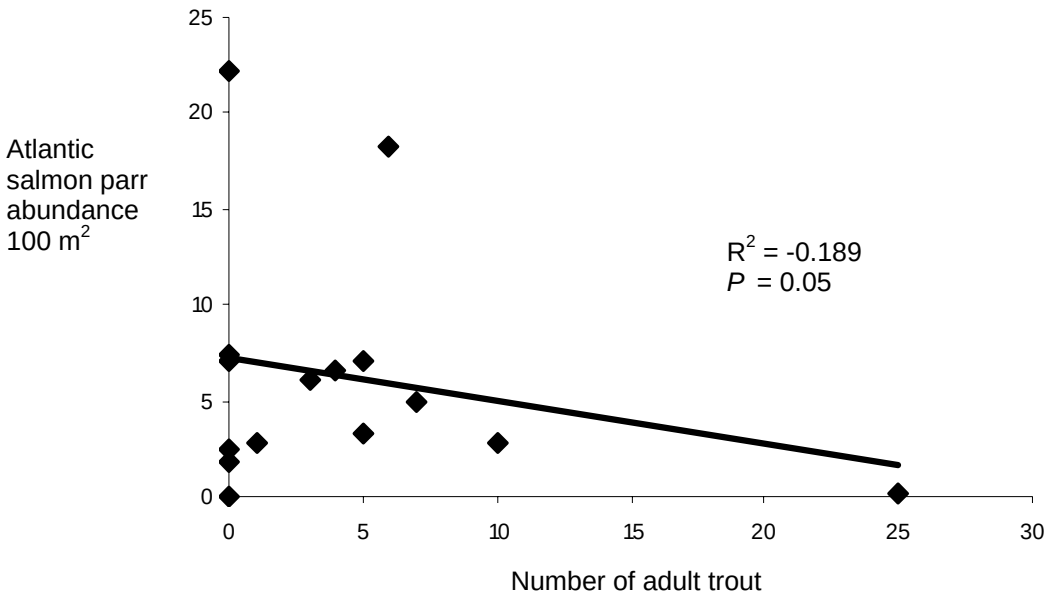


Figure 3.4 Relationship between adult brown trout and Atlantic salmon parr in the River Frome, July 1999.

3.2.7 Relationship between adult Atlantic salmon and parr abundance

For the years 1997, 1998 and 1999, the total number of adult salmon (from fish counter data) that entered the river varied between years, and the totals were 1326, 1426, 863 adults per year, respectively. Adults spawning in year χ would give rise to parr in year χ +1. There was no relationship between the number of Atlantic salmon adults the previous year and juvenile abundance the following year.

3.3 Discussion

3.3.1 Atlantic salmon parr abundance

Sites were sampled at the same time (within two weeks) each year, except for the autumn sampling for the year 2000. Autumn samples were carried out in September for the first two years, but this was delayed until October 2000 due to flooding of the river in early autumn. It was possible that the flooding could have affected fish behaviour and caused greater dispersal of juveniles in the autumn. However there was no evidence for this from the abundance or mark-recapture estimates. Parr were detected at most of the sites in all sample times and it was sites at the extremes of the range (lowest downstream near the tidal limit, furthest upstream and a small tributary) where juveniles were sometimes not detected. It is possible that the furthest upstream or small tributary sites were not accessible by spawning adults or that sites with no parr did not have suitable habitat for parr.

Parr abundance varied between sites within the River Frome from less than one individual to maximum of 50 parr 100 m^2. This is similar to that found in other rivers. In the nearby Hampshire chalk rivers, an Environment Agency survey found up to 13.5 parr 100 m^2 in the River Test and up to 35.2 parr 100 m^2 in the River Itchen (Longley 2007). In the River Claw, the average density of 0+ parr was 4 individuals 100 m^2, for the River Inney the average density was 32 individuals, in the River Ottery average density was 43 individuals and for the River Tamar average density was 2 juveniles 100 m^2 (Cornish river data provided by Environment Agency Cornwall Area). A number of factors affect juvenile density so it is difficult to state if density in any particular river is higher or lower than it should be. For example in rivers with low adult population size it might be expected that juvenile density would be reduced. Where rivers have been artificially stocked, density would be expected to be higher: Galvin *et al.* (1994) found up to 314 juveniles per site in the River Shannon, Ireland, but since the 1960s this river was stocked with over 2 million salmon at various life cycle stages.

3.3.2 Prediction of parr abundance from habitat characteristics

The expectation was that parr abundance would be related to local stream habitat features, but there was no relationship for Atlantic salmon parr the River Frome. Many of the sites had higher observed abundance than predicted, and some of these were much higher than expected (up to 1000% higher). This would seem to indicate that the habitat was ideal for salmonids, however, this is suspicious, because the often actual number of parr at these sites was low. In typical upland rivers, a trend of increased habitat quality upstream would be expected (e.g. upstream water faster flowing water, more oxygenated) but this was not the case for the Frome.

HABSCORE does not take into account all factors that influence salmon abundance in the river, such as predators and factors that influence adult site selection. These factors could be operating at a scale larger than the individual site selected and may also have a stochastic effect on parr abundance at a site, e.g. a pollution incident would cause juvenile mortality and reduce abundance. If 0+ parr have not dispersed far from the natal site, their location may be more strongly associated with adult spawning site location. Another factor is that abundance-dependent competition may occur among juveniles at a younger stage than anticipated and the population may be at a high equilibrium or a low equilibrium.

Prediction of abundance is an important management tool for small populations, by identifying sites that are below carrying capacity. A site with observed abundance equal to expected abundance is at carrying capacity, and it would be argued that any action to increase the number of juveniles would be a waste of resources. However, sites below carrying capacity could potentially benefit from habitat improvement. If a large number of sites in a river were below carrying capacity then this river would be targeted for conservation efforts. This approach assumes that the sites have relatively high habitat quality, but in natural rivers it may be low habitat quality that caused the reduction in salmon. If the habitat were heterogeneous it would also be important to consider the role of suitable but unoccupied patches, because this would affect models of carrying capacity.

3.3.3 HABSCORE limitations

HABSCORE is an empirical, statistical representation of the association between habitat features and abundance. The variables selected for these models were those expected to determine abundance for salmonid juveniles, but a drawback of HABSCORE is that it was developed for upland rivers and therefore is not necessarily applicable to chalk streams. Given that chalk streams are an important habitat for salmon, re-calibration of HABSCORE specifically for chalk streams, including the factors which are actually predictive, would be very useful. Due to the problems of HABSCORE for predicting juvenile density, it may be suggested that other habitat quality models, for example Physical Habitat Simulation System (PHABSIM, Milhous *et al.* 1999) and Numerical Habitat Model (NHM) be used, however, these were also developed for upland rivers. PHABSIM has been has been tested in chalk stream habitat (Elliot *et al.* 1999) and applied to natural Atlantic salmon populations (Guay *et al.* 2000).

3.3.4 Juvenile dispersal within the river

Almost all Atlantic salmon juveniles captured in the River Frome were 0+ parr, which met the expectation that in this river almost all juveniles are able to grow fast enough to smolt after one year. This confirms predictions from studies in other rivers (Mills 1989, Fontaine *et al.* 1997 and Beacham and Dempson 1998).

It would appear that limited parr dispersal from the site occurs between July and October, thus dispersal takes place later in the year. Mark-recapture estimates show parr had high site fidelity at some sites, although not all. The mark-recapture was a relatively unsophisticated method - the parr were clipped on either left or right at alternate sites, so if an individual had moved by more than one site, this would be detected as site fidelity and would be an underestimate of migration.

Atlantic salmon parr are able to move actively to different sites, but it is not fully understood what prompts individuals to move. Riley (2002) investigated downstream movement of parr between October and March in the River Itchen and found this was random with regard to sunset and time of maximum daily water temperature,

suggesting the environmental cue that initiate movement may be different outside the spring smolt period.

Parr can demonstrate strong attachment to site. In an artificial stream, juveniles returned to the original site when displaced by 5 m (Huntingford *et al.* 1998). Salmonids can actively associate with kin (Brown and Brown 1996 and Mjolnerod *et al.* 1999) and it may be that juveniles were taking advantage of the benefits of association with kin. In kin selection theory, individuals gain inclusive fitness through the reproduction of related individuals and benefit from association with related individuals. The theory suggests that association with kin will increase fitness due to reduced acts of aggression between kin. Altruism or reduced aggressiveness is favoured when rb-c>0, where c is the fitness cost to the altruist of the action, b is the fitness benefit to the recipient and r is the relatedness (Hamilton 1971).
Although it was outside of the remit of this research to investigate factors such as kin selection, it was important to be aware of the degree of dispersal because;

a) it is possible that the life history of juvenile Atlantic salmon in chalk streams is different to the life history of parr in other rivers and it should not be assumed that the findings in other rivers hold for chalk streams and;

b) it is important to understand the potential for juvenile population structure as these samples were used to infer adult population structure (this is discussed in Chapter 4.).

3.3.5 The effect of predators on juveniles

Adult brown trout were found to negatively affect the number of juvenile Atlantic salmon in the River Frome in July of one year. The smallest adult trout are around 20cm long, therefore, by the autumn the average juvenile salmon length of around 10cm will mean that the salmon are no longer a prey target for adult trout. There are approximately 100 brown trout per ha in the Frome, which are large enough to eat salmon parr Hilton *et al.* (2001). No pike were detected at time of sampling but the Frome does contain pike (Masters *et al.* 2002 and Beaumont *et al.* 2005). Pike have a large home range, over several km (Masters *et al.* 2002), which may account for their non-presence in a 100m stretch. Pike may be important predators of juvenile salmon. In data used in the Hilton *et al.* (2001) model, it was assumed that 5% of pike diet is

of salmonid parr but up to 60% of pike diet is of salmon smolts in April (peak smolt run). Eel was detected in the Frome, but no relationship was found between eel and parr abundance. It was estimated that there are approximately 3460 (+/- 1643) eels per hectare in the Frome and that one eel eats between 1 and 4 salmon parr per year (Hilton *et al.* 2001). Thus, eel could potentially have a large impact on parr. Juvenile size may be important for predator avoidance, although this relationship is not clear-cut. Size-selective predation occurs juveniles if predators actively target larger juveniles and it is possible that larger juveniles suffer greater mortality from predators in flood conditions (Metcalfe *et al.* 1998 and Good *et al.* 2001).

3.3.6 Relationship between number of adults and juvenile abundance

It was expected that a larger number of spawning Atlantic salmon adults would result in a greater juvenile abundance, but no relationship between adult number and juvenile number was found. This is not a reliable conclusion, as the juvenile data was only for three years and a longer data set for juveniles would be needed to thoroughly test this hypothesis and refine stock-recruitment models. From knowledge of salmon life history it would be expected that there would be many more juveniles in the river than adults, by a factor of 100-10,000 depending on life history stage. It would not be possible to measure every parr in a river, however, it is possible to count smolts as they migrate downstream prior to leaving the river. Using resistivity counter and video surveillance (located at site 3-ES, grid reference SY870867), smolts were counted in the River Frome and in April and May (the peak smolt run) 3083 smolts were detected migrating downstream to sea (Pinder *et al.* 2003).

Hilton *et al.* (2001) proposed two models to aid management of Atlantic salmon in chalk streams. The first model used data for survival at each life history stage and can be used to predict number of eggs from the number of spawning adults or vice versa. It is assumed that if adult numbers are increased there is sufficient spawning area available for any additional adults and also that the main factor affecting egg survival is clean gravel. The model does not take density dependence into account. Survival data were available for some of the some of the life-history stages but not all. The second model investigated the effects of predators on juvenile salmon. The Hilton *et al.* (2001) model had high variability in the outputs, however, the estimates

produced with the assumption of 'dirty' gravel produced figures close to the actual data and the predation model appeared to be even less reliable. One of the conclusions from the paper was that there was a paucity of data from salmon rivers to calibrate even simple models.

3.3.7 Management to increase number of juveniles

Augmenting sites with low carrying capacity assumes that increased juveniles will lead to increased number of adults. Although increased juvenile density may appear to be desirable for increasing the population, this may lead to increased competition at the juvenile stage. Juveniles are territorial (Huntingford *et al.* 1998) and will compete for food with other salmon and also with juvenile trout. Parr density is related to survival at various life-cycle stages. In an artificial enclosure, juvenile salmon were stocked at densities between 0.16 and 1.33 m^2. A dominance hierarchy was present and it was found that even at lower densities the territories overlapped (Armstrong *et al.* 1999 and Griffiths and Armstrong 2002). Territorial attachment is strong, for example, Armstrong *et al.* (1999) found that Atlantic salmon juveniles at high density (1.33 m^2) (this is high compared with the wild) did not move to neighbouring areas, even when sites were cleared of competitors. Thus, it is not necessarily absolute density which is important but behaviour and space use.

If the number of adults could be predicted from number of juveniles, this could be applied to conservation monitoring and management i.e. if juvenile numbers fell below a set threshold, action could be initiated to increase juvenile numbers, resulting in increased adult returns.

3.4 Conclusions

The main findings were:

- Habitat quality was not a predictor of Atlantic salmon parr abundance in the River Frome.
- Movement of Atlantic salmon parr from natal sites occurred later in the year (after October) than was expected.
- Adult brown trout were found to decrease juvenile Atlantic salmon abundance at one sample time only.

The River Frome, having the characteristics of chalk streams such stable flow and stable water temperature, does provide good Atlantic salmon habitat and the abundance of juveniles detected was similar to other rivers. However, on a site-by-site basis, the habitat quality scores were very variable and high quality sites did not necessarily have more juveniles. For future studies, if a detailed assessment of habitat is required then this can be measured using the HABSCORE system, but if the main objective is to determine juvenile abundance, reliable data are best obtained from quantitative electric fishing. This is time-consuming and has to be weighed against the need to limit disturbance to populations.

Habitat quality is not the only factor that can influence population size: predators can affect juvenile density. Juvenile abundance in the River Frome was not so low as to cause alarm, although there is still an incomplete knowledge of the stock-recruitment pattern of juveniles compared to number of returning adults. Even if the habitat quality in a river is generally high, and there are sufficient juveniles, population decline may still occur and factors such as reduced genetic variation, bottlenecks and population structure may be involved. These are addressed in the following chapters.

The hypothesis stated that ‘abundance of juvenile Atlantic salmon in a chalk stream is related to habitat quality’. This hypothesis was rejected.

Chapter 4

Genetic variation and spatial population structure of Atlantic salmon in the River Frome

Abstract

Microsatellites were used to estimate genetic variability and population structure in Atlantic salmon parr at 15 sites, in summer and autumn over three consecutive years. Moderate genetic variability was found in all the populations and there was no evidence for a past genetic bottleneck. Homozygote excess was detected for all loci, but null alleles did not account for all of this. There was no evidence for linkage between any loci (with only three exceptions of individual comparisons). Significant differentiation between sites was found and F_{ST} was between 0.023 and 0.03. The level of genetic variability and population structure were similar to that found in previously published studies. There was evidence for isolation by distance in two out of six sample times. Older parr were not assigned to the site where they were caught, indicating that they had migrated between sites in the river and were therefore not used in the estimates of population structure.

4.1 Introduction

As discussed in the previous chapter, differences in habitat quality between sites were found in the River Frome, but no relationship between habitat quality and site position in stream was detected and there was no relationship between habitat quality and juvenile abundance. The next aspect of the research was to investigate genetic variation and population structure, in the context of small and declining populations. Microsatellites were used to estimate genetic variability to compare between sites within the River Frome and, using data from previously published studies, to compare variability of Atlantic salmon in the River Frome with other rivers. It might be expected that, given no obvious pattern of habitat structuring, small distances between sites and no barriers to migration, that no significant spatial genetic differentiation would be detected. However, previous studies have detected differentiation between Atlantic salmon at sites within one river (Sections 4.1.3 and 4.1.4 for details), thus the hypothesis was set that 'there is significant genetic differentiation between Atlantic salmon within one river'.

4.1.1 Genetic diversity

The amount and distribution of genetic diversity are influenced by biology of the species. Diversity is important in small and declining populations because low genetic diversity is both a result of and a cause of population decline and can lead to population extinction (see Chapter 1, section 1.4.). Genetic diversity can be measured using number of alleles or allelic richness per locus and by observed heterozygosity (note, these are not independent measures). In published studies on Atlantic salmon, the number of alleles detected per locus varied between loci, as is expected, because some loci are inherently more variable than others (Table 4.1). The number of alleles detected per locus is dependent to a certain extent on sample size, due to the presence of many low frequency alleles in natural populations (Nei 1987). An adjustment, allelic richness, was made to take sample size into account (Chapter 2, section 2.3.2). Allelic richness is constrained between 1, low diversity and 2, high diversity. Allelic richness was calculated using published data for other rivers to compare diversity between loci and between rivers. In published studies of Atlantic salmon, observed heterozygosity varied between loci and between studies (Table 4.2). Some of these

differences will be a reflection of inherent differences in variability between the loci, the characteristics of the study population (for example some of the published studies may be from declining populations or where there has been a bottleneck) and most importantly, differences in sample sizes.

Table 4.1 Number of alleles for Atlantic salmon in other rivers.

Locus	*Sample size*	*Number of alleles*	*Reference*
Ssa 171	343 173	33 28	Garant et al. 2000 Fontaine et al. 1997
Ssa 197	181 343 145	20 19 14	Fontaine et al. 1997 Garant et al. 2000 Beacham and Dempson 1998
Ssa 202	171 113 343	20 16 16	Fontaine et al. 1997 Beacham and Dempson 1998 Garant et al. 2000
Ssa 289	55	6	McConnell et al. 1995
Ssosl 85	150 176 343	16 11 15	Nielsen et al. 1999 Fontaine et al. 1997 Garant et al. 2000

Table 4.2 Observed heterozygosity, from published data, of the microsatellites used in this study.

Locus	*Observed heterozygosity (Ho)*	*Reference*
Ssa 171	0.6- 0.9	Fontaine et al. 1997, Tessier et al. 1997, Tessier and Bernatchez 1999
Ssa 197	0.4 -0.8	Beacham and Dempson 1998, Fontaine et al. 1997, Tessier et al. 1997, Tessier and Bernatchez 1999
Ssa 202	0.4-0.8	Beacham and Dempson 1998, Fontaine et al. 1997, Nielsen et al. 1999
Ssa 289	0.3 -0.9	Beacham and Dempson 1998
Ssosl 85	0.7 - 0.96	Fontaine et al. 1997, Garant et al. 2000

4.1.2 Homozygote excess due to drop-out

The data were checked for scoring errors and allele non-amplification (known as drop-out) which would lead to false homozygotes. Inaccurate estimation of homozygosity could potentially bias estimates of population structure. However, the tests would indicate where potential errors occurred and would give the potential to correct the data if needed.

4.1.3 Spatial population structure in Atlantic salmon

Adult Atlantic salmon return to the river of origin to spawn with a high degree of fidelity. This is reflected in total genetic differentiation between N. America and Europe (Stahl 1998 and King *et al.* 2001). The Baltic Sea drainage forms a distinct cluster from both the European and N. American fish (Stahl 1998). The main factor controlling differentiation between rivers is believed to be the tendency of Atlantic salmon to home to the natal river to spawn, allowing differentiation between rivers to occur over time. Most previous studies of Atlantic salmon population structure have focussed on assessment of genetic variation *between rivers*. Estimates of Atlantic salmon genetic differentiation between rivers are lower than between-continent estimates, although founder effects could affect the rivers sampled and it may be expected that adult Atlantic salmon are more likely to 'stray' to a nearby river. Between-river differentiation for anadromous populations was between 0.3% and 23% (Figure 4.1). One of the studies that reported 23% differentiation (Nielsen *et al.* 1996) used mitochondrial markers (mitochondria have a small effective population size and are more susceptible to genetic drift, thus higher differentiation is expected).

4.1.4 Population structure of Atlantic salmon within a river

Life history and behavioural tactics such as time of return, mate choice and site selectivity, combined with habitat structure will control within-river spatial genetic population structure. The potential of structuring of Atlantic salmon within a river was posited by Heggberget and Lund (1986). At the within-river scale, fewer studies have been carried out and it is not yet established if adult Atlantic salmon return to specific sites within the river to spawn. Previous studies show within-river variation

is generally lower than between-river differentiation, but values are in the lower range of those detected for between-river variation. Differentiation between populations within rivers of 0.1% to 3% has been detected in previous studies (Figure 4.1).

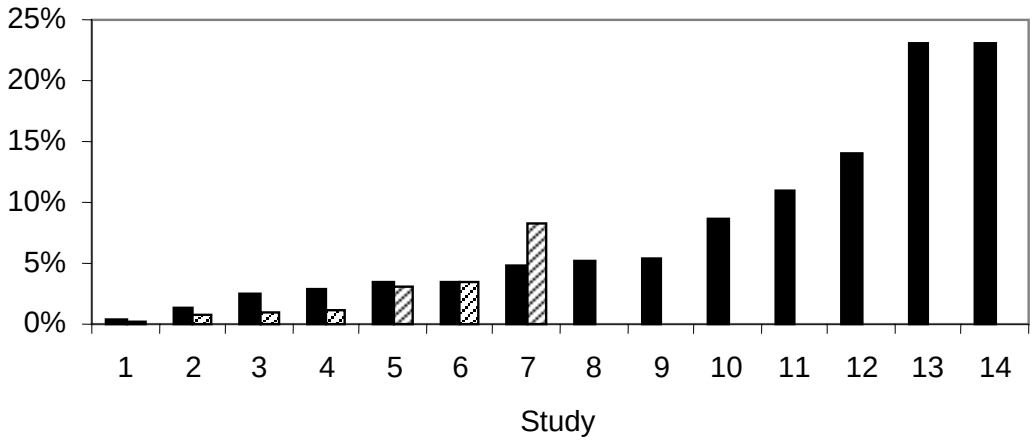


Figure 4.1 Genetic differentiation between anadromous Atlantic salmon between rivers (black bars) and within rivers (hatched bars). Between river data from Stahl 1983, Fontaine *et al*. 1997, O'Connell *et al*. 1995, King *et al*. 2001, Galvin *et al*. 1996, McElligott and Cross 1991, Stahl 1998, McConnell *et al*. 1997, Verspoor *et al*. 1999, McConnell *et al*. 1995, Verspoor *et al*. 2002, Nielsen *et al*. 1996, Bourke *et al*. 1997. Within river data from Verspoor *et al*. 1991, Jordan *et al*. 1992, Garant *et al*. 2000, Beacham and Dempson 1998, Galvin *et al*. 1994, McElligott and Cross 1991 and Spidle 2001.

4.1.5 Isolation by distance

Isolation by distance is a correlation between genetic differentiation between sites and geographic distance between sites, with the expectation that sites further away

from each other are less similar because there are fewer migrants between distant sites. Isolation by distance is more likely to be detected in resident populations, where the rate of migration reduces with increasing distance, but this is not necessarily the case for migratory species where the breeding population is in effect reconstituted each year and where any individual could potentially breed with any other individual anywhere in the system.

Between-rivers, isolation by distance would be expected for migratory species if there is a degree of homing to the natal river and differentiation between rivers would be expected to be related to distance between rivers and the length of time the populations had been established. Isolation by distance in Atlantic salmon in adjacent rivers was detected by McConnell *et al.* (1997), Bourke *et al.* (1997) and King *et al.* (2001). In contrast, McElligott and Cross (1991) and Nielsen *et al.* (1996) reported no relationship between genetic and geographic distance. A pattern of isolation by distance should be more noticeable in a linear system. Castric and Bernatchez (2003) analysed brook charr in 52 rivers along the Atlantic coast of Canada and detected a strong relationship between genetic differentiation and increasing northwards distance. It is important to bear in mind the biology of the species when making assumptions about isolation by distance: brook charr have limited marine movements and it is assumed that fish colonised rivers moving northwards since the last ice age.

Significant genetic differentiation between sites was detected in 7 previously published studies that have assessed population structure within rivers (Figure 4.1), but a significant correlation between genetic and geographic distance between sites within a river was detected only in one of these 7 studies (isolation by distance was detected by Verspoor *et al.* 1991). Isolation by distance within a river is not necessarily expected for Atlantic salmon because of their ability to move between sites within the river. Detection of isolation by distance with the juvenile samples would support an assumption of population structuring in the adult population.

4.1.6 Bottleneck

Current diversity may have been affected by a past demographic and/or genetic bottleneck. When a population undergoes a reduction in size, heterozygosity excess can occur, i.e. observed heterozygosity is larger than the expected heterozygosity from the number of alleles found in the sample, if the population were at equilibrium (Cornuet and Luikart 1996). Degree of heterozygote excess is related to the amount of time since the bottleneck, the effective population size before and after the bottleneck, the mutation rate and the sample size. The effects of bottlenecks on heterozygosity are expected to last for thousands of years after the recovery of population size (Nei 1987), therefore it is expected that the observed heterozygosity would be lower than expected heterozygosity obtained from the current population size. To test for heterozygote excess, the relationship between the observed number of alleles and expected heterozygosity is determined. This can be done by a) determination of the proportion of loci with heterozygosity excess significantly larger than expected or b) determining if the average of standardised differences between observed and expected heterozygosity is significantly different from zero (Cornuet and Luikart 1996). Populations with significant heterozygosity excess may have been through a recent bottleneck, although heterozygosity excess can also be caused by dissortative mating.

4.1.7 Movement of older parr from original site

The age structure of juvenile Atlantic salmon in the River Frome was noted at time of sampling. It was expected that the abundance of older parr in the River Frome would be low because the river is towards the south of the range, and being a ground-water fed chalk stream, the water temperature is expected to be stable and relatively warm, allowing most juveniles to smolt after one year. Older parr can move from the natal site and if movement had occurred, this would influence estimates of population structure. One aim of this research was to investigate if 1+ Atlantic salmon parr moved from their original sites in the River Frome, and, if they do so, to determine how far they move and if there was a trend for downstream movement, by assignment of 1+ parr to a site of origin.

Assignment of individuals to populations has many applications (Waser and Strobeck 1998). Assignment has been successfully applied to questions in fish biology such as determination of parentage, mixed-stock analysis and assignment of adults caught at sea to a river of origin. It is useful to assign offspring to parents to investigate heritability of traits or success of different genotypes in farmed or stocked situations. Estoup *et al.* (1998) used parentage assignment to investigate relationships and facilitate selection for desirable traits in farmed rainbow trout. Parentage analysis was applied to Atlantic salmon in an American river, where salmon were extirpated 150 years ago, to investigate the success of different genetic types (Letcher and King 1999). In this river, most adults were blocked from returning to the natal sites to spawn, natural reproduction was negligible and therefore over the past 30 years fish were stocked. Assignment of adult fish can be used to investigate the river or lake of origin. This was neatly illustrated by Primmer *et al.* (2000) to exclude an Atlantic salmon caught in a fishing competition from the lake in which the competition was carried out. The winning fish was actually purchased on a market and thus the angler was prosecuted. Stock assignment is crucial in commercial marine fisheries and can also be applied on a smaller scale. For example, Hansen *et al.* (2001) analysed the contribution from domesticated brown trout to anadromous brown trout in two stocked populations with no genetic data available prior to stocking.

Several assignment methods have been developed. Rannala and Mountain (1997) used an assignment method to calculate an individual's most likely population of origin that was less sensitive to deviation from the true allele frequency in the reference population, for example, if there is a small sample size. Genetic distance methods can also be used, by calculating the genetic distance between individuals and reference populations and assigning the individual to the population with the lowest distance. Davies *et al.* (1999) cautioned that phylogenetic approaches to assignment of individuals to populations are less reliable if the reference populations were relatively recently established. The most useful assignment method, where the aim was to assign juveniles to a population of origin, appeared to be that devised by Paetkau *et al.* (1995) for polar bear population structure. Using the test, an individual can either be assigned to or rejected from a group on the basis of genetic similarity. In this situation, an individual is assigned to the population in which that individual's genotype is most likely to occur. This determines how indicative an individual's

genotype is of the population from which it was sampled. The assignment test is performed by calculating the expected frequency of each individual's genotype in each population and subsequently assigning each individual to the population which had the highest expected genotype frequency.

4.1.8 Hypothesis and aims

Hypothesis:

There is significant genetic differentiation between Atlantic salmon within one river, despite small geographic distance between sites and no barriers to migration.

The main aim was to estimate genetic differentiation between sites and determine if isolation by distance occurred.

The second aim was to estimate the level of genetic variation, because low variation can be related to population decline.

The third aim was to determine if older parr had moved from the original site.

4.2 Results

4.2.1 Genetic diversity- number of alleles

The five loci used to screen the population for genetic variability were all polymorphic and the number of alleles detected (at all sites) per locus was between 5 and 13. The most highly polymorphic loci were Ssa 197 and Ssosl 85, having 13 alleles each. Twelve alleles were detected at locus Ssa 171 seven alleles were detected at locus Ssa 202. The least polymorphic locus was Ssa 289 with 5 alleles (for data for each site at each locus at each sample time, see Appendix Tables 4 to 9).

Allelic richness standardised per individual (ARi) varied between loci. No site had particularly low ARi, at any sample time (Figure 4.2). Highest over all loci ARi was found (mean over three summer samples) at site 1-WH, ARi 1.76 and the lowest ARi (mean over three autumn samples) was 1.58 at 9-TN.

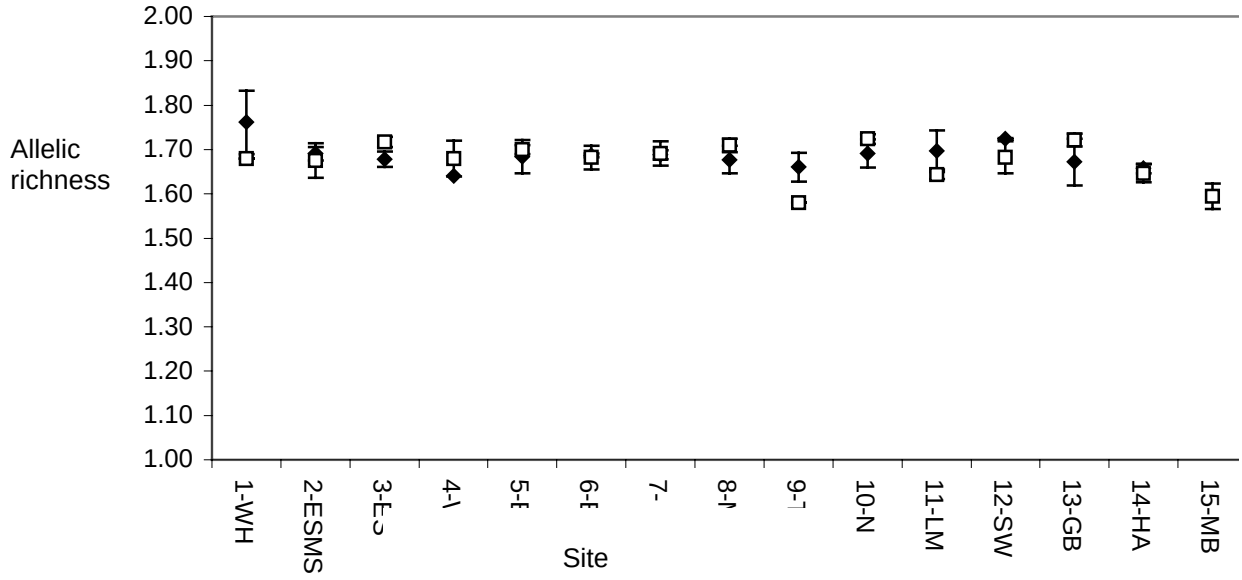


Figure 4.2 Allelic richness over all loci per site for three years, summer (diamonds) and autumn (squares), for Atlantic salmon in the River Frome.

4.2.2 Allelic richness using data from published studies

Published allele frequency data were used to estimate ARi per locus for Atlantic salmon in Canadian and European rivers (Table 4.3). ARi varied between loci and between study and the lowest values were for Ssa 289 in three North American and two European rivers from McConnell *et al.* (1995) data. At loci Ssa 197, Ssa 171, Ssosl 85 and Ssa 202, the estimates of allelic richness in this study were lower than allelic richness estimates for every other river. At locus Ssa 289 estimates of allelic richness in this study were within the range of estimates obtained from other studies.

Table 4.3 Allelic richness for Atlantic salmon in other rivers

Locus	*Allelic richness*	*Study*	*Reference*
Ssa 171	1.84 to 1.913	seven Canadian rivers	Fontaine et al.1997
Ssa 197	1.856 (in 1986) 1.866 (in 1992) 1.825 to 1.912 1.904 (in 1996) 1.928 (in 1997)	three tributaries, Canadian river, seven Canadian rivers	Beacham and Dempson 1998, Fontaine et al.1997, Garant et al.2000
Ssa 202	1.875 (in 1986) 1.849 (in 1992) 1.838 and 1.91 1.857 (in 1996) 1.889 (in 1997)		Beacham and Dempson 1998, Fontaine et al.1997, Garant et al.2000
Ssa 289	1.305, 1.262, 1.547, 1.756 and 1.544 1.638 (in 1986) 1.075 (in 1992)	three North American and two European rivers	McConnell et al.1995, Beacham and Dempson 1998
Ssosl 85	1.743 to 1.887 1.869 (in 1996) 1.879 (in 1997) 1.736 (in 1930s) 1.76 (in 1989) 1.869 (in 1989)	Danish river, Scottish river, Swedish river	Fontaine et al.1997, Garant et al.2000, Nielsen et al.1999

4.2.3 Genetic diversity- observed heterozygosity

Moderate heterozygosity across loci was detected at each site for summer and autumn (Figures 4.3 and 4.4). For summer samples, highest Ho was 0.72 at site 12-

SW and the lowest Ho was 0.54 at sites 3-ES and 11-LM. For autumn samples the highest value across all loci was Ho 0.70 at site 4-WO and the lowest value was Ho 0.52 at site 15-MB. (Appendix Tables 4 to 9 for Ho and Ht for each site per locus for six sample times).

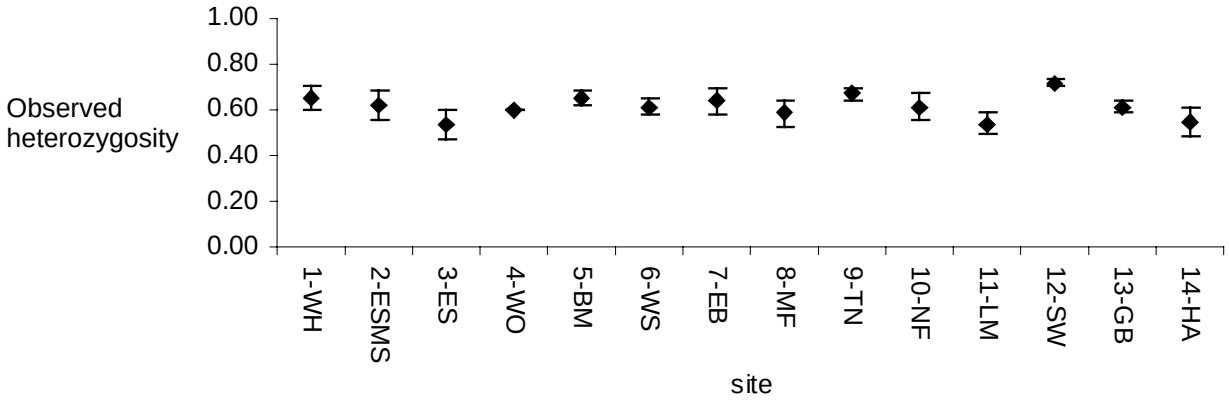


Figure 4.3 Observed heterozygosity (Ho) over all loci (mean over three sample years) per site for summer.

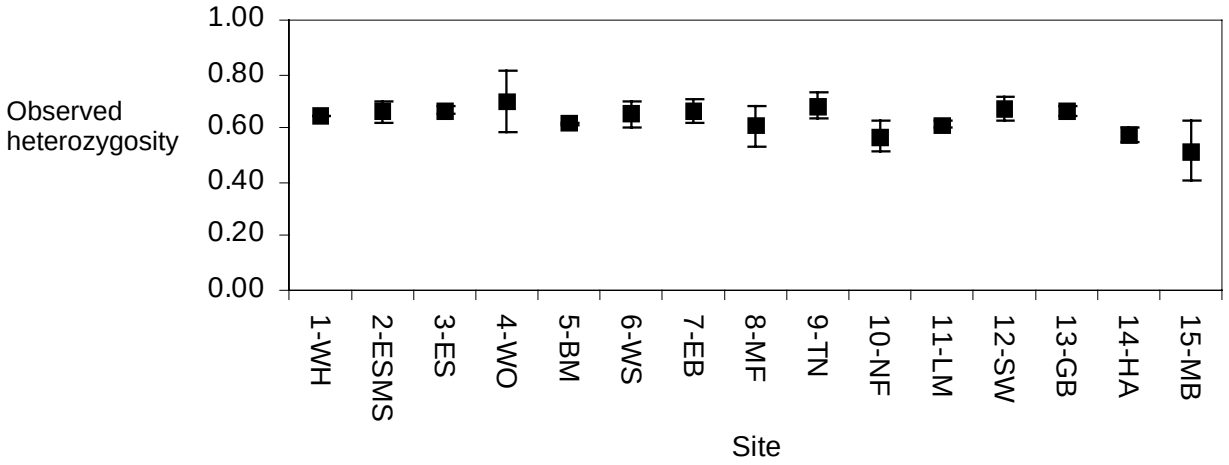


Figure 4.4 Observed heterozygosity (Ho) over all loci (mean over three sample years) per site for autumn.

4.2.4 Stutter and nulls

No evidence for stutter was detected at any locus at any population at any time, with the single exception of locus Ssa 289 at Pop 1 in July 1998. No evidence for large allele dropout was detected at any locus at any population at any sample time, with the single exception of Ssa 289 at population 3 in July 2000. There was evidence for homozygote excess at all loci, and although not all indications of homozygote excess were attributable to potential null alleles, high proportions were. Loci were affected by homozygote excess at between 3/34 and 11/34 of the populations tested (Table 4.4). The checks were carried out for July 1998, July 1999 and July 2000. Thirty-four populations were analysed in total as the program was not able to complete analysis on sites with small sample size). For loci Ssa 171 and Ssa 202, all excess homozygotes were explained by potential nulls. At loci Ssa 197 and Ssosl 85, of the 8/34 populations with excess homozygotes three of these populations per locus were *not* due to nulls. At Ssa 289, of 11 populations with excess homozygotes, four of these populations were *not* due to nulls.

Most sites were affected by nulls in at least one locus and at least one sample time. Site 3 had the highest incidence of null alleles, with nulls at Ssosl 85 in all 3 years, nulls at Ssa 202 in 2 years and a null at Ssa 197 in one year.

Table 4.4 Incidence of homozygote excess and potential null alleles for microsatellite loci.

Locus	*Ssa 171*	*Ssa 197*	*Ssa 202*	*Ssa 289*	*Ssosl 85*
Number and (%) of populations with excess homozygotes	3/34 (8.8%)	8/34 (23.5%)	4/34 (11.8%)	11/34 (32.3%)	8/34 (23.5%)
Number and (%) of populations with potential nulls	All excess homozygotes due to potential nulls	5/34 (14.7%)	All excess homozygotes due to potential nulls	7/34 (11.8%)	6/34 (8.8%)

4.2.5 Linkage

Linkage between the microsatellite loci was tested using the program Linkdos in Genepop. There was no evidence for linkage between any locus and any other locus at any of the six sample times with the following three exceptions: in July 1998, Ssa 171 and Ssa 197 had a X^2 of 42.40 $P = 0.006$, in September 1998, Ssa 202 and Ssa 171 had a X^2 of 37.67 $P = 0.01$, in July 1999, Ssa 202 and Ssa 171 had a X^2 of 39.1 $P = 0.027$. Some statistically significant linkages would be expected by chance when a large number of tests are carried out.

4.2.6 Population structure – F statistics over all sites per sample time

F statistics were estimated over all loci, over all sites for each sample time. At each sample time significant F_{IT} (total non-random mating) was detected. Significant F_{ST} (non-random mating between sites) was detected at each sample time. For the summer samples, F_{ST} was between 0.023 and 0.047 and for the autumn samples, F_{ST} was between 0.03 and 0.041. Significant F_{IS} was detected at each sample time (Table 4.5).

Table 4.5 F statistics over all sites for Atlantic salmon in the River Frome.

Sample time	F_{IT}	F_{ST}	F_{IS}
July 1998	0.195***	0.023***	0.176***
September 1998	0.157***	0.041***	0.121***
July 1999	0.078***	0.03***	0.049**
September 1999	0.085***	0.04***	0.046*
July 2000	0.106***	0.047***	0.062***
October 2000	0.075***	0.03***	0.046*

Significance $P = 0.0001$***, $P = 0.001$**, $P = 0.01$*

4.2.7 F_{IS} per site

F_{IS} (non-random mating within sites) was estimated for each site at each sample time per locus and over all loci for summer (Figure 4.5) and autumn (Figure 4.6). For

summer samples, most F_{IS} values were between zero and +0.4, however, in autumn there were negative as well as positive values. There were differences between years, between sites and between summer and autumn but there were no clear trends. Not all positive F_{IS} values were significant: in July 1998 six sites had significant positive F_{IS}, in September 1998 and July 2000 two sites per year were significant. There were no significant values at any other sample time (Table 4.6). (Data for each locus per site for the six sample times, Appendix Tables 10 to 15).

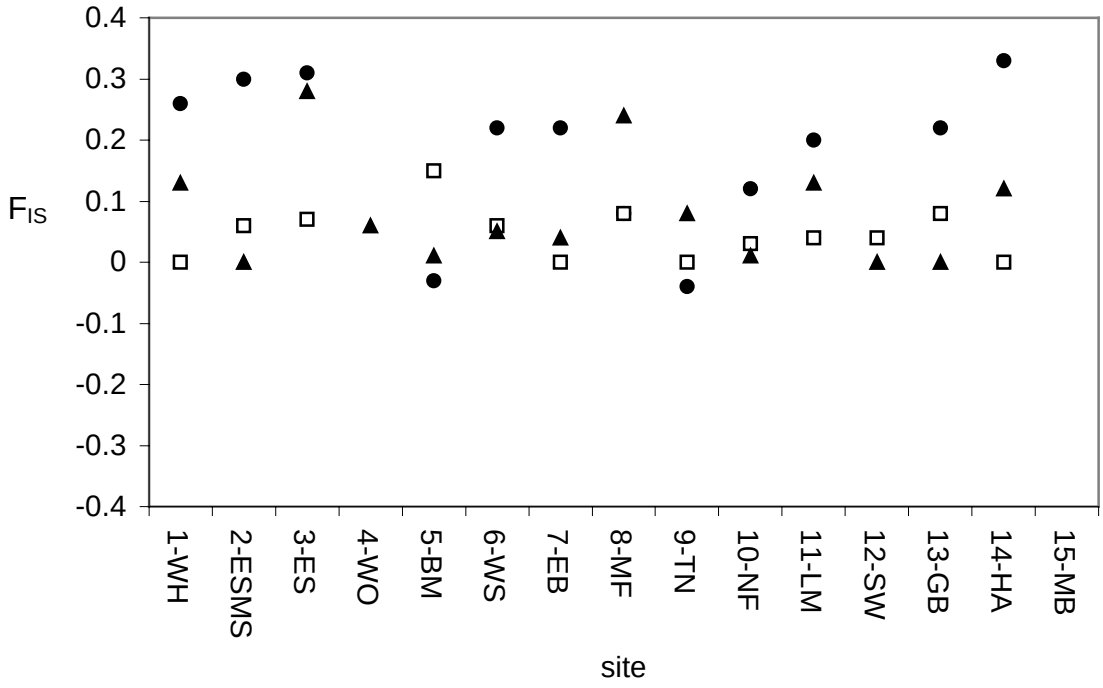


Figure 4.5 F_{IS} per site for summer 1998 (circles), 1999 (squares) and 2000 (triangles).

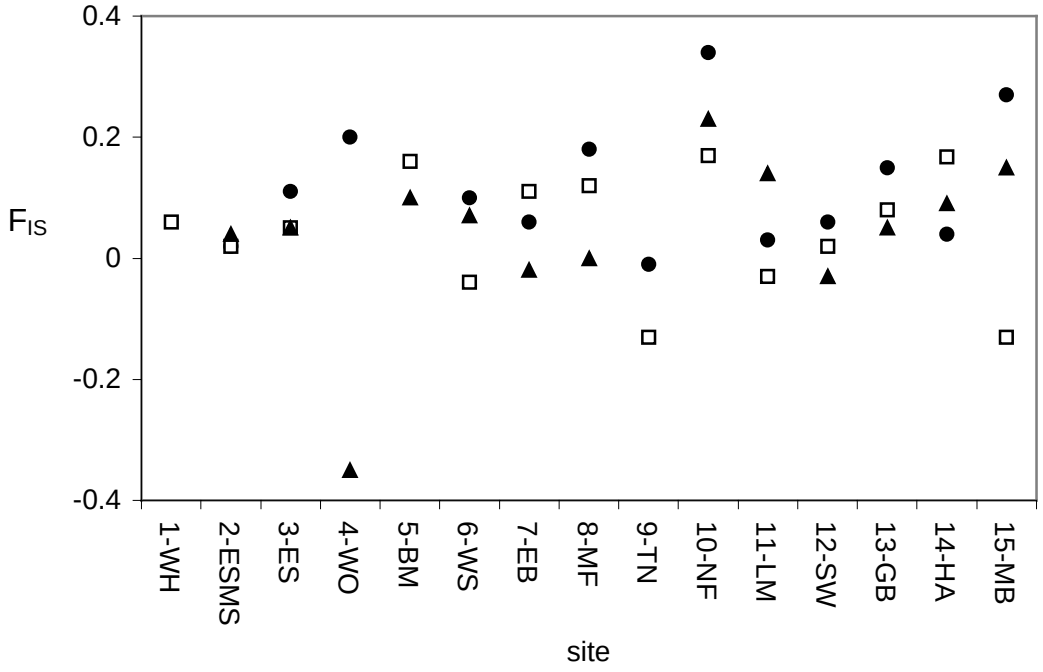


Figure 4.6 F_{IS} per site for autumn in three years 1998 (circles), 1999 (squares) and 2000 (triangles).

Table 4.6 F_{IS} values significant at $P = 0.05$, after Bonferroni correction (July 1998, September 1999 and October 2000). There were no significant values at any other times.

Site	*July 1998*	*September 1998*	*July 2000*
1-WH			
2-ESMS	*		
3-ES	*		*
4-WO			
5-BM			
6-WS	*		
7-EB	*		
8-MF		*	*
9-TN			
10-NF		*	
11-LM	*		
12-SW			
13-GB			
14-HA	*		
15-MB			

* significant at $P = 0.05$, after Bonferroni correction

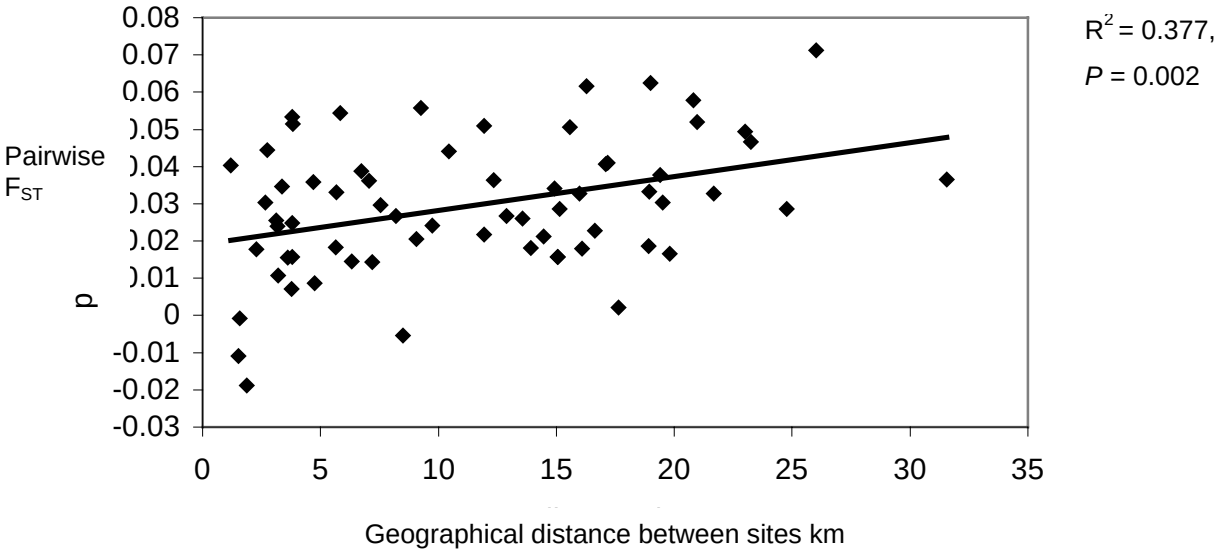
4.2.8 Pairwise F_{ST}

Genetic differentiation was estimated between each pair of sites for each sample time (Appendix Tables 16 to 21). Not all comparisons were significant after Bonferroni correction and the number that were significant varied between years and between summer and autumn with no clear trend. Four comparisons were significant in July 1998, 12 comparisons were significant in September 1998, 25 comparisons were significant in July 1999, 11 were significant in September 1999, 30 were significant in July 2000 and 26 were significant in October 2000. Of the significant comparisons, the actual sites involved varied between years and between seasons. The highest significant value was 0.1427 between site 13-GB and site 3-ES.

4.2.9 Isolation by distance

Geographic distance between sites was plotted against genetic distance (F_{ST}) between sites for each year in summer and autumn (Figure 4.7) and a significant correlation (isolation by distance) occurred in two out of six sample times, in summer 1999 and autumn 2000.

a) July 1999



b) October 2000

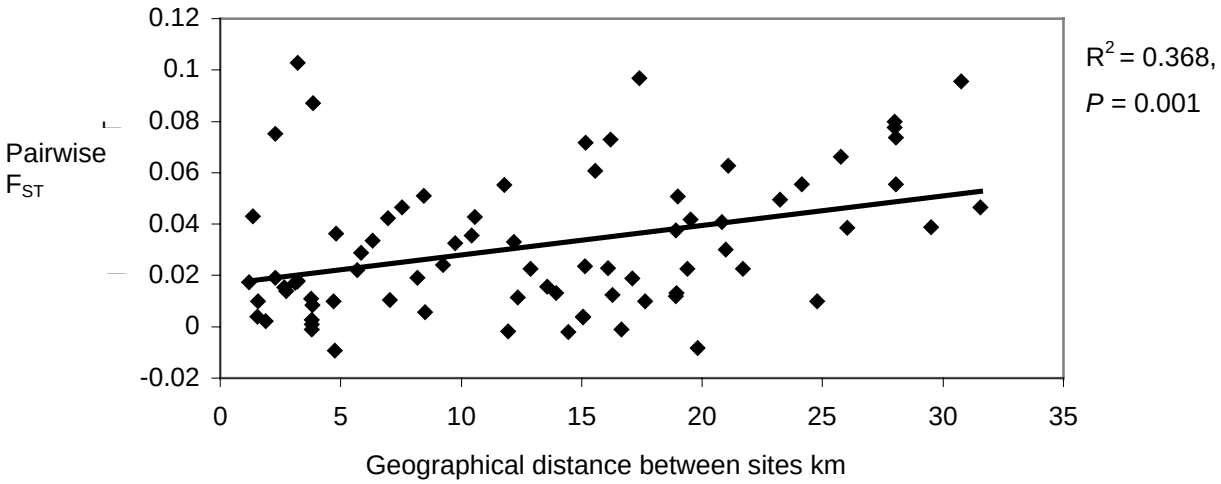


Figure 4.7 Pairwise F_{ST} against geographical distance for Atlantic salmon in the River Frome for a) summer 1999 and b) autumn 2000.

4.2.10 Bottleneck

The program *BOTTLENECK* was used to test the three summer sample times (JY98, JY99 and JY00) for genetic bottlenecks. There was no evidence for a genetic bottleneck with just one exception in July 1998, which had evidence for a genetic bottleneck at site 5 only, under the infinite alleles model.

4.2.11 Number of 1+ parr

A small number of 1+ parr were captured in the River Frome but no parr older than 1+ were captured. No juveniles older than 0+ were detected in July 1998 or in October 2000. Where 1+ parr where detected, the percentage was low, between 0.34% in September 1999 and 4.44% in July 1999 (Table 4.7).

Table 4.7 Atlantic salmon 1+ parr in the River Frome.

Sample time	*Number of 1+ juveniles*	*% 1+ juveniles*
Summer 1998	none	0
Autumn 1998	10	3.38
Summer 1999	14	4.44
Autumn 1999	1	0.34
Summer 2000	5	1.38
Autumn 2000	none	0

4.2.12 Assignment of 1+ parr to natal site

None of the 1+ parr samples were assigned to just one site (Table 4.8). In total, eight 1+ parr *were* assigned to the site where they were caught, but these 1+ parr were not assigned exclusively to this site and some were assigned to ten sites in total.

Table 4.8 Probability of assignment of 1+ parr to sample site and adjacent site

1+ parr sample site	*Probability of assignment to sample site*	*Probability of assignment to adjacent site*
1+ parr sampled July 1999 to reference 0+ parr July 1998		
4-WO	0/8	1/8
4-WO	0/8	2/8
4-WO	0/9	2/9
6-WS	1/9	2/9
6-WS	1/7	1/7
7-EB	1/9	2/9
7-EB	1/5	1/5
7-EB	1/5	2/5
10-NF	1/9	1/9
10-NF	1/8	0/8
1+ parr sampled September 1999 to reference 0+ parr July 1998		
3-ES	0/3	0/3
1+ parr sampled July 2000 to reference 0+ parr July 1999		
6-WS	0/2	0/2
6-WS	1/6	2/6
6-WS	0/4	1/4
4-WO	0/10	2/10
4-WO	0/7	2/7

Probability of assignment 10000 simulations at a rejection level of $P = 0.01$

4.3 Discussion

4.3.1 Molecular analysis

No automated sequencing facility was available at the laboratory where the research was carried out and it was deemed too expensive to screen the samples using an automated sequencer at another institute, therefore silver stain was used to detect alleles differentiated by size on polyacrylamide gels. Silver staining allowed sizing and scoring of alleles at equivalent accuracy to that obtained from an automated sequencer and silver staining was much cheaper to set up and run. Silver staining removed safety issues associated with radionucleotide labelling, but did involve the use of formaldehyde, which was used in a fume cupboard. Although the silver staining method was found to be adequate for this study, for future studies it would be preferable to use an automated detection system and multiplex the microsatellites to speed throughput.

4.3.2 Number of primers, performance and statistical power

Initially 17 primers were selected for testing, however, it was not intended to use all 17 to screen all the samples. The reason for selecting a large number to start was to ensure a useful set could be obtained from these. Using a smaller number of microsatellites is not problematic if the markers are sufficiently variable to differentiate individuals or populations on the desired scale, with low standard error. If the loci selected were not sufficiently variable to investigate small-scale population structuring then more loci should be optimised to increase the power.

Primers cloned from other species did not perform as well in Atlantic salmon as primers cloned from Atlantic salmon. Brown trout primers were tested in this study but it was found that although the product was polymorphic, the alleles had such a high degree of stutter that they were not scorable. *Oncorhynchus* sp. are less closely related to Atlantic salmon and *Oncorhynchus* primers tested in this study gave a monomorphic product. The limited use of cross-species amplification was not

surprising and was not a particular problem for this analysis of Atlantic salmon because there were still Atlantic salmon primers that did provide a polymorphic, scorable product.

Primer information from this study (details of previous use, heterozygosity and use in related species) was contributed to the international meeting of the 'SALGEN' working group on Atlantic salmon genetic population structure to be published by the Atlantic salmon Trust (www.atlanticsalmontrust.org). The aim was to promote co-ordination of Atlantic salmon primer use for all studies and development of a standardised set for international use. If a standardised set of primers were agreed this would make comparison of genetic variation between rivers more straightforward. Since the literature survey was carried out many more studies have used microsatellites to investigate population structure in Atlantic salmon (for example Spidle 2001, Tessier 2000 and Vasemagi, Gross *et al.* 2001). It would be very interesting to able to analyse data obtained in different rivers in a meta-population study. In order to do this, laboratories would need a standard primer set, calibrated to standard samples and even if all laboratories were using the same automated sequencers, there may be still be differences between runs.

4.3.3 Genetic variability of Atlantic salmon

The number of alleles detected in the Frome was lower than number of alleles detected in studies of Atlantic salmon in other rivers and allelic richness was lower than in other rivers. It is possible that higher allelic richness estimates in other rivers were due to the larger adult population size, but this is not possible to state with certainty because adult census population size was given in only one of these studies (adult population between 1520 and 2271 individuals, Fontaine *et al.* 1997). Heterozygosity in this Frome was generally equivalent to that found at the same loci in other studies of Atlantic salmon. The loci were selected to be heterozygous to provide high power for population structure analysis.

Genetic variation was expected to be low in the River Frome because the population had declined, but in contrast the River Frome currently has a larger population than

many other rivers. In addition to population size the time since the population was founded and degree of isolation from other rivers will affect genetic variation. The maintenance of genetic variation in the River Frome could be due to adults straying from other rivers. Although it might be expected that strays would come from the nearest rivers such as the Piddle, there are currently no published studies which have characterised the stock for each river, thus it was not possible to identify the offspring of possible strays into the study river. For conservation purposes, there is no 'target' level of genetic variation set for large healthy populations, thus it is difficult to determine if the level of variation detected here is sufficient to maintain the population.

4.3.4 Homozygote excess

No evidence for stutter was detected at any locus at any population at any time, with the single exception of the dinucleotide locus Ssa 289 at site 1- WH in July 1998. No stutter was detected at the other dinucleotide locus used in this study (Ssosl 85). Stutter bands are expected more at dinucleotide loci due to polymerase slippage during replication. If the stutter bands of the two alleles overlap then it is not possible to score the allele size accurately, and this primer would be rejected. Sufficient published Atlantic salmon microsatellite primers were available, therefore, if stutter bands were a problem, dinucleotide loci could be avoided. No evidence for large allele dropout was detected at any locus at any population at any sample time, with the single exception of Ssa 289 at population 3 in July 2000. Thus large allele dropout was not accountable for homozygote excess.

Homozygote excess can be caused by null alleles, and whilst not all homozygote excess is caused by null alleles, in this study potential null alleles were quite common (affecting up to 15% of the sample populations and affecting all loci to some degree). Primers Ssa 171, Ssa 197, Ssa 202, Ssa 289 and Ssosl 85 were used to screen the samples. These loci have been used in previous studies of Atlantic salmon and some have found some evidence for homozygote excess, but other studies found no evidence of homozygote excess. For loci Ssa 171, Ssa 202 and Ssa 197, Fontaine *et al.* (1997) found some evidence for heterozygote deficiency at one site. At locus Ssa 197 this could have been due to the high number of alleles compared with the

sample size. At loci Ssosl 85, Nielsen *et al.* (1997) detected a significant heterozygote deficiency. Nielsen *et al.* (2001) detected deviation from Hardy-Weinberg at Ssosl 85 at two populations and Ssa 202 in one population. King *et al.* (2001) found heterozygote deficiency for locus Ssa 197 in one population. Vasemagi *et al.* (2001) found no deviation from Hardy Weinberg equilibrium at Ssa 202, Ssa 197 or Ssa 171. No significant deviation from Hardy-Weinberg equilibrium was found by Beacham and Dempson (1998) for loci Ssa 197, Ssa 202 and Ssa 289, nor by Nielsen *et al.* (1999) at loci Ssosl 85, nor by King *et al.* (2001) at locus Ssa 289.

No previous studies have suggested that non-amplification was a cause of deviation from expected allele frequencies at the loci used. In these studies, deviation from expected Hardy-Weinberg frequencies was estimated using exact tests, e.g. Raymond and Rousset (1995) and not using Micro-Checker. It is possible to adjust the heterozygote frequencies using the program Micro-Checker, however the adjusted genotypes are ordered according to allele size and do not correspond to the original sample numbers thus these data can not then be used to perform multi-locus genotypic analysis in other applications. The presence of null alleles will affect the estimates of relatedness.

Allele non-amplification will make the population appear to be less variable and may lead to assumptions about low variability, past bottlenecks and hence fitness. In addition, false homozygotes may affect estimation of population structure and relatedness. Null alleles are not only found in fish: for example in a study of red deer (*Cervus elephus* L.*)*, Pemberton *et al.* (1995) found three out of 16 loci had non-amplifying alleles. Null alleles are likely to be found in many populations, although few studies specifically test for them. If a problem with null alleles was suspected, the non-amplifying alleles could be revealed by reducing the primer annealing temperature to allow some primer mis-binding or by re-designing primers to avoid mutation sites. DNA adjacent to a microsatellite may accumulate mutations, therefore, primers should be designed slightly away from the microsatellite region.

Microsatellite allele size homoplasy

With all markers there will be a proportion of homozygotes (i.e. individuals that have alleles of the same size) that are not ‘true’ homozygotes because the alleles are not

identical by descent –proteins and restriction fragments with different sequences may band at the same position on a gel. Microsatellites are probably less prone to this problem, but with forward and back mutations, alleles with the same repeat number may not be identical by descent.

Allele size homoplasy occurs when alleles of identical size are not identical by descent. Alleles may be identical in state, having exactly the same sequence but may not be identical by descent (Estoup *et al.* 2002). In addition, alleles may be the same size but have different sequences and are therefore definitely not identical by descent. Homoplasy will result in lower genetic variation being detected in the population than the true level of variation.

To determine if alleles of the same size have different sequences (and therefore are not identical by descent) proportion of the alleles can be sequenced. It is of course not possible to detect alleles that are identical in state but not identical by descent in this way. Allele size homoplasy due to different sequences may be quite prevalent in population studies. For example, van Oppen *et al.* (2000) found 13 additional alleles after sequencing in cichlids and Angers *et al.* (2000) found 8 alleles among 3 electromorphs in a freshwater snail. No studies have yet reported homoplasy in Atlantic salmon, however, given that homoplasy occurs in *Oncorhynchus* sp. (Ardren *et al.* 1999) it is possible that it occurs in Atlantic salmon.

Homoplasy can also affect the estimation of population structure. Estoup *et al.* (2002) assessed the effect of mutation rate, mutation model, effective population size and time of divergence between populations on size homoplasy and concluded that for many ecological situations the large amount of variability at microsatellite loci compensates for any homoplasy. Only the infinite alleles model assumes that identity in state equals identity by descent. Use of the stepwise mutation model rather than the infinite alleles model will also compensate for homoplasy. Allele size homoplasy is expected to be greater with increasing time of divergence between lineages. Given that microsatellites have previously been sufficiently variable to discriminate between populations of Atlantic salmon on a small spatial scale then it is anticipated that size homoplasy, whilst potentially present, would not form a significant problem in this research.

4.3.5 Linkage

There was very limited evidence for linkage at any of the loci used. Only three out of the 60 comparisons had any evidence for linkage and two of the significant occasions were between Ssa 171 and Ssa 202. Studies of Atlantic salmon in other rivers have detected some evidence for linkage disequilibrium for the loci used in this study. In a study of Atlantic salmon, Spidle (2001) found that in a few samples, loci Ssa 197 and Ssosl 85 were linked and also in a few samples that loci Ssa 171 and Ssa 197 were linked. The linkage was ascribed to differences in effective population size between sites. No association was found between loci Ssa 171, Ssa 197 and Ssa 202 by O'Reilly *et al.* (1996) and also no evidence was found for linkage between Ssa 171, Ssa 197 and Ssa 289 by Beacham and Dempson (1998). If loci are found to be linked it can be a useful tool for population genetics. Estoup *et al.* (2000) used juxtaposed microsatellites to investigate population mixing in brown trout. Juxtaposed microsatellites are defined as being two microsatellite arrays less than 200 bp but more than 20 bp apart. Juxtaposed markers would only need to be used if the markers selected were not sufficiently variable to differentiate between populations.

Salmonids are of tetraploid origin (Allendorf and Thorgaard 1984 and Johnson *et al.* 1987) and appear to have retained 50-75% of the loci as duplicates (Bailey *et al.* 1978). This can cause problems when using microsatellites. Primers cloned for a microsatellite locus will amplify two or more sites if the locus has had a historical replication and the primer binding sites have not mutated. This can lead to scoring difficulties. If the primer binds two sites, an individual fish may have one, two, three or four bands per locus. Four bands would indicate that the individual is heterozygous at both sites. For individuals with one, two or three bands the situation is more complicated. One band may indicate that both sites are homozygous or that non-amplification of alleles has occurred, two bands may indicate two homozygotes or two identical heterozygotes or non-amplification of alleles of different heterozygotes and three bands could be one homozygote and a heterozygote or two heterozygotes with non-amplification of one allele. Although methods have been proposed to deal with replicated loci (Waples and Aebersold 1990) it is preferable to

reject any such loci from a screening set if possible. Duplication has not previously been reported for any of the loci used in this study.

4.3.6 Bottlenecks

Atlantic salmon underwent a severe demographic bottleneck in the Frome in 1850, and also a less severe demographic bottleneck occurred in 1989 when the census size dropped from 4000 to 1000 adults. The demographic bottlenecks may have resulted in reduction of genetic variation, but, using data from three consecutive years, there was no evidence for a genetic bottleneck in Atlantic salmon in the Frome (with the exception of just one population at one sample time). It can be concluded that the Frome population was not suffering from a bottleneck, thus the demographic bottlenecks of 1850 and 1989 were not severe and had no lasting adverse effects of genetic variation. No conclusive evidence for genetic bottlenecks has been reported in any salmonid study. A potential population bottleneck in Atlantic salmon was proposed by Nielsen *et al.* (1997) in a comparison of genetic variation in adult scale samples (from 1930s) to current variation in juveniles, but the authors suggested that using juvenile samples to compare to adult samples was not reliable. Tests for bottlenecks rely on the assumption of no population substructure and no immigration (Cornuet and Luikart 1996). Significant population structuring within river was detected and it is certainly possible that salmon from other rivers, such as the Test and Itchen in Hampshire, could have strayed into the Frome. This would introduce new alleles to the population and would serve to mitigate the effects of any bottleneck and maintain genetic diversity. From these data it can be concluded it is unlikely that a genetic bottleneck played a role in the decline of Atlantic salmon in the River Frome.

4.3.7 Atlantic salmon population structure

Low but highly significant differentiation was detected between sites in one river with no barriers to migration, but not all sites were significantly differentiated from each other. It was important to investigate differentiation between sites, because it is possible that there was unique genetic diversity held in these populations. Previous studies of differentiation between the River Frome and its neighbouring rivers have

been carried out using allozymes. Russell and James (1995) used 5 allozyme markers and found no genetic differentiation between the River Frome and its nearest river, the Piddle. However, one of the loci used was MEP-2, which is known to be under selection in other systems, and two loci were monomorphic in both populations, therefore the assessment relies on just two markers and was not therefore statistically powerful. It is likely that allozyme markers used were not sufficiently variable to have the power to detect population structure. Microsatellite markers are highly variable and have been used previously to discriminate Atlantic salmon populations on a within river scale. The extent of genetic differentiation between sites in this research was within the range expected when compared with the extent of differentiation between sites in other rivers of similar size.

There are few external factors likely to have influenced population structuring in the River Frome (this is in contrast to most other UK rivers, Hendry *et al.* 2003): there are no barriers to migration, the river is small and there has been no hydroelectric development, thus population structuring would not be disrupted due to adults being unable to reach spawning grounds. Many U.K. rivers with Atlantic salmon have been stocked with fish from other rivers in the past, sometimes from other countries (Aprahamian *et al.* 2003), which could disrupt population structure. Escapees from aquaculture can cause genetic disruption, however, it is very unlikely that there has been any past or current disruption of genetic processes from aquaculture escapees entering the River Frome, as the nearest farms are in Scotland. There have been no reports from anglers of capture of escapee adults (evidence from CEH Fish Biology Department). Previous stocking was identified as a potential disruptor of population structure by Stahl (1983), Galvin *et al.* (1994) and, within river, by Spidle (2001). The pattern of genetic differentiation between sites may be related to barriers to migration, such as waterfalls and hydroelectric stations. For example a relationship between genetic differentiation and barriers were found by Galvin *et al.* (1994) and Garant *et al.* (2000) for within-river salmon populations.

4.3.8 Isolation by distance

A significant positive relationship was found between geographical distance between sites and genetic distance between sites for Atlantic salmon in summer 1999 and

autumn 2000. Isolation by distance was detected in summer 1999, but was not detected in autumn 1999, suggesting that the pattern of differentiation between sites had started to disintegrate as juveniles dispersed more by autumn (although there was still significant population structure in autumn 1999). It was however, unexpected to find isolation by distance in an autumn sample (2000), without isolation by distance being detected in the corresponding summer sample. Atlantic salmon can move between sites and within a river, it is not necessarily the case that straying will occur more often between neighbouring rivers or sites, thus isolation by distance is not necessarily expected. It is possible that adjacent sites contained the offspring of the same parents. It is known that some adults undertake multiple mating, sometimes across sites that are separated by 5 km. Many of the sites in this study were much closer than that and therefore it was not expected to detect isolation by distance in a river as small as the Frome, with very small geographic distances between sites of less than 50 km. In published studies of Atlantic salmon within one river, isolation by distance was detected in just one out of seven studies (Verspoor *et al.* 1991). Detection of isolation by distance would be strong evidence for population structuring a population.

4.3.9 Habitat structure and population structure

Habitat structure can influence population structure, for example, in classic granite rivers the dendritic stream ordering may play a role in structuring, because a) higher quality habitats would be expected to occur upstream and b) adult salmon might be able to locate specific sites within the river to spawn, that is, home to the site where they were born (Massen and Welters, 2002).

In granite rivers, adults typically return to the river in two stages, a spring migration and an autumn migration, with no fish returning in the summer because this is when the river would be impassable due to low flow. In these rivers, this could cause structuring because fish that return earlier in the year could potentially migrate to the best spawning sites or further upstream. Chalk rivers, however, have a relatively stable flow throughout the year (i.e. do not have the low summer flow associated with other rivers) and in the Frome adults return throughout the spring, summer and

autumn (data from fish counter). Thus structuring due to distinct spring and autumn returns would be unlikely in a chalk stream.

4.3.10 Assignment of older parr

It was not possible to assign any of the 1+ parr exclusively to a site of origin. In fact 1+ parr appeared to be able to be assigned to a large proportion of the available populations (up to ten). The advantage of assignment tests is that identification of individuals and estimation of recent gene flow or migration can be carried out without relying on the assumption of genetic equilibrium (this is not necessarily expected in recently-founded populations) (Davies *et al.* 1999).

The power of microsatellites to assign individuals accurately depends on the number of offspring, loci and alleles sampled. Bernatchez and Duchesne (2000) developed multivariate analytical models to predict the probability of assigning offspring to parental couples as a function of population size, number of loci and allelic diversity. Given sufficient allele diversity, a relatively low number of alleles are required to achieve high allocation success. There was no significant gain in allocation success with allele diversity greater than 6-10 alleles per locus. Neff (2000) provided a model that requires knowledge of the genotypes of putative parents, genotypes of the offspring and allele frequency in the breeding population but does not require sampling of all the potential parents in order to estimate parentage. When parents are incompletely sampled, there are similar benefits from increasing the number of loci and the number of offspring sampled (Neff *et al.* 2000).

The number of loci may have affected the ability of the program to assign individuals. More loci and more alleles would make the assignment test more powerful and may have resulted in more precise assignment. Bernatchez and Duchesne (2000) carried out simulations and found that a relatively low number of alleles were required to achieve high allocation success. For example, in the simulation, 7 loci with an average of 13 alleles would be required to reach an allocation success in a population of 300 possible parents (Bernatchez and Duchesne 2000). In this study 5 loci with an average of 8 alleles were used. The power of microsatellites to assign offspring to parents was analysed by Villanueva *et al.*

(2002) who found that nine loci with 5 alleles or six loci with 10 alleles were sufficient to assign parents with certainty to 99% of fish resulting from either 100 or 400 crosses. Letcher and King (2001) used microsatellite genotypes of mating adults to identify offspring in a mixture. First generation offspring were assigned to the correct family using four loci with 7 alleles per locus.

Samples from juveniles were used to estimate population structure and, because older juveniles may have moved from the site of origin, then using these samples would affect the estimation of genetic differentiation, when using juveniles to estimate structure among returning adults. In the River Frome, older parr were a very small percentage of the total number of juveniles thus older parr were removed from estimates of population structure, without detriment to sample size.

4.4 Conclusions

The main findings were:

- a moderate level of heterozygosity was detected at each sample site
- low but significant genetic differentiation between sites was detected in summer and autumn for three consecutive years
- isolation by distance was detected at two out of the six sample time, despite very small distances between sites, of less than 50 km
- older parr could not be assigned to a site of origin.

The fact that genetic differentiation was detected between sites within the river indicates that population structuring can occur on a small spatial scale. There was no pattern of habitat differences between sites, therefore genetic differentiation between sites was not correlated with habitat structure. The presence of older parr in a sample could affect estimates of spatial population structure as it is likely that older parr will have moved from the natal site. The lack of a relationship between genetic isolation by geographical distance at all sample times may indicate that sites were selected on factors other than adults returning strictly to the natal site to spawn.

The hypothesis ‘there is significant genetic differentiation between Atlantic salmon in one river, despite small distance between sites and no barriers to migration’ was accepted.

Chapter 5

Temporal stability of spatial population structure

Abstract

Temporal stability of spatial population structure was tested using Atlantic salmon from 11 sites in three consecutive years, in the River Frome. No significant genetic variation between years was detected using Analysis of Molecular Variance (AMOVA). A new test (*Q*-test, R.T Clarke unpublished) was used to explicitly test if particular sites were more similar over time. The test was valid even if some of the sites were affected by non-random sampling of juveniles. Mean relatedness for Atlantic salmon parr was low per site. Although particular sites had some full-sib pairs, across all the sites the percentage of full-sibs comparisons was low (<8.5%). No temporal stability of spatial population structure was detected in the River Frome. Significant temporal stability of spatial population structure was detected using published data from Atlantic salmon at 7 sites sampled in two consecutive years, in a Canadian river.

5.1 Introduction

As discussed in the previous chapter, significant spatial genetic differentiation between sites was detected in Atlantic salmon the River Frome in both summer and autumn in three consecutive years. Differentiation was between 2.3 and 3%, which is comparable to that found in other studies, of 0.1% to 3% (Verspoor *et al*. 1991, Jordan *et al*. 1992, Garant *et al*. 2000, Beacham and Dempson 1998, Galvin *et al*. 1994, McElligott, Cross 1991 and Spidle 2001). Isolation by distance was detected at two out of six sample times.

Detection of significant differentiation in three consecutive years and detection of isolation by distance would appear to be strong evidence for within-river population structuring in Atlantic salmon. The next step was to determine if temporal stability of spatial population structure occurred. If temporal stability of spatial population structure is detected, this indicates that the fish spawned at specific sites within the river and that this is stable over time. This could lead to increased genetic differentiation between sites, to adaptation to site and in extreme cases to speciation.

The possibility that the sampling system affected estimates of spatial population structure was investigated. In this study, fin-clip samples taken from juveniles were used to estimate genetic differentiation between sites. The important assumption under Hardy-Weinberg (required for the F statistics) was that these were random samples. This would not be the case if the parr were related at sibling level (see section 5.1.3).

5.1.1 Temporal genetic variation in salmonids

Previous studies, for example Consuegra *et al.* (2002) using Atlantic salmon historic scale and bone samples, detected low genetic variation between years, even over long time scales. Long term genetic variation in Atlantic salmon in Iberian rivers (Spain) was assessed by analysis of thirty Atlantic salmon vertebrae from between 3250 to 41000 years before present (Pleistocene era) (Consuegra *et al.* 2002). The ancient samples did not cluster with either current samples from geographically-close sites or with other European rivers, thus it was concluded that there may have been

significant changes in genetic structuring over this time. Studies using archive scale samples to investigate genetic variation in Atlantic salmon over a 20-100 year time period generally show that Atlantic salmon allele frequencies are relatively stable over time, for example Martinez *et al.* (2001) compared fish in a Spanish river from 1970s to 1997 and found that local fish were more similar over this time period than stocked individuals. Few collections of old scales exist and old scale sample sizes tend to be very small, so the conclusions could be unreliable.

In this study, the temporal scale was very short, i.e. between consecutive years. As differentiation had not been detected in other studies, even over long time periods, it was not expected to detect significant temporal variation. Aspects of the life history such as repeat spawners and overlapping generations due to different age at spawning, will tend to reduce genetic drift over time. (Not all adults survive spawning, thus the breeding population will have a percentage of adults which have bred previously. The percentage of previous breeders is between 7% and 43% and varies between rivers, Fleming 1998). If most adults die after spawning, and all fish bred in their cohorts it would be expected that genetic drift between years would be higher). The size of the breeding population will also affect the amount of stochastic variation between years and smaller populations will be more prone to this.

5.1.2 Spatio-temporal population structure in Atlantic salmon

Previous studies of Atlantic salmon spatial and temporal genetic variation found lower temporal differentiation than spatial genetic variation and temporal genetic variation was often non-significant. For example, Jordan *et al.* (1992) used juvenile samples from different year classes to investigate spatial and temporal genetic variation in three rivers. Variance among rivers was low (1.6%) and variance among tributaries in river was low (0.7%). Among year classes within tributaries variance was low at 1.2%. Most of the variance was within year classes (96.4%). Galvin *et al.* (1996) studied Atlantic salmon in the River Shannon, Ireland between 1991 and 1993 and found only one site had temporal genetic differentiation and that temporal differentiation between year classes was low and non-significant. Beacham and Dempson (1998) analysed Atlantic salmon in the Conne River and found no fluctuation in allele frequency over time was found for any of the rivers. Nielsen *et*

al. (1997) compared samples from 1930s with a currently (1989) endangered Danish population of Atlantic salmon, and concluded that temporal allele frequency change was lower than spatial structuring. Samples from 1913 compared with samples from 1989 also exhibited few temporal differences but there was evidence for the persistence of population structuring (Nielsen *et al.* 1999). Tessier and Bernatchez (1999) compared genetic variation, using archive scale samples, in four landlocked populations of Atlantic salmon over three to five generations and found that within-site temporal genetic variation was lower than between-site variation.

Garant *et al.* (2000) surveyed Atlantic salmon at seven sites within one river, the River Sainte-Marguerite, Canada, and sampled over two consecutive years (1996 and 1997). Variation held between tributaries, among sites, between temporal samples within sites, among individuals within samples was tested and the number of loci with significant differences in allele frequency was tested. Total among-sites variation was 3.4%. No significant variation due to grouping in tributaries was found. Same site- different year differentiation explained 2.5% of the variance and 0.9% of the variance was spatial (with temporal variance removed). Thus, the Garant study found similar levels of spatial genetic variation to this study (3.4% in the Canadian river, compared with 2.3 – 3% in the River Frome).

Although previous studies looked at both spatial genetic variation and temporal genetic variation in Atlantic salmon, no previous study specifically tested for temporal stability of spatial structuring. Although the design of the Garant study was similar to this study, there are conceptual differences between the two, with regards to the reasoning and conclusions (see section 5.3.1 for details).

5.1.3 Juvenile relatedness and non-random sampling

As mentioned in Chapters 3 and 4, the degree of dispersal of parr from their natal site is critical when using juveniles to infer adult breeding structure. Structuring would only be detected therefore if a) adults had mated non-randomly and b) the juveniles had not become randomly dispersed through the river by the sample time. If genetic differentiation between sites were detected, this would be an indication that adults spawned at specific sites within the river. Spatial genetic differentiation was detected

at each sample time, therefore the parr had not become randomly distributed across the whole river. However, at the other extreme, there was a possibility that the samples taken per site were the offspring off just one adult pair and thus the juveniles would all be from one family.

Sampling one family per site would contravene the assumptions of the application of F statistics to estimate population structure, i.e. that the samples in each site should be a true representation of the total group at that site. In species with non-random distribution of related individuals, sampling a few families may lead to biased estimates of population allele frequencies (Hansen *et al.* 1997). Sampling small numbers of families per site will decrease the effective sample size and will artificially inflate the statistical significance of differentiation between sites. Even in sites where a reasonable number, e.g. 30- 60 samples, were obtained, it is possible that these could all be the offspring of just one adult pair.

For example, in the case shown in Figure 5.1, sampling the n = 18 juveniles at site a and n = 18 juveniles at site b, would lead to estimation of genetic differentiation between sites. This estimation of genetic differentiation would be true reflection of the differentiation between the two adult pairs but it is the significance of the differentiation (due to the sample size) which is erroneous.

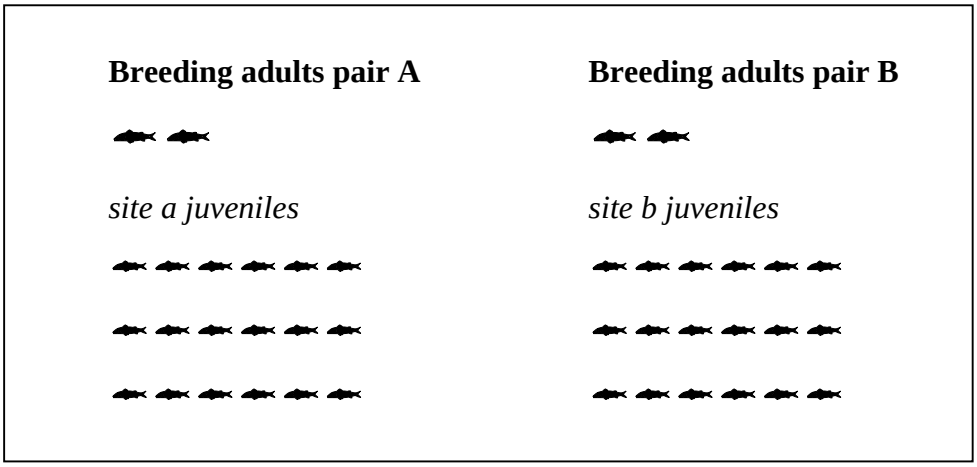


Figure 5.1 Non-random sampling due to sampling families.

The above example is an extreme case and it was not expected to literally find one family per site. The aim was to estimate the degree of relatedness in the samples per

site to determine if sampling families per site caused erroneous statistical significance of estimates of differentiation.

5.1.4 Estimation of relatedness

If a small number of families were sampled at each site, then there would be limited confidence in the inferences of spatial population structure of adults using these samples. Non-random sampling can be investigated by estimation of relatedness between juveniles within sites. Individuals in a population will have a pedigree relatedness of either full sibs, half-sibs or unrelated. Statistics to estimate pairwise relatedness use data from co-dominant genetic markers such as microsatellites and assume that the loci are not under selection. Researchers have investigated different methods of combining data from multiple loci and multiple alleles (Pamilo 1984, Pamilo 1989 and Queller and Goodnight 1989) and the difference between regression and identity by descent to estimate relatedness (Lynch and Ritland 1999).

When estimating relatedness between individuals it is important to define the group which will be used as the background level of allele sharing (Lynch and Lande 1998, Lynch and Ritland 1999). Given a situation of estimating relatedness between parents and offspring, if the potential parents do not share any alleles, then it is clear which parent is the mother and which is the father. However, if parents do share alleles then it is important to estimate the allele frequency in the entire population. Estimator bias is related to the number of loci and allele frequency.

The Queller and Goodnight (1989) method is generally applied to population data due to the way that data from multiple loci and multiple alleles are used because bias due to sampling small numbers of groups can be corrected and because between-individuals relatedness can be estimated. Relatedness is related to F statistics in the following way: $r = 2F_{ST}/ 1+F_{IT}$ (see Chapter 4 for explanation of F statistics). The program Fstat uses the F statistics to estimate relatedness. Queller and Goodnight (1989) stated that the approach is broadly similar, however, the disadvantage of the F statistics approach is that it does *not* permit within-sample (sample = site) estimation (relatedness between individuals).

5.1.5 Temporal stability of population structure in the River Frome

No previous estimate of spatio-temporal population structure had been carried out for the River Frome. Atlantic salmon juveniles were sampled at 15 sites on the River Frome over three years but at some sites there were not enough fish thus these sites were not used in the analysis of temporal variation. Altogether, nine sites were suitable to estimate spatio-temporal population structure, these were sites 1-WH, 2-ESMS, 3-ES, 5-BM, 6-WS, 7-EB, 10-NF, 11-LM and 13-GB. Sampling a large number of sites, over a small spatial scale and over three years was intended to allow high power to detect stability of spatio-temporal genetic variation, if it were present. Juveniles sampled in the autumn of each sample year were not used in the analysis of spatio-temporal genetic variation because it is possible that by the autumn these fish may have migrated between sites.

5.1.6 Estimation of temporal genetic variation

It is important to use appropriate statistical tests for temporal changes in allele frequency to differentiate stochastic processes from deterministic changes. Standard chi-squared tests could be applied to temporal data but there are problems associated with the standard tests due to drift and limited sample size but, with historic samples, this problem cannot be over come by going back to take more samples. Waples (1989) developed models to take genetic drift into account. Using data from *Oncorhynchus* sp., Waples and Teel (1990) found that annual fluctuation in population allele frequency due to drift was expected to be several percent. The magnitude is determined by the number of breeders each year rather than the age structure. The effect of breeding structure and effective population size was further investigated by Waples (1990) who suggested that effective population size (Ne) is equivalent to the number of breeders (Nb) multiplied by age at spawning. The equation can be used in tests of variance of allele frequency, loss of heterozygosity and loss of alleles to predict the rate of genetic change of the population as whole.

5.1.7 Estimation of spatio-temporal genetic variation: analysis of variance approach

Analysis of variance is used to determine the contribution of various factors to total variance. Analysis of Molecular Variance (AMOVA) is used to partition molecular variance: the total variance is partitioned in a hierarchy between spatial and temporal factors. Previous studies of Atlantic salmon have employed analysis of variance to investigate the amount of differentiation over time compared with the amount of differentiation between sites (Beacham and Dempson 1998, Garant *et al.* 2000 and Jordan *et al.* 1992).

5.1.8 Estimation of spatio-temporal genetic variation: a test of the temporal stability of population structuring

The AMOVA approach does not explicitly test if particular sites are stable over time. A key assumption of AMOVA is that temporal replication of samples from the same site provides an estimate of the sampling error between sites at one time, however, due to the mating system of the species it is possible that biologically meaningful differentiation occurs between years and thus differentiation over time should not be ignored.

The *Q* test approach is as follows. If there is a tendency for spatial structure to occur and to be stable over time, then individuals sampled at the same site are likely to be more similar to each other than individuals sampled at different sites at different times. The differentiation between the same sites sampled at two time points is compared with differentiation between different sites across time. Using different sites at different times as a control takes into account allele frequency change over time and is valid even if non-random sampling of individuals per site was inadvertently carried out. In Atlantic salmon, detection of stable spatio-temporal differentiation would indicate that adult fish returned to the natal sites to spawn.

To explicitly test the stability of differentiation within sites over time, a statistic was devised by Ralph Clarke at CEH. The program to carry out the test is available at www.ceh.ac.uk/products_services/software/mantime. The test is valid even if non-

random sampling of individuals per site was inadvertently carried out. The test requires the genetic differentiation between all sites and within sites over time to be estimated. The test can be used with any estimator of genetic difference such as genetic distance, F_{ST} and R_{ST}.

The test is as follows:

Let n denote the number of populations sampled in both years 1 and 2. Let D_{jk} denote the estimate of genetic distance between site j based on the individuals sampled in year 1 and site k based on the individuals sampled in year 2 (j , k = 1,...,n)

Rank all of the n^2 values of D_{jk} (rank 1 = smallest D_{jk} value)

Calculate:

Q_W = mean rank of the values D_{jj} (j = 1,...,n) obtained for the genetic distances between years at same site

Q_B = mean rank of the values D_{jk} ($j \neq k$, j,k = 1,...,n) obtained for the genetic distances between years at different sites

The test statistic, Q, is then given by

$$Q = \frac{Q_W - Q_B}{(n \times n/2)}$$

Q has been scaled to vary between –1 and +1, with negative values indicating that genetic distances within sites between years are on average less than genetic distances between sites between years. In the most extreme case, if all genetic distances between years at the same site are less than all genetic distances between years and different sites, then Q = -1. Under the null hypothesis of no real difference between sites, genetic distances between the same site will, on average, be the same as genetic differences between sites, and hence Q = 0.

The statistical significance of Q is obtained by a Mantel-type randomisation test. Under the null hypothesis of no difference between sites, the data for sites in year 2 will not be correlated with the data for the same sites in year 1. Therefore the null distribution of Q is obtained by repeatedly randomly re-ordering the sites in year 2 (say m = 10000 times) and re-calculating Q. The one-sided test probability is the proportion of randomisation distribution values of Q which are less than or equal to the observed data value of Q.

Estimation of temporal stability of genetic variation would indicate the stability of spatial population structure and can also be used to investigate aspects of adult Atlantic salmon life history and behaviour, such as homing to sites within the river. If temporal stability of spatial population structure was detected this would indicate adults homed to natal sites to spawn. A new test of the temporal stability of spatial population structure was proposed, which can be used even if there is suspicion that non-random sampling or allele non-amplification affected samples. If temporal stability of spatial population structure is detected then this indicates that the adults returned non-randomly to sites within the river to spawn.

5.1.9 Hypothesis and aims

Hypothesis:

Temporal stability of spatial population structure of Atlantic salmon occurs within a river.

The **main aim** was to estimate temporal genetic differentiation and to determine if temporal stability of spatial population structure occurred.

The **second aim** was to estimate relatedness between individuals within a site. It was not expected that a high incidence of relatedness would be detected, however, it was important to test for this as relatedness would affect the conclusions made about spatial population structuring.

5.2 Results

5.2.1 Analysis of molecular variance

To compare the amount of genetic differentiation between sites to genetic variation over time, genetic variance was partitioned using the AMOVA test. This was carried out for data from Atlantic salmon in the River Frome for the 11 sites common to all three summer sample times (JY98, JY99 and JY00) (Table 5.1). Summer samples only were used.

Variance due to differences between years (among groups) was non-significant (F_{CT} -0.0117^{NS}). Among populations (sites) within years, variance was low, 0.67%, but significant (F_{SC} 0.0092, *P* 0.001). Among individuals within populations, variance was low, 1.92% and non-significant (F_{IS} 0.0265^{NS}). Most of the variance was held within-individuals 98.7% but this was non-significant (F_{IT} 1.3614^{NS}).

Table 5.1 Analysis of variance for Atlantic salmon at 11 sites over three consecutive years in the River Frome.

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*% variation*
Among groups	2	-15.19	F_{CT} -0.0177	-1.29
Among populations within groups	30	55.67	F_{SC} 0.0092	0.67
Among individuals within populations	786	1111.65	F_{IS} 0.0265	1.92
Within individuals	819	1115.00	F_{IT} 1.3614	98.70
Total	1637	2267.13	1.3793	

5.2.2 Pairwise F_{ST} between sites over time

For Atlantic salmon in the River Frome, pairwise F_{ST} between sites over time was estimated for the 9 sites common to all sample times. For July 1998-July 1999, 5/9 same site over time comparisons were significant (Table 5.2) but for July 1999-July 2000, only one same site over time comparison was significant (Table 5.3).

Table 5.2 Pairwise F_{ST} between July 1998 and July 1999 for Atlantic salmon juveniles at sites on the River Frome.

Site	*July98 1-WH*	*July98 2-ESMS*	*July98 3-ES*	*July98 5-BM*	*July98 6-WS*	*July98 7-EB*	*July98 10-NF*	*July98 11-LM*	*July98 13-GB*
July99 1-WH	0.0655	-0.0199	-0.0289	0.0385	-0.0093	-0.0133	0.0198	-0.0216	0.0539 *
July99 2-ESMS	0.0338	0.0056	0.0181	0.0467 *	0.0125 *	0.0179 *	0.0424 *	0.0364	0.0411 *
July99 3-ES	0.0173	0.0041	0.0024	0.0296 *	-0.0131	0.0009	0.0377	0.0184	0.0138
July99 5-BM	0.0046	0.024 *	0.0234	0.0344 *	0.0118 *	0.019 *	0.0352	0.0217	0.0068 *
July99 6-WS	0.0075	0.0036 *	0.0034	0.0307 *	0.0093 *	0.0003 *	0.0099	0.047 *	0.0278 *
July99 7-EB	0.0111	-0.0031	0.018	0.0295	0.0253 *	0.0143 *	0.0332	0.0568 *	0.0459 *
July99 10-NF	0.0419	0.0227 *	0.016	0.0356 *	0.0153 *	0.0177 *	0.0372	0.0244	0.017 *
July99 11-LM	0.0913	0.0291 *	0.0348	0.0379 *	0.0379 *	0.0304 *	0.056 *	0.0409 *	0.0989 *
July99 13-GB	-0.0193	0.0164	0.0132	0.04 *	0.0083	0.0157 *	0.006 *	0.0205	0.0027 *

P values obtained after 23100 permutations, adjusted 5% level for multiple comparisons was 0.000216, significant comparisons marked *

Table 5.3 Pairwise F_{ST} between July 1999 and July 2000 for Atlantic salmon juveniles at 9 sites on the River Frome.

Site	*July99 1-WH*	*July99 2-ESMS*	*July99 3-ES*	*July99 5-BM*	*July99 6-WS*	*July99 7-EB*	*July99 10-NF*	*July99 11-LM*	*July99 13-GB*
July00 1-WH	-0.0189	0.0159	-0.0055	-0.0055	-0.0055	-0.0047	0.0021	0.0078	0.0081
July00 2-ESMS	-0.0207	0.0324	0.0259	0.0146	0.011	0.0205	0.0181 *	0.0398 *	0.0284 *
July00 3-ES	0.074	0.0759	0.0722	0.0585	0.0456	0.0684	0.0745 *	0.1063 *	0.0147
July00 5-BM	-0.0037	0.0076	-0.0073	-0.0013	0.0161	0.0235	0.0164	0.0516	0.0291
July00 6-WS	0.0536	0.0256	0.0034	0.0129	0.0228	0.0462	0.0399	0.082	0.0287
July00 7-EB	0.0061	0.0136	0.0007	0.0072	0.0048	0.0082	0.0112	0.0279	0.0107
July00 10-NF	-0.0127	0.056	0.0216	0.0198	0.0328	0.0416	0.0153	0.0511 *	0.0346
July00 11-LM	0.0109	0.0411	0.0163	0.0234	0.0076	0.0044	0.029 *	0.0197	0.0354
July00 13-GB	0.0046	0.0799	0.0559	0.0637	0.0646	0.0438	0.0346	0.0452 *	0.0786 *

P values obtained after 23100 permutations, adjusted 5% level for multiple comparisons was 0.000216, significant comparisons marked *

Table 5.4 Pairwise F_{ST} between Atlantic salmon at 7 sites over two consecutive years, River Sainte-Marguerite, Canada. Data from *Table 3* (Garant *et al.* 2000). All F_{ST} values were significant after Bonferroni correction at 0.0006.

Site code and year	*PR27 1997*	*PR81 1997*	*PR58 1997*	*NO05 1997*	*NE06 1997*	*NE28 1997*	*XA01 1997*
PR27 1996	0.0129	0.0175	0.0305	0.0432	0.0192	0.0201	0.0305
PR81 1996	0.0194	0.026	0.0369	0.0483	0.0255	0.0323	0.0222
PR58 1996	0.0232	0.0184	0.038	0.0411	0.0257	0.038	0.0296
NO05 1996	0.0363	0.0477	0.0837	0.0396	0.0582	0.0464	0.0728
NE06 1996	0.0187	0.0174	0.0392	0.0423	0.0169	0.0296	0.0270
NE28 1996	0.0077	0.0128	0.0417	0.0269	0.014	0.0192	0.0193
XA01 1996	0.0296	0.0409	0.0445	0.0549	0.029	0.0291	0.0352

5.2.3 Spatio-temporal genetic variation in Atlantic salmon in the River Frome

For Atlantic salmon in the River Frome, Q was not significant for either time comparison. For the comparison 1998-1999, $Q = -0.022^{NS}$ and for the comparison 1999-2000, $Q = -0.047^{NS}$. This indicated that there was no temporal stability of spatial population structure for these samples.

5.2.4 Spatio-temporal genetic variation in Atlantic salmon in River Sainte-Marguerite

The Q test was applied to published data from Atlantic salmon at seven sites within the Sainte-Marguerite river in Canada (Table 5.4), sampled in consecutive years (Garant *et al.* 2000). Genetic distances $\{D_{jk}\}$ were based on similar size (46-50) samples of fry from each site and calculated using Weir and Cockerham's (1984) estimator of pairwise F_{ST}. For these data the observed value of Q was –0.259, P = 0.021. Thus, significant temporal stability of spatial population structure was detected.

5.2.5 Relatedness between juvenile Atlantic salmon

Atlantic salmon full sibs are related at r = 0.5 and half sibs at r = 0.25. Low mean relatedness per site was detected. Over all loci relatedness values per site were all lower than r = 0.016 (data for each site, Appendix Table 22 and 23). There was no trend of similar relatedness values at particular sites over time or of higher relatedness at specific sites (Figure 5.2).

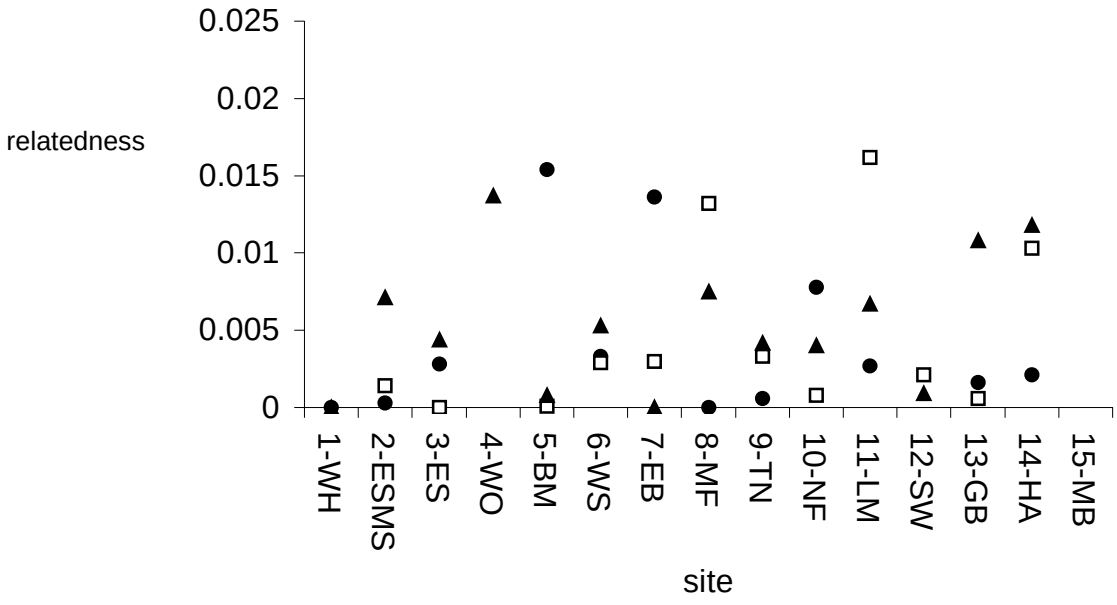


Figure 5.2 Mean relatedness of Atlantic salmon juveniles per site for summer 1998 (circles), 1999 (squares) and 2000 (triangles).

Although mean relatedness per site was low in each case, it is possible that some between-individual pairwise comparisons were higher than 0.5, indicating full sibs. The between-individual relatedness was estimated for each site for each sample time. Although there were some sites that appeared to have a high number of full-sib comparisons, the percentage of full-sib comparisons compared with all comparisons was low (from a maximum of 5.6% in July 1999 to a maximum of 8.4% in July 2000) (Figure 5.3). There was no trend of greater percentage of highly related individuals at particular sites.

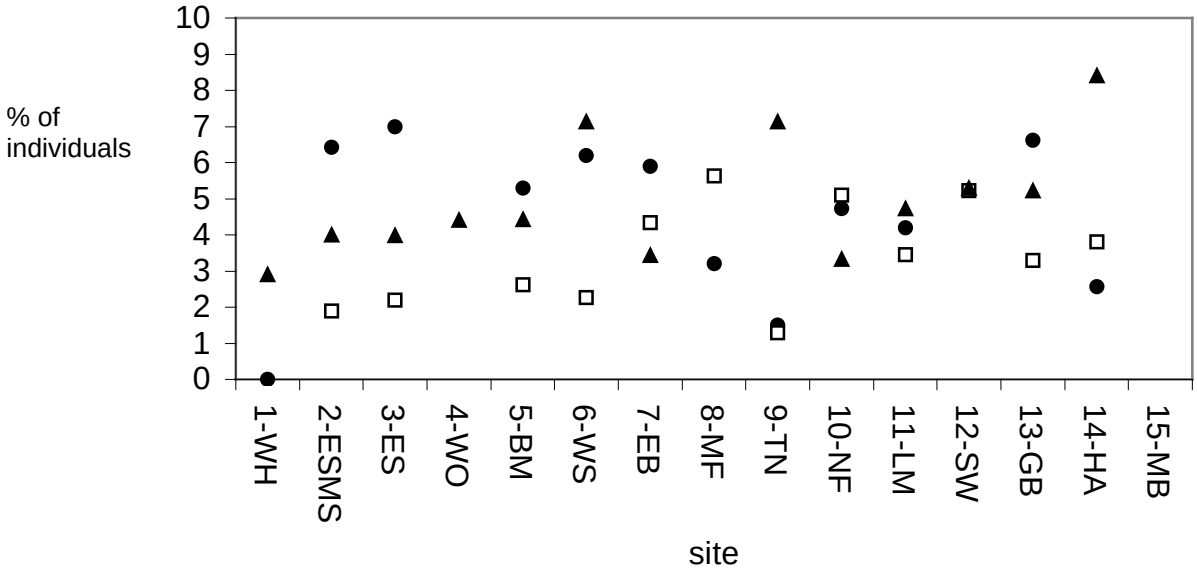


Figure 5.3 Percentage of individuals related at r = 0.5 or over per site, for summer 1998 (diamonds), 1999 (squares) and 2000 (triangles).

5.3 Discussion

5.3.1 Spatio-temporal variation in Atlantic salmon

Analysis of variance indicated that there was low, non-significant genetic variation over three consecutive years for Atlantic salmon in the River Frome. Low genetic variation between years would be expected in populations which are relatively large and that have overlapping generations, because this would reduce drift. The adult population size in the River Frome was around 1000 (Welton *et.al.* 1999) and, based on the assumptions of the extinction vortex (Frankham *et al.* 2002), it can be assumed that the River Frome population is sufficiently large for genetic variation to be stable over time. In addition, the breeding population will be composed of overlapping generations due to differences in age at spawning, precocious parr and repeat adult spawners. In the River Frome, although repeat spawning does occur, the total amount of repeat spawning is less than 3%. In the River Frome, 6.9% of 2 sea winter fish and 28.4% of 3 sea winter fish were repeat spawners, but the proportion of multi sea winter fish was less than 10% (data for 1991-1995) (Welton *et. al.* 1999).

Temporal stability of the spatial pattern of genetic variation in Atlantic salmon was estimated with the assumption that detection of spatio-temporal stability could have real biological meaning. This is in contrast to the assumptions of Garant *et al.* (2000), which, although similar in sampling design (7 sites, over two consecutive years), focussed on between-year variance purely as a test of the accuracy of the spatial structuring estimates. Garant stated that variance between years was ‘noise’, which may have led to possible over-estimation of spatial genetic differentiation. Garant found low but significant spatial genetic structure was still apparent after accounting for variance among temporal replicates and the amount of variance due to what was termed ‘temporal instability or random sampling error’ was three times the spatial component. In contrast, the approach of this study was not to discount temporal changes as ‘noise’ but to look specifically if there was a tendency for the same sites to be more similar over time. The *Q*-test was used to do this (this cannot be done using AMOVA). No temporal stability of spatial genetic structure was found in either year-to-year comparison, using 11 sites on the River Frome but temporal

stability of spatial population structure was found for Atlantic salmon using the published data from Garant. Detection of stability of the pattern of genetic differentiation over time indicates that the adults spawned at specific sites and that this was stable over time.

5.3.2 Relatedness

Relatedness between individuals was detected and a proportion of the samples at each site were related 0.5 or above, but relatedness was low (the highest detected was r = 0.016). To my knowledge, no other studies have investigated relatedness in Atlantic salmon natural (unstocked) populations. The relatedness detected in this study was similar to that found for 1+ brown trout parr in a natural river (1+ parr were related at r = 0.009 and 2+ parr were related at r = 0.223, Carlsson and Carlsson 2002). Hansen et al. (1997) found that average relatedness among individuals within groups was r = 0.17. (Higher relatedness in 2+ trout may be accounted for by mature individuals selecting specific sites to spawn).

Relatedness may have been overestimated in this study, due to contravention of certain assumptions, such as that the *adults* were from a large, randomly mating population and were unrelated. In small populations inbreeding may have occurred in the past therefore breeding adults have a higher chance of containing alleles that are identical by descent (and thus the juveniles will too). In small populations, the number of adults mating at each site is likely to be low and, if adults do return with high fidelity to specific sites within the river to spawn, it is possible that the adults will breed with relatives. In this situation, higher relatedness would be expected in the offspring and this would be a true reflection of the nature of the system.

The relatedness program did not include a Bonferroni correction for a large number of comparisons, but if this could have been performed it is possible that the incidence of statistically significant pairwise relatedness would be lower. It was not possible to use simulations to establish the standard error of relatedness estimates in this study because it was not possible to type true full sibs. Simulations to estimate the standard error of full and half sibs (full sib estimated values may be between, e.g. 0.47 and 0.55) (Blouin *et al.* 1996). If the confidence limits between full and half sib overlap,

then there is a danger of type I (e.g. half sib classified as full sib) or type II (e.g. full sib classified as half sib) statistical errors. To run the simulation, data for known true full and half sibs would be required. If it *were* possible to correctly identify sibs and half-sibs the maternal genotype could be inferred and then the population structure could be investigated based on the inferred genotypes.

5.3.3 Non-random sampling

As already mentioned in Chapter 4, population structure would be detected using juvenile samples, if a) the adults were structured and the b) juveniles were not randomly dispersed between sites. If the juveniles *had* dispersed randomly between sites then no structuring would have been detected at all even if the adults had been structured. It may of course be possible that there was no structuring in the adult generation and any detection of structure was spurious: if juveniles were in family groups then estimation of population structuring due to family sampling cannot be distinguished from population structuring in the adult breeding stage. Previous studies of salmonid population structure have recognised the potential problem of sampling families and have taken various steps to address it. For example, Jordan *et al.* (1992) sampled over a large area (2 km) to ensure that samples were not from one family. Sampling over large areas defeats the object of detecting spatial population structure on a small scale so this was not useful for this study. Studies have included juveniles from different cohort years per site (Beacham and Dempson 1998 and Spidle 2001), however different cohorts may not always be present. The most appropriate ways of sampling juveniles to infer population structure is discussed further in Chapter 6.

5.4 Conclusions

The main findings were:

- No significant genetic variation was detected between years.
- No temporal stability of spatial population structure was detected for Atlantic salmon in the River Frome, however, significant temporal stability was detected for fish in a Canadian river. This is evidence that Atlantic salmon home to specific sites within the river.
- Some Atlantic salmon parr (between six and nine months after hatching) in the River Frome within a site were found to be closely related. This was likely to be due to limited dispersal and may have affected estimation of spatial genetic differentiation, due to non-random sampling.

This is the first time that temporal stability of spatial population structure within one river has been demonstrated for Atlantic salmon, using data from a published Canadian study and this would strongly suggest that Atlantic salmon in this river returned to specific sites to spawn. Temporal stability of spatial population structure was not detected in the chalk stream, the River Frome, however.

The hypothesis that 'temporal stability of population structure occurred, within one river' was rejected for Atlantic salmon over three consecutive years, in the River Frome.

Chapter 6

Main conclusions

6.1 Introduction

The research investigated Atlantic salmon biology and population structure within a chalk stream, the River Frome in Dorset, UK. The three hypotheses tested were:

1. Abundance of juvenile Atlantic salmon in a chalk stream is related to habitat quality.
2. There is significant genetic differentiation between Atlantic salmon within one river, despite small geographic distance between sites and no barriers to migration.
3. Temporal stability of spatial population structure of Atlantic salmon occurs within a river.

6.2 Conclusions on estimation of population size and population structure

Throughout the research the methods were assessed for suitability and this was discussed in the preceding chapters. Issues that are potentially important for future studies are discussed in this chapter.

6.2.1 Prediction of adult population size from juvenile abundance

It is important to know that any management actions to increase the number of juveniles will actually result in an increased number of adults returning to the river to breed, thus an accurate stock-recruitment model would be a useful management tool. The current models devised by Hilton *et al.* (2001) using data from the River Frome were not found to be predictive and therefore it could be suggested that a way to

improve accuracy of the models would be to incorporate data from Atlantic salmon in other chalk streams.

6.2.2 Use of juveniles to infer adult population structure

Juveniles are used to infer adult population structure, especially for populations of conservation concern where disrupting adults is not permitted. There main limitations to sampling juveniles are;

a) samples may include families and thereby contravene the assumptions of random mating within a site and causing potential overestimation of differentiation between sites
b) samples including older parr could led to underestimation of population structure if older parr had moved from the natal site.

Sampling on a small spatial scale can mean that samples contain sib and half sib-related individuals. It could be argued that sibling pairs should be removed from the estimates of F statistics, because including siblings may bias the data and lead to erroneous conclusions about the degree and significance of differentiation between sites. In a large, randomly-mating population where the incidence of full-sibs in the sample is expected to be low, full-sibs could be removed without prejudice to the sample size. In small and declining populations, higher relatedness may be expected in the juveniles if there are a small number of adults. In the River Frome, no site contained literally just one family and percentage of full-sibs was moderate (between 5.6% and 8.4%). The full-sib pairs were retained within the samples, because it can be argued that these full-sibs were a true representation of the adult mating system. In addition, samples were retained because the *Q*-test of temporal stability of spatial population structure is valid even if there is a possibility that some families were contained in the samples.

Sampling parr of different year classes has been used to attempt to overcome potential sampling of families. This has drawbacks, however, because significant genetic differentiation between Atlantic salmon juvenile year classes within one river has been detected (Galvin et al. 1994, Galvin et al. 1996). If parr had dispersed from their natal site this would tend to reduce the estimate of differentiation. On a practical

level, whilst most rivers are expected to have juveniles of different age classes, in chalk streams and southerly rivers where parr can grow fast enough to smolt after one year, there may only be 0+ parr present. In this study, 0+ parr only were used to infer population structure because there were very few 1+ captured and the 1+ parr could not be assigned to a site of origin, thus were assumed to have moved from their natal site. In addition, if the older parr were precocious, this would tend to reduce the actual level of differentiation between sites (if the precocious parr bred at a different site to the natal site). It was not possible to determine if 1+ parr captured in the Frome were precociously mature, because this can only be determined by dissection.

Current work suggests that avoidance of sampling family groups is the most important factor when using juveniles and this can be overcome by sampling older juveniles (J. Stevens, pers.comm.).

6.2.3 Habitat structure and population structure

The thesis investigated population structure of Atlantic salmon in one river with small distances between sites and no barriers to migration. In addition, the river was a chalk stream which is relatively homogenous compared to classic dendritic rivers. Although chalk streams generally provide good habitat for salmon and a stable environment in terms of water temperature and flow rate through the year, habitat quality in the River Frome (as estimated by HABSCORE which is not appropriate for chalk streams) varied between sites and also varied over time. Detection of stable spatio-temporal genetic variation in the Canadian river but not in the River Frome could be due to differences in habitat structure between the two rivers. It is possible that the Canadian river (being a ‘classic’ salmon river) could have a dendritic structure, with stable habitat at each site and differences in habitat between sites. Thus, in this river, adults could potentially return to sites with specific environmental conditions or to the natal site. Castric *et al.* (2001) suggested that tributary structure could be important in controlling spawning site selection. Over time, homing to specific sites could lead to increasing differentiation between sites and in the extreme case, to speciation.

Population structuring could also be related to behavioural differences between individuals, which in turn may be related to the physical characteristics of the river. For example, in many rivers, there are differences in the predominant time of upriver migration between grilse and multi-sea winter fish (Rogan *et al.* 1993). In classic rivers, larger fish may ascend the river earlier in the year and migrate furthest upstream. In granite rivers summer flow rates are likely to be low, which can prevent fish from ascending thus a bimodal return pattern is observed. This is not likely to occur in the Frome however, because chalk streams are fed from groundwater and thus tend to have a stable flow rate throughout the year (Berry 1992), so adult Atlantic salmon can ascend the river throughout the year. Other behavioural factors which serve to reduce the extent of differentiation include multiple mating and sneak mating by precociously mature parr. Multiple spawning is common in Atlantic salmon because females may have more than one redd and adult males may fertilise eggs over a large distance of river. In a natural stream, over 50% of adults contributed to more than one redd, up to 6 redds per female were observed and males were observed to contribute at up to 7 redds, up to 5 km apart (Taggart *et al.* 2001). In addition to multiple mating between adults, precocious male parr would generate half sibs (Fleming 1998).

6.3 Conclusions on salmon conservation

6.3.1 Protection and maintenance of genetic integrity

Genetic variation in Atlantic salmon in the River Frome was moderate and consistent across all sites at each sample time and there was no evidence for a genetic bottleneck. Thus, it could be concluded that low genetic diversity was not the cause of population decline in this system, although the relationship between past population size and genetic diversity would of course be better investigated using longer-term historic samples (see Section 6.5).

One of the motivating factors in conservation of genetic diversity is the ‘extinction vortex’ (Frankham *et al.* 2002). The vortex suggests that inbreeding in small populations leads to reduction of genetic variation, which leads to reduced ability of the population to adapt to evolutionary change, which leads to further decline and

eventually extinction. Maintenance of genetic diversity is a target for many species conservation plans, but it is not proven that low diversity necessarily leads to reduced fitness and there is no current cut-off point for the level of diversity which is deemed to be acceptable. In some populations it might not be possible to recover the maximum genetic diversity because local population sizes are too low or because the genetic diversity has been irretrievably lost from the wider population. What is perhaps more useful to know is the level of genetic variability compared to population size for the species. Frankham *et al.* (2002) suggested a population size of 1000 individuals is sufficient for a sustainable population - this is approximately the current size of the Atlantic salmon population of the River Frome (Welton *et. al.* 1999). Thus, rather than suggest targets for percentage heterozgosity for Atlantic salmon within the River Frome, a more tangible and easy to measure target could be maintenance of the population at, at least, the current population size (see section 6.3.2).

It is not just the level of genetic variation that is important but also the distribution of genetic variation. The degree of population structure in Atlantic salmon between sites in the River Frome was equivalent to other rivers and although the amount of differentiation detected within rivers is not generally very high (less than 5 %), even this small amount of structure is important because it reflects the mating structure of the adults. No temporal stability of spatial population structure was detected for Atlantic salmon in the River Frome, however, significant temporal stability was detected for fish in a Canadian river. This shows that genetic differences between years should not be discounted purely as ‘noise’ and may indicate real biological differences.

Populations can become locally-adapted to site through natural selection acting on genetic variation and increasing the fitness of individuals in a particular environment (Carvalho 1993). Loss of sites or loss of the population at that site could mean loss of unique genetic diversity and loss of adapted populations therefore legislation to protect distinct genetic units of a species has been suggested. Protection for genetically distinct units could be given official status via Evolutionarily Significant Units (ESUs) (Fraser and Bernatchez 2001). However, there is no set amount of differentiation which defines an ESU for legislative or management purposes. The

low level of genetic differentiation between sites within the Frome would mean that it is unlikely that this would meet targets for differentiation, even if this legislation were brought into practice in the UK.

In addition to protecting genetic units, a management option is to enhance the population using selected stocking. This has been used in the past in rivers where salmon have been completely lost or were very low. This was not appropriate for the Frome as no sites had a particularly low juvenile stock and more importantly, significant genetic differentiation was detected between sites which could potentially be disrupted. Thus, although maintenance of genetic integrity is important it is only a part of the conservation picture and maintenance of the habitat and population size are just as important.

6.3.2 Conservation of population size

One of the highest-impact conservation efforts has been the persuasion of governments to set strict quotas on removal of adults at sea (enforced by NASCO), backed by the voluntary buy-out of some commercial sea fisheries in a campaign led by the North Atlantic Salmon Fund. In 1990s the Faroe Islands and Greenland Fisheries were co-operatively bought out. This not only protected the salmon spawning in the rivers of these countries but potentially benefits Atlantic salmon from all Atlantic rivers. In N.E. England, 78% of nets were bought out at a cost of £3.34 million, saving 50,000 salmon. In Northern Ireland, 70% of nets were bought out at a cost of £2 million, reducing the commercial catch from 12,000 to 3,500 salmon. The current focus in this area is on the commercial fisheries off the South Irish Coast, where it is thought that many fish destined for rivers on the south coast of England are captured. Currently, the North Atlantic Salmon Fund aim is to buy out these fisheries, which may have a direct impact to increase the population size of Atlantic salmon in the Frome.

Any action to increase Atlantic salmon in river must be weighed against possible damage to other species. For example, there may be pressure to reduce brown trout because juveniles can compete for resources and adults are a predator of salmon parr. Some brown trout populations have anadromous as well as resident forms originating

from the same river and sea trout are afforded conservation status and thus conservation plans must take into account the need to protect both species.

6.3.3 Protection and maintenance of habitat quality

High habitat quality was found at some sites on the River Frome but it was not possible to identify specific sites or regions of the river that required improvement as the habitat quality scores varied between sites and also over time. Thus it might be suggested that small-scale management of specific sites would be a waste of resources and it would be better to concentrate on water quality as this will benefit all sites in the river.

Atlantic salmon need clean water, with good oxygen content to survive and the European Water Framework Directive, implemented in 2000, is one of the most important pieces of legislation that will help to deliver this in European rivers (www.defra.gov.uk/environment/water/wfd/index.htm). The Directive identifies pollution potential from particulates, pesticides, solids, oxygen depleting, heavy metals, hydrocarbons and temperature (www.euwfd.com/html/source_of_pollution_-_overview.html). The target of 2015 for all rivers to have achieved 'good' status will benefit Atlantic salmon and may lead to further recovery of some populations.

In addition to the potential for improved water quality, Atlantic salmon should also benefit from the EU Habitats Directive which aims to 'promote the maintenance of biodiversity by requiring Member States to take measures to maintain or restore natural habitats and wild species at a favourable conservation status, introducing robust protection for those habitats and species of European importance' (www.jncc.gov.uk/page-1374). Full implementation of the Directive should ensure habitats which are important for Atlantic salmon, such as chalk streams, receive protection and this should also indirectly conserve genetic variation.

At a more local scale, Atlantic salmon are a Priority species under the 2007 Biodiversity Action Plan (www.ukbap.org.uk), and as the criteria and targets are developed and made available this will provide the incentive for Atlantic salmon to

be given priority in Local Action Plans. Currently, the Hampshire Avon is the only chalk stream to be designated as a Special Area of Conservation for Atlantic salmon. Chalk rivers are themselves recognised as a Priority Habitat (www.ukbap.org.uk) and many the actions to restore chalk rivers (maintain the characteristic plants and animals of chalk rivers, including their winterbourne stretches, restore all rivers notified as SSSI to favourable condition and restore important non-SSSI rivers to favourable condition) will directly benefit salmon. Although differentiation between rivers was not tested as part of this current research, it is highly likely that Atlantic salmon in the River Frome and other chalk streams are significantly differentiated from other rivers. If this were found to be the case, this might warrant increased effort in protection of chalk streams and the salmon populations in them.

6.4 Outputs from the research

The main data outputs were detailed habitat data and a comprehensive set of allele frequency data for Atlantic salmon juveniles at a large number of sites over three consecutive years. These data could be used as a baseline to determine changes over time, for example if the population size were to increase or decrease significantly it would be interesting to look at allele frequency change to see how this related to population size. Data for genetic variability and genetic differentiation between sites could also be used within a meta-population study of Atlantic salmon in the UK to investigate potential founder effects and migration between rivers (although of course, studies would have to use the same microsatellite markers to compare across studies). The juvenile density survey will provide a useful baseline which can be used to assess the impact of any future management action to enhance juvenile numbers.

The main statistics output from this research was the test of temporal stability of spatial population structure- this could potentially be applied to other species of conservation interest with migratory populations, such as birds and butterflies, and can be used to infer fidelity of individuals to specific sites, whether in a structured or apparently homogenous landscape. This may strengthen the case for conservation of units of these species and/or the habitat that they require, to maintain the genetic integrity of the population as a whole.

6.5 Further work

There are many questions remaining about both adult and juvenile Atlantic salmon ecology and behaviour and the influence of this on population structure that could be investigated. For example, movement of Atlantic salmon parr from natal sites occurred later in the year (after October) in the chalk stream than was expected. To determine exactly when dispersal occurs, it would be necessary to electric fish later in the year, perhaps at two-monthly intervals, or to pit-tag and radio-track individuals. Whilst it would be possible to do either of these, it would be labour intensive and the Environment Agency would be unlikely to permit this in the Frome, to reduce any potential risk to the juveniles. Whilst it was outside of the remit of this research to directly investigate adult salmon, the behaviour of the adults when they return to spawn will influence population structure. Thus it would be interesting to investigate the:

- influence of adult age at return on population structure i.e. do grilse and multi-sea winter fish select different sites to spawn?
- influence of adult age at return on fitness- in some rivers there appears to be a shift in life history type from mostly multi-sea winter fish to predominantly grilse. Whilst external factors, such as fishing pressure or availability of food at sea could influence this, it could also potentially be a selected response.

Long term genetic variation could be assessed using archive scale samples. Scale samples have been collected from fish caught by sport anglers in the River Frome, from 1963 to current. This is a comprehensive data set and, together with the long-term data on census size, offers a unique opportunity to investigate allele frequency changes over time and the relationship between genetic variability and population size.

The motivation behind continued research on Atlantic salmon behaviour and population genetics is that populations are declining and therefore work is needed to find out more about the populations to protect them effectively. However, the fact that salmon are a protected species means it is often difficult to obtain permission to carry out the sorts of experiments that would be most useful to understand aspects of

behaviour and populations may be too small to provide reliable data. For Atlantic salmon, the Environment Agency is understandably cautious about repeatedly electric fishing the same fish (as this could cause stress to the fish) and impeding adults to tag (this is not permitted in some rivers where there are few adults).

To minimise disruption to declining Atlantic salmon populations, brown trout (which generally have more stable populations) could be used as a model. Brown trout have a life history where some individuals are resident (mature in river) and some individuals are anadromous. The cue to migrate to sea could be environmental or genetic. If it were possible to tag and genetically assess juvenile trout, and track them through their life cycle, it would be possible to investigate genetic differences between individuals that mature in river and ones that go out to sea. The trade-off between greater risk of dying at sea and assumed higher fecundity on return could be assessed. Many of the microsatellites used for salmon can also be used for trout, though of course, caution may be needed in interpreting allele frequency data where brown trout and Atlantic salmon occur in the same stretch, as they can interbreed.

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Appendix

Appendix Table 1 Habitat quality score (HQS), parr abundance 100 m^2 and observed abundance as a percentage of the number predicted by HABSCORE. Where observed abundance equals predicted (100%), the site is at carrying capacity. Data for Atlantic salmon parr in River Frome, Dorset UK, sampled in summer and autumn for 3 consecutive years. Site map Figure 2.1 for site locations.

Appendix Table 1 a

July 1998 Site	*Number of fish caught*	*Grid reference*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	9	SY885863	/	0.04	/
2-ESMS	18	SY860867	10.56	1.76	17
3-ES	24	SY874865	19.81	5.04	51
4-WO	0	SY855868	2.94	0	/
5-BM	29	SY855867	2.68	8.8	328
6-WS	52	SY832872	4.24	7.65	180
7-EB	76	SY824875	16.66	11.7	70
8-MF	58	SY806894	0.7	14.7	2100
9-TN	10	SY771871	10.97	0.04	25
10-NF	30	SY739907	41.47	1.72	4
11-LM	34	SY738901	12.9	7.65	26
12-SW	21	SY726898	4.71	3.43	73
13-GB	16	SY708908	3.16	1.54	49
14-HA	14	SY676916	41.54	0.82	2
15-MB	1	SY641937	34.03	0.19	1

Appendix Table 1 b

September 1998 Site	*Number of fish caught*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	0	/	0	/
2-ESMS	18	6.55	5.52	84
3-ES	10	3.62	3.12	86
4-WO	10	0.68	4.24	624
5-BM	3	1.44	1.32	92
6-WS	20	5.31	6.59	124
7-EB	54	5.25	15.08	287
8-MF	57	3.69	13.69	371
9-TN	4	8.07	0.01	48
10-NF	10	53.63	4.18	8
11-LM	48	15.14	9.65	64
12-SW	10	0.91	4.27	469
13-GB	14	11.5	7.45	65
14-HA	10	27.09	2.12	8
15-MB	27	6.59	3.72	56

Appendix Table 1 c

July 1999 Site	*Number of fish caught*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	5	/	0.03	/
2-ESMS	14	10.17	6.09	143
3-ES	9	1.23	1.74	82
4-WO	3	1.16	2.76	217
5-BM	31	2.46	22.13	379
6-WS	26	2.08	7.43	810
7-EB	21	2.29	2.42	79
8-MF	49	3.7	7.11	470
9-TN	17	4.55	3.31	49
10-NF	53	38.17	18.32	7
11-LM	36	20.74	6.56	32
12-SW	17	4.85	7.12	336
13-GB	13	4.38	4.92	39
14-HA	20	7.86	2.82	13
15-MB	1	4.67	0.11	19

Appendix Table 1 d

September 1999 Site	*Number of fish caught*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	17	/	0.09	/
2-ESMS	13	4.19	6.01	60
3-ES	34	12.02	9.85	141
4-WO	3	1.51	3.27	238
5-BM	13	1.66	6.29	900
6-WS	11	12.47	4.78	324
7-EB	30	0.59	4.78	116
8-MF	21	0.54	2.54	192
9-TN	17	17.7	8.7	73
10-NF	33	177.1	12.75	48
11-LM	31	23.66	7.52	32
12-SW	21	2.03	6.82	147
13-GB	12	10.96	4.29	112
14-HA	11	15.08	1.95	36
15-MB	30	13.41	2.54	2

Appendix Table 1 e

July 2000 Site	*Number of fish caught*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	18	/	6.82	/
2-ESMS	31	87.02	10.11	50
3-ES	26	6.94	6.67	570
4-WO	28	7.4	24.01	1072
5-BM	31	13.94	33.35	929
6-WS	28	9.55	12.26	2004
7-EB	30	21.09	49.69	255
8-MF	30	4.79	10.82	894
9-TN	7	45.84	8.7	0
10-NF	20	122.2	6.47	22
11-LM	30	35.62	11.81	141
12-SW	29	14.55	29.97	776
13-GB	25	2.88	8.55	1140
14-HA	30	111.1	6.3	24
15-MB	Not fished	/	/	/

Appendix Table 1 f

October 2000 Site	*Number of fish caught*	*HQS*	*Abundance 100m^2*	*% observed to predicted parr abundance*
1-WH	0	/	0	/
2-ESMS	17	8.25	7.66	361
3-ES	27	1.89	9.39	3238
4-WO	13	6.75	8.19	431
5-BM	24	13.39	10.05	266
6-WS	15	2.6	10.13	988
7-EB	31	44.77	6.62	104
8-MF	31	3.1	7.2	857
9-TN	8	12.87	3.65	99
10-NF	14	96.7	1.7	7
11-LM	30	204.4	7.79	17
12-SW	15	2.64	5.79	793
13-GB	20	10.92	7.59	240
14-HA	12	36.68	3.74	40
15-MB	30	54.1	3.72	28

Appendix Table 1 g Percentage recapture of Atlantic salmon parr at each site, in three consecutive years

Site	*% recapture Sept 1998*	*% recapture Sept 1999*	*% recapture Oct 2000*
1-WH	/	/	/
2-ESMS	35	90	12
3-ES	13	40	0
4-WO	0	0	9
5-BM	13	3	8
6-WS	13	28	3
7-EB	10	30	4
8-MF	4	14	6
9-TN	22	0	43
10-NF	4	9	10
11-LM	23	14	10
12-SW	43	30	15
13-GB	44	23	24
14-HA	57	0	24
15-MB	/	/	/

Appendix Table 2 Genetic differentiation between anadromous Atlantic salmon populations *between rivers.*

Level of differentiation detected	*River system*	*Marker*	*Reference*
0.3%	9 Baltic rivers	allozymes	Stahl 1983
1.3%	7 populations in Quebec	microsatellites	Fontaine et al. 1997
2.5%	Welsh rivers	allozyme and mtDNA	O'Connell et al. 1995
2.98% 5.28%	North American rivers European rivers	microsatellites	King et al. 2001
3.4%	River Shannon system Ireland	allozymes	Galvin et al. 1996
3.4%	Rivers in Southern Ireland	allozymes	McElligott and Cross 1991
4.9%	Between rivers	allozymes	Stahl 1998
5.4%	15 populations Nova Scotia and Canada	microsatellites	McConnell et al. 1997
8.57%	European rivers	RFLP of mtDNA	Verspoor et al. 1999
11%	N. American and European	microsatellites	McConnell et al. 1995
14%	Bay of Fundy, Eastern Canada	mtDNA bp mutations	Verspoor et al. 2002
23%	8 European rivers	mtDNA	Nielsen et al. 1996
23%	14 European rivers	allozyme	Bourke et al. 1997

Appendix Table 3 Genetic differentiation between anadromous Atlantic salmon populations *within* rivers.

Level of differentiation detected	*River system*	*Geographic distance between sites*	*Marker*	*Reference*
0.1 %	five sites in the Kyles of Sutherland system, Scotland	20 to 40 km	allozymes	Verspoor et al. 1991
0.7 %	7 sites on the River Tweed, Scotland	20 to 50 km	allozymes	Jordan et al. 1992
0.9 %	Sainte-Marguerite River, Canada	5 to 50 km	microsatellites	Garant et al. 2000
1.1 %	Conne River, Newfoundland	Not stated	microsatellites	Beacham and Dempson 1998
3.09 %	11 tributaries of the River Shannon, Ireland	10 to 200 km	allozymes	Galvin et al. 1994
3.4 %	9 tributaries of the River Blackwater, Ireland	not stated	allozymes	McElligott and Cross 1991
8.0 %	3 sites on the Penobscot River in Maine, U.S.A.	not stated	microsatellites	Spidle 2001

Appendix Table 4 Genetic variability of Atlantic salmon at sites within the River Frome, July 1998.

Site# locus	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*13-GB*	*14-HA*	*Over all sites*
N total	9	18	24	29	52	76	58	10	30	34	16	14	370
Ssa 171													
N	3	16	18	28	48	61	57	10	30	32	15	11	329
N_A	4	7	7	6	9	10	7	4	7	8	5	5	11
Ht	0.83	0.75	0.80	0.77	0.79	0.80	0.81	0.70	0.74	0.82	0.69	0.57	0.77
Ho	0.67	0.63	0.83	0.82	0.79	0.74	0.84	0.70	0.60	0.88	0.73	0.46	0.72
Ssa 197													
N	9	17	24	28	49	72	50	10	30	33	15	12	349
N_A	7	7	7	10	8	11	11	5	10	9	8	6	13
Ht	0.89	0.84	0.824	0.79	0.83	0.84	0.86	0.73	0.85	0.81	0.80	0.8	0.83
Ho	0.67	0.42	0.5	0.89	0.55	0.67	0.68	0.70	0.63	0.48	0.87	0.25	0.6
Ssa 202													
N	2	15	20	9	38	66	45	7	24	12	3	5	246
N_A	2	4	4	3	5	6	5	3	4	5	3	3	6
Ht	0.50	0.69	0.64	0.58	0.67	0.67	0.62	0.52	0.54	0.80	0.58	0.75	0.65
Ho	0.50	0.73	0.40	0.44	0.63	0.50	0.69	0.71	0.50	0.58	0.67	0.6	0.58
Ssa 289													
N	5	17	18	25	47	56	38	9	0	0	2	10	227
N_A	4	3	3	4	4	5	5	4	NA	NA	2	2	5
Ht	0.85	0.43	0.21	0.32	0.47	0.32	0.59	0.61	NA	NA	1	0.34	0.51
Ho	0.2	0.06	0.11	0.36	0.11	0.18	0.34	0.56	/	/	0	0.2	0.22
Ssosl 85													
N	9	17	23	27	47	71	58	9	29	32	13	10	345
N_A	8	6	9	7	8	7	6	5	7	8	6	7	11
Ht	0.88	0.80	0.82	0.59	0.66	0.72	0.80	0.74	0.78	0.76	0.82	0.84	0.79
Ho	0.89	0.65	0.43	0.63	0.60	0.54	0.81	0.78	0.83	0.59	0.79	0.7	0.68

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht), observed heterozygosity (Ho) per locus per population

#Site 4-WH, no fish were captured at this site; Site 14; juveniles were captured at this site but they were not genotyped; Site 15-MB only one fish was captured at this site.

Appendix Table 5 Genetic variability of Atlantic salmon at sites within the River Frome, September 1998.

Site# locus	*2-ESMS*	*3-ES*	*4-WO*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*	*Over all sites*
N total	18	10	10	20	54	57	4	10	48	10	14	10	27	306
Ssa 171														
N	18	4	2	19	50	45	2	10	43	8	12	9	16	244
N_A	6	4	3	6	7	6	4	6	8	4	6	6	6	11
Ht	0.74	0.79	0.75	0.84	0.80	0.77	1.0	0.82	0.80	0.74	0.73	0.77	0.79	0.80
Ho	0.78	1.0	1.0	0.68	0.84	0.69	1.0	0.90	0.77	1.00	0.92	0.56	0.38	0.75
Ssa 197														
N	15	6	6	14	48	47	1	9	38	10	7	7	0	216
N_A	7	8	7	7	10	11	1	3	9	3	5	6	NA	13
Ht	0.79	0.95	0.92	0.81	0.71	0.82	NA	0.67	0.75	0.71	0.77	0.87	NA	0.82
Ho	0.80	0.67	0.83	0.79	0.56	0.70	0	0	0.68	0.60	0.86	0.57	/	0.68
Ssa 202														
N	15	4	4	11	30	39	3	6	32	9	7	8	8	183
N_A	3	2	4	3	5	5	3	4	5	4	4	3	4	6
Ht	0.35	0.50	0.83	0.70	0.70	0.60	0.67	0.82	0.74	0.76	0.79	0.42	0.65	0.72
Ho	0.40	0.75	0.50	0.45	0.70	0.54	1.00	0.50	0.66	0.44	0.43	0.50	0.50	0.59
Ssa 289														
N	15	10	6	15	32	38	4	10	44	8	14	10	10	228
N_A	3	4	3	3	4	4	3	3	4	2	4	3	1	5
Ht	0.48	0.63	0.32	0.19	0.23	0.44	0.63	0.68	0.15	0.23	0.73	0.278	0	0.45
Ho	0.47	0.20	0.33	0.2	0.23	0.45	0.50	0.20	0.14	0.25	0.14	0.3	0	0.26
Ssosl 85														
N	18	10	4	15	50	47	3	10	39	6	13	10	26	261
N_A	5	4	4	6	7	8	4	3	7	4	5	5	7	8
Ht	0.69	0.74	0.83	0.78	0.76	0.8	0.83	0.66	0.77	0.70	0.74	0.72	0.80	0.75
Ho	0.56	0.60	0.25	0.87	0.68	/	0.67	0.80	0.87	0.67	0.85	1.0	0.77	0.69

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht) and observed heterozygosity (Ho) per locus per population
#Site 1-WH, N =1; Site 3-BM N=3.

Appendix Table 6 Genetic variability of Atlantic salmon at sites within the River Frome, July 1999.

Site # locus	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*Over all sites*
N total	5	14	9	31	26	21	49	17	53	36	17	13	20	335
Ssa 171														
N	5	14	9	31	26	21	49	16	53	36	17	12	20	329
N_A	6	6	5	7	6	6	8	6	10	8	6	7	7	11
Ht	0.83	0.81	0.78	0.74	0.74	0.83	0.74	0.73	0.83	0.83	0	0.63	0.75	0.78
Ho	1.0	0.86	0.78	0.74	0.69	0.90	0.59	0.75	0.87	0.92	0.82	0.67	0.90	0.79
Ssa 197														
N	5	14	9	31	26	21	49	16	53	35	16	13	19	326
N_A	6	8	5	11	10	10	10	9	11	8	9	6	10	15
Ht	0.9	0.84	0.78	0.81	0.83	0.79	0.86	0.86	0.86	0.84	0.67	0.86	0.85	0.86
Ho	1.0	0.86	0.67	0.74	0.88	0.71	0.82	0.75	0.70	0.55	0.88	0.62	0.74	0.74
Ssa 202														
N	5	14	9	31	26	21	49	17	53	35	17	13	20	330
N_A	3	3	4	4	4	4	5	5	6	5	4	4	5	7
Ht	0.73	0.65	0.74	0.72	0.63	0.68	0.72	0.64	0.75	0.75	0.50	0.65	0.73	0.71
Ho	0.8	0.64	0.78	0.58	0.69	0.71	0.78	0.82	0.85	0.83	0.82	0.92	0.90	0.77
Ssa 289														
N	4	14	9	31	26	20	49	16	49	35	17	9	20	319
N_A	1	3	3	5	4	3	4	3	5	5	4	3	3	5
Ht	0	0.51	0.56	0.61	0.45	0.45	0.32	0.68	0.50	0.16	0.50	0.57	0.19	0.47
Ho	0	0.71	0.78	0.35	0.5	0.45	0.43	0.38	0.37	0.17	0.29	0.11	0.15	0.36
Ssosl 85														
N	3	13	9	29	26	21	41	16	46	36	16	10	20	307
N_A	3	6	4	5	6	7	5	5	6	5	5	5	6	9
Ht	0.75	0.78	0.72	0.74	0.805	0.80	0.80	0.72	0.69	0.71	0.67	0.76	0.71	0.75
Ho	1.0	0.31	0.33	0.66	0.5	0.81	0.46	0.75	0.74	0.72	0.69	1.0	0.60	0.64

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht) and observed heterozygosity (Ho) per locus per population
Site 4-WH N=3,; Site15-MB N= 1

Appendix Table 7 Genetic variability of Atlantic salmon at sites within the River Frome, September 1999.

Site# locus	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*	*Over all sites*
total N	17	13	34	13	11	30	21	17	33	31	21	12	11	30	296
Ssa 171															
N	9	12	23	13	9	21	17	17	24	25	20	10	8	11	221
N_A	5	6	7	5	7	5	6	4	7	7	6	7	5	5	12
Ht	0.73	0.83	0.82	0.72	0.85	0.75	0.57	0.76	0.83	0.83	0.67	0.84	0.80	0.63	0.81
Ho	0.78	1.0	0.96	0.80	1.0	0.71	0.76	0.65	0.79	0.96	1.00	0.70	1.00	0.91	0.86
Ssa 197															
N	14	7	19	12	10	27	21	14	1	19	18	11	7	15	297
N_A	7	7	8	10	9	9	8	9	2	7	8	7	4	6	12
Ht	0.79	0.70	0.85	0.91	0.86	0.81	0.88	0.86	NA	0.83	0.83	0.84	0.69	0.81	0.83
Ho	0.71	0.57	0.58	0.83	0.50	0.74	0.76	1.0	1	0.74	0.94	0.82	0.43	0.60	0.74
Ssa 202															
N	9	10	21	10	10	8	17	15	24	20	21	9	7	13	195
N_A	4	4	4	4	3	5	4	4	5	4	4	5	4	4	5
Ht	0.67	0.68	0.65	0.73	0.68	0.77	0.71	0.76	0.74	0.66	0.83	0.67	0.75	0.73	0.73
Ho	0.78	0.80	0.48	0.50	0.90	0.75	0.58	1.00	0.67	0.65	0.86	0.78	0.71	0.85	0.72
Ssa 289															
N	17	13	33	13	11	14	21	16	25	17	20	12	9	17	240
N_A	4	4	4	3	4	4	4	3	5	2	3	4	3	2	5
Ht	0.48	0.71	0.64	0.59	0.40	0.62	0.56	0.70	0.55	0.06	0.83	0.43	0.22	0.45	0.54
Ho	0.18	0.38	0.67	0.31	0.45	0.43	0.38	0.38	0.20	0.06	0.15	0.33	0.22	0.53	0.35
Ssosl 85															
N	14	11	32	13	11	27	21	17	33	28	21	12	11	20	273
N_A	5	5	6	4	6	5	4	3	5	7	6	5	4	4	8
Ht	0.76	0.81	0.73	0.64	0.85	0.65	0.54	0.67	0.74	0.68	0.67	0.80	0.71	0.56	0.72
Ho	0.79	0.91	0.81	0.62	0.91	0.56	0.57	0.65	0.70	0.71	0.71	0.67	0.27	0.70	0.69

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht) and observed heterozygosity (Ho) per locus per population
#Site 4-WH N=3

Appendix Table 8 Genetic variability of Atlantic salmon at sites within the River Frome, July 2000.

Site# locus	*1-WH*	*2-ESMS*	*3-ES*	*4-WO*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*Over all sites*
Total N	18	31	26	28	31	28	30	30	7	20	30	29	25	30	377
Ssa 171															
N	17	31	24	28	29	22	27	30	7	20	30	29	25	19	352
N_A	8	6	7	6	8	6	7	8	4	7	8	7	9	8	11
Ht	0.80	0.73	0.81	0.79	0.76	0.68	0.77	0.75	0.81	0.67	0.74	0.71	0.74	0.71	0.77
Ho	0.71	0.77	0.83	0.93	0.79	0.73	0.85	0.63	0.71	0.7	0.63	0.93	0.84	0.68	0.76
Ssa 197															
N	18	30	26	28	31	28	30	29	7	20	29	29	25	29	373
N_A	9	10	7	9	8	10	10	8	7	8	8	8	9	8	16
Ht	0.85	0.85	0.79	0.81	0.78	0.79	0.85	0.87	0.78	0.84	0.87	0.77	0.81	0.86	0.86
Ho	0.89	0.90	0.69	0.79	0.74	0.75	0.80	0.79	0.71	0.75	0.79	0.83	0.80	0.76	0.79
Ssa 202															
N	17	30	18	27	29	23	25	29	7	19	29	27	23	19	332
N_A	5	4	5	3	4	4	5	4	4	5	4	4	4	4	5
Ht	0.70	0.66	0.40	0.70	0.66	0.70	0.69	0.79	0.69	0.80	0.67	0.63	0.70	0.73	0.72
Ho	0.65	0.60	0.22	0.59	0.65	0.65	0.60	0.69	0.71	0.84	0.69	0.81	0.83	0.79	0.66
Ssa 289															
N	5	29	23	26	22	12	29	23	7	10	23	25	12	5	262
N_A	3	4	4	4	5	3	4	4	1	2	4	5	1	2	5
Ht	0.4	0.36	0.68	0.31	0.61	0.60	0.45	0	0.57	0.19	0.32	0.44	0	0.4	0.39
Ho	0.2	0.38	0.35	0.23	0.68	0.33	0.34	0.22	0	0.2	0.22	0.44	0	0	0.31
Ssosl 85															
N	12	29	18	24	26	23	29	16	2	19	16	29	22	23	313
N_A	4	6	4	4	7	6	5	4	4	4	4	6	5	4	10
Ht	0.74	0.72	0.69	0.56	0.78	0.65	0.72	1.0	0.76	0.65	0.64	0.65	0.59	0.41	0.71
Ho	0.58	0.76	0.33	0.46	0.69	0.78	0.76	0.06	1.0	0.63	0.06	0.69	0.36	0.53	0.60

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht) and observed heterozygosity (Ho) per locus per population
#Site 15-MB was not fished at this time

Appendix Table 9 Genetic variability at of Atlantic salmon at sites within the River Frome, October 2000.

Site# locus	*2-ESMS*	*3-ES*	*4-WO*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*	*Over all sites*
Total N	17	27	13	24	15	31	31	14	30	15	20	12	30	308
Ssa 171														
N	17	26	11	23	15	28	25	12	23	15	18	12	28	280
N_A	6	7	3	6	5	6	9	6	6	7	8	6	5	9
Ht	0.76	0.75	0.54	0.65	0.78	0.79	0.78	0.69	0.81	0.81	0.81	0.76	0.44	0.74
Ho	0.65	0.73	0.91	0.61	0.73	0.82	0.80	0.50	0.61	0.93	0.89	0.83	0.40	0.68
Ssa 197														
N	16	27	12	24	15	31	31	14	29	15	19	9	30	299
N_A	8	9	4	10	9	8	8	8	8	6	8	6	7	12
Ht	0.76	0.82	0.65	0.76	0.85	0.82	0.85	0.86	0.87	0.78	0.85	0.88	0.81	0.84
Ho	0.88	0.81	0.92	0.67	0.80	0.87	0.94	0.79	0.97	0.67	0.84	0.89	0.80	0.85
Ssa 202														
N	13	22	13	22	13	24	27	14	30	15	19	9	28	278
N_A	5	5	4	4	4	6	5	5	5	3	5	4	4	6
Ht	0.71	0.71	0.52	0.70	0.63	0.77	0.74	0.68	0.72	0.60	0.75	0.67	0.61	0.71
Ho	0.77	0.68	0.57	0.82	0.54	0.79	0.74	0.71	0.67	0.80	0.63	0.44	0.61	0.69
Ssa 289														
N	17	25	11	23	15	30	29	9	27	15	9	7	28	272
N_A	2	4	3	3	3	5	5	3	3	4	2	3	4	5
Ht	0.37	0.45	0.56	0.54	0.30	0.56	0.56	0.63	0.41	0.65	0.47	0.48	0.49	0.50
Ho	0.35	0.40	0.82	0.30	0.33	0.57	0.48	0.22	0.30	0.40	0.44	0.43	0.32	0.41
Ssosl 85														
N	17	27	12	24	14	31	31	14	29	15	20	12	30	303
N_A	5	5	4	5	5	5	5	5	5	4	5	8	6	8
Ht	0.77	0.75	0.72	0.81	0.71	0.72	0.76	0.75	0.51	0.53	0.74	0.62	0.56	0.71
Ho	0.59	0.67	0.83	0.71	0.64	0.68	0.74	0.57	0.38	0.67	0.65	0.50	0.37	0.62

Sample size (N), number of alleles (N_A), expected heterozygosity (Ht) and observed heterozygosity (Ho) per locus per population
#Site 1-WH no fish were captured at this site; Site 9-TN, although fish were captured at this site these samples were not genotyped.

Appendix Table 10 Atlantic salmon in the River Frome, F_{IS} per site per locus and over 5 loci, July 1998.

Site# *Locus*	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*13-GB*	*14-HA*
N total	9	18	24	29	52	76	58	10	30	34	16	7
Ssa 171												
N	3	16	18	28	48	61	57	10	30	32	15	11
F_{IS}	0.2	0.17	-0.04	-0.06	0	0.08	-0.05	0	0.19	-0.07	-0.06	0.2
Ssa 197												
N	9	17	24	28	49	72	50	10	30	33	15	12
F_{IS}	0.25	0.51*a	0.39*a	-0.14	0.34*a	0.20*a	0.21***	0.04	0.25**	0.40*a	-0.09	0.69*a
Ssa 202												
N	2	15	20	9	38	66	45	7	24	12	3	5
F_{IS}	0	-0.07	0.37*	0.23	0.06	0.25*	-0.11	-0.36	0.06	0.27	-0.14	0.2
Ssa 289												
N	5	17	18	25	47	56	38	9	/	/	2	10
F_{IS}	0.77**	0.87*a	0.48	-0.13	0.73*a	0.44*a	0.42*a	0.09*	NA	NA	1.0	0.42
Ssosl 85												
N	9	17	23	27	47	71	58	9	29	32	13	10
F_{IS}	-0.02	0.19	0.47*a	-0.07	0.10	0.26*a	-0.01	-0.05	-0.07	0.21*	0.06	0.17
F_{IS} over all loci	0.26**	0.30*a	0.31*a	-0.03	0.22*a	0.22*a	0.08**	-0.04	0.12*	0.20*a	0.22	0.33*a

P value based on 12500 randomisations *P* 0.05 *, *P* 0.01 **, *P* 0.001***. Indicative adjusted nominal level (5%) was 0.0004, *P* 5% adjusted *a
#Site 4-WH, no fish were captured at this site; Site 14; juveniles were captured at this site but they were not genotyped; Site 15-MB only one fish was captured at this site.

Appendix Table 11 Atlantic salmon in the River Frome, F_{IS} per locus and over 5 loci, September 1998.

Site# *Locus*	*2-ESMS*	*3-ES*	*4-WO*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*
N total	18	10	10	20	54	57	4	10	48	10	14	10	27
Ssa 171													
N	18	4	2	19	50	45	2	10	43	8	12	9	16
F_{IS}	-0.06	-0.26	-0.33	0.19	-0.06	0.11	0	-0.10	0.04	-0.35	-0.25	0.28	0.52*a
Ssa 197													
N	15	6	6	14	48	47	1	9	38	10	7	7	/
F_{IS}	-0.01	0.3	0.09	0.03	0.20**	0.14*	NA	1.0*a	0.09	0.15	-0.19	0.34	NA
Ssa 202													
N	15	4	4	11	30	39	3	6	32	9	7	8	8
F_{IS}	-0.14	-0.5	0.4	0.35	0.01	0.10	-0.5	0.39	0.11	0.41	0.46	-0.19	0.23
Ssa 289													
N	15	10	6	15	32	38	4	10	44	8	14	10	10
F_{IS}	0.03	0.68**	-0.05	-0.05	0.06	-0.02	0.2	0.71**	0.10	-0.08	0.80*a	-0.08	NA
Ssosl 85													
N	18	10	4	15	50	/	3	10	39	6	13	10	26
F_{IS}	0.2	0.19	0.7*	-0.12	0.10	0.47*a	0.2	-0.21	-0.13	0.05	-0.14	-0.39	0.04
F_{IS} over all loci	0.02	0.11	0.20	0.1	0.06	0.18*a	-0.01	0.34*a	0.03	0.06	0.15	0.04	0.27**

P value based on 14400 randomisations. Indicative adjusted nominal level (5%) was 0.00042. *P* 0.05 *, *P* 0.01 **, *P* 0.001***, *P* 5% adjusted *a.
#Site 1-WH, N =1; Site 3-BM N=3.

Appendix Table 12 For Atlantic salmon in the River Frome, F_{IS} per locus per population and over 5 loci, July 1999.

Site# locus	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*
N total	5	14	9	31	26	21	49	17	53	36	17	13	20
Ssa 171													
N	5	14	9	31	26	21	49	16	53	36	17	12	20
F_{IS}	-0.14	-0.07	0	0	0.06	-0.16	0.25***	-0.01	-0.06	-0.14	-0.12	0.15	-0.2
Ssa 197													
N	5	14	9	31	26	21	49	16	53	35	16	13	19
F_{IS}	-0.11	-0.02	0.14	0.09	-0.04	0.1	-0.02	0.13	0.19**	0.39*a	-0.02	0.28*	0.13
Ssa 202													
N	5	14	9	31	26	21	49	17	53	35	17	13	20
F_{IS}	-0.10	0.01	-0.06	0.2	-0.1	-0.05	-0.2	-0.15	-0.14	-0.11	-0.28	-0.42	-0.24
Ssa 289													
N	4	14	9	31	26	20	49	16	49	35	17	9	20
F_{IS}	NA	-0.39	-0.4	0.42**	-0.1	-0.01	0.22*	-0.16	0.26**	-0.05	0.57***	0.81**	0.21
Ssosl 85													
N	3	13	9	29	26	21	41	16	46	36	16	10	20
F_{IS}	-0.33	0.6*a	0.54	0.11	0.38***	-0.01	0.18	0.07	-0.07	-0.01	0.04	-0.31	0.15
F_{IS} over all loci	-0.17	0.06	0.07	0.15***	0.06	-0.03	0.08*	0	0.03	0.04	0.04	0.08	-0.02

P value based on 13200 randomisations. Indicative adjusted nominal level (5%) was 0.00045. *P* 0.05 *, *P* 0.01 **, *P* 0.001***, *P* 5% adjusted *a
Site 4-WH N=3, Site15-MB N= 1

Appendix Table 13 Atlantic salmon in the River Frome, F_{IS} per locus per population and over 5 loci, September 1999.

Site# locus	*1-WH*	*2-ESMS*	*3-ES*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*
N total	17	13	34	13	11	30	21	17	33	31	21	12	11	30
Ssa 171														
N	9	12	23	13	9	21	17	17	24	25	20	10	8	11
F_{IS}	-0.07	-0.21	-0.16	-0.07	-0.18	0.04	0.08	-0.14	0.05	-0.16	-0.31	0.17	-0.24	-0.45
Ssa 197														
N	14	7	19	12	10	27	21	14	1	19	18	11	7	15
F_{IS}	0.09	0.19	0.32**	0.08	0.42**	0.08	0.02	-0.14	NA	0.11	-0.1	0.02	0.38	0.26*
Ssa 202														
N	9	10	21	10	10	8	17	15	24	20	21	9	7	13
F_{IS}	-0.16	-0.18	0.27	0.32	-0.33	0.03	0.21	-0.4	0.09	0.01	-0.12	-0.17	0.48	-0.16
Ssa 289														
N	17	13	33	13	11	14	21	16	25	17	20	12	9	17
F_{IS}	0.63**	0.46	-0.05	0.48	-0.14	0.31	0.14*a	0.33	0.63*a	0	0.78*a	0.22	-0.032	-0.18
Ssosl 85														
N	14	11	32	13	11	27	21	17	33	28	21	12	11	20
F_{IS}	-0.03	-0.13	-0.11	0.05	-0.07	0.14	0.17	-0.21	0.06	-0.06	-0.07	0.17	0.62**	-0.24
F_{IS} over all loci	0.06	0.02	0.05	0.16*	-0.04	0.11*	0.12*	-0.13	0.17***	-0.03	0.02	0.08	0.167	-0.13

P value based on 12600 randomisations *P* 0.05 *, *P* 0.01 **, *P* 0.001***. Indicative adjusted nominal level (5%) was 0.00048, *P* 5% adjusted *a.
#Site 4-WH N=3

Appendix Table 14 Atlantic salmon in the River Frome, F_{IS} per locus per population and over 5 loci, July 2000.

Site# Locus	*1-WH*	*2-ESMS*	*3-ES*	*4-WO*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*9-TN*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*
N total	18	31	26	28	31	28	30	30	7	20	30	29	25	30
Ssa 171														
N	17	31	24	28	29	22	27	30	7	20	30	29	25	19
F_{IS}	0.12	-0.06	-0.02	-0.31	-0.05	-0.07	-0.10	0.19	0.05	-0.04	0.15	-0.15	-0.14	0.04
Ssa 197														
N	18	30	26	28	31	28	30	29	7	20	29	29	25	29
F_{IS}	-0.05	-0.06	0.12	-0.03	0.05	0.05	0.06	0.03	0.18	0.10	0.12	-0.07	0.01	0.11
Ssa 202														
N	17	30	18	27	29	23	25	29	7	19	29	27	23	19
F_{IS}	0.08	0.08	0.44	0.05	0.01	0.07	0.14	0.01	0.09	-0.06	0.31*	-0.18	-0.18	-0.08
Ssa 289														
N	5	29	23	26	22	12	29	23	7	10	23	25	12	5
F_{IS}	0.5	-0.06	0.49***	0.48**	-0.12	0.44	0.23	0.30	NA	-0.06	0.10	0.23	NA	1
Ssosl 85														
N	12	29	18	24	26	23	29	16	2	19	16	29	22	23
F_{IS}	0.21	-0.07	0.51**	0.29	0.12	-0.2	-0.05	0.89*a	0	0.02	-0.06	0.09	0.39**	-0.27
F_{IS} over all loci	0.13*	-0.03	0.28*a	0.06	0.01	0.05	0.04	0.24*a	0.08	0.01	0.13**	-0.03	0	0.12

P value based on 12600 randomisations. Indicative adjusted nominal level (5%) was 0.00048. *P* 0.05 *, *P* 0.01 **, *P* 0.001***, *P* 5% adjusted *a.
#Site 15-MB was not fished at this time

Appendix Table 15 For Atlantic salmon in the River Frome, F_{IS} per locus per population and over 5 loci, October 2000.

Site# *locus*	*2-ESMS*	*3-ES*	*4-WO*	*5-BM*	*6-WS*	*7-EB*	*8-MF*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*
N total	17	27	13	24	15	31	31	14	30	15	20	12	30
Ssa 171													
N	17	26	11	23	15	28	25	12	23	15	18	12	28
F_{IS}	0.14	0.02	-0.68	0.07	0.06	-0.05	-0.03	0.28	0.25*	-0.15	-0.09	-0.1	0.11
Ssa 197													
N	16	27	12	24	15	31	31	14	29	15	19	9	30
F_{IS}	-0.16	0	-0.42	0.13	0.06	-0.06	-0.1	0.09	-0.11	0.15	0.01	-0.02	0.02
Ssa 202													
N	13	22	13	22	13	24	27	14	30	15	19	9	28
F_{IS}	-0.08	0.04	-0.04	-0.18	0.14	-0.02	0	-0.05	0.09	-0.33	0.16	0.34	0
Ssa 289													
N	17	25	11	23	15	30	29	9	27	15	9	7	28
F_{IS}	0.05	0.12	-0.48	0.43*	-0.13	-0.01	0.14	0.64*	0.35*	0.39	0.06	0.1	0.35
Ssosl 85													
N	17	27	12	24	14	31	31	14	29	15	20	12	30
F_{IS}	0.23	0.11	-0.15	0.13	0.09	0.06	0.02	0.24	0.3	-0.26	0.12	0.19	0.34**
F_{IS} over all loci	0.04	0.05	-0.35	0.10	0.07	-0.02	0	0.23**	0.14**	-0.03	0.05	0.09	0.15**

P value based on 12000 randomisations *P* 0.05 *, *P* 0.01 **, *P* 0.001***. Indicative adjusted nominal level (5%) was 0.0005, *P* 5 % adjusted *a.
#Site 1-WH no fish were captured at this site; Site 9-TN, although fish were captured at this site these samples were not genotyped.

Appendix Table 16 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, July 1998.

Site	*1. WH*	*2. ESMS*	*3. ES*	*5. BM*	*6. EB*	*7. WS*	*8. MF*	*9. TN*	*10. NF*	*11. LM*	*13. GB*	*14. HA*
1. WH												
2. ESMS	-0.0019											
3. ES	0.0206	0.0018										
5. BM	0.0461	0.0274	0.0186									
6. EB	0.031	0.0229	0.0118	0.0149*								
7. WS	0.0257	0.0079	-0.0055	0.0092	0.0005							
8. MF	-0.0004	0.0144	0.0105	0.0548*	0.0306*	0.0253*						
9. TN	0.0198	0.0078	0.0397	0.0652	0.0422	0.038	0.0317					
10. NF	-0.0343	0.0101	0.0084	0.0368	0.0374	0.0162	0.016	0.0267				
11. LM	0.0334	0.0305	0.0333	0.0607	0.035	0.0376	0.0447	0.0196	0.042			
13. GB	-0.0578	0.0171	0.0079	0.0523	0.0238	0.0228	0.0116	0.0309	-0.001	0.0388		
14. HA	0.0439	0.0119	0.0265	0.0758	0.0299	0.0322	0.0559	0.0493	0.0514	0.0207	0.0547	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 17 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, September 1998.

Site	*2-ESMS*	*3-ES*	*4-WO*	*6-EB*	*7-WS*	*8-MF*	*10-NF*	*11-LM*	*12-SW*	*13-GB*	*14-HA*	*15-MB*
2-ESMS												
3-ES	0.0953											
4-WO	0.0262	0.0815										
6-EB	0.0909	0.0907	0.0033									
7-WS	0.0643*	0.0855	0.0019	0.0151								
8-MF	0.0289	0.0422	-0.0057	0.0191	0.0163							
10-NF	0.1145*	0.0205	0.0632	0.0727	0.0665*	0.052*						
11-LM	0.0705*	0.0589	0.014	0.0216*	0.0146*	0.0248*	0.0642					
12-SW	0.1046*	0.1091	-0.0001	0.0269	0.0256*	0.0489*	0.0849*	0.0458				
13-GB	0.0902	0.0341	0.0397	0.0578	0.0684	0.043*	-0.0015	0.0689	0.0985			
14-HA	0.053	0.0871	0.0281	0.0457	0.0574	0.0261	0.0896	0.0408	0.1024	0.0541		
15-MB	0.0713	0.087	-0.0238	-0.0008	0.0041	0.0185	0.109	0.005	0.041	0.0916	0.0477	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 18 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, July 1999.

Site	*2. ESMS*	*3. ES*	*5. BM*	*6. EB*	*7. WS*	*8. MF*	*9. TN*	*10. NF*	*11. LM*	*12. SW*	*13. GB*	*14. HA*
2. ESMS												
3. ES	-0.0188											
5. BM	0.0157	-0.0109										
6. EB	0.0157	0.0086	0.0107									
7. WS	0.033*	0.0248	0.0177	-0.0008								
8. MF	0.0241	-0.0055	0.0146	0.0255	0.0358							
9. TN	0.0205	0.0144	0.0183	0.0156	0.0347	0.0388						
10. NF	0.0304*	0.0022	0.0179*	0.0267*	0.0213*	0.0268*	0.0341*					
11. LM	0.0624*	0.0406	0.0506*	0.0363*	0.0181*	0.0558*	0.0327*	0.0303*				
12. SW	0.0579*	0.0333	0.0366	0.0261	0.0286	0.0441	0.0409	0.0444*	0.0403			
13. GB	0.0328	0.0166	0.0187	0.0157	0.0227	0.0217	0.051	0.0071	0.0514	0.024		
14. HA	0.0713*	0.0286	0.0467*	0.0378*	0.052*	0.0616*	0.0494*	0.0297*	0.0362*	0.0544*	0.0534	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 19 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, September1999.

Site	*1. WH*	*2. ESMS*	*3. ES*	*5. BM*	*6. EB*	*7. WS*	*8. MF*	*9. TN*	*10. NF*	*11. LM*	*12. SW*	*13. GB*	*14. HA*	*15. MB*
1. WH														
2. ESMS	-0.0044													
3. ES	-0.007	-0.0128												
5. BM	-0.0074	0.0111	0.0145											
6. EB	-0.0133	0.0043	0.0049	0.0181										
7. WS	0.0027	0.0077	0.0195	-0.0055	0.0094									
8. MF	0.0159	0.0295	0.0372*	0.0158	0.0023	-0.0091								
9. TN	0.0865	0.0796	0.0716*	0.0403	0.0795	0.0224	0.0604*							
10. NF	0.0017	0.0094	0.0126	0.0434	0.0025	0.0371	0.0341	0.1085						
11. LM	0.0332	0.0742	0.0379	0.0743	0.0091	0.0586	0.0423*	0.1134*	0.0597					
12. SW	0.0714*	0.0539	0.0723*	0.0397	0.0515	0.0283	0.0451	0.0798*	0.086	0.1157				
13. GB	-0.0171	0.0257	0.0136	0.0235	-0.0145	0.0102	0.0212	0.0834	-0.0048	0.0419	0.0609			
14. HA	0.0126	0.0497	0.0427	0.0436	0.0199	0.0169	0.0197	0.0863	0.0191	0.0379	0.0746	0.0297		
15. MB	0.0479	0.0606	0.0594	0.0473	0.065	0.0233	0.0595*	0.0756*	0.0919	0.0843	0.0706*	0.058	0.0325	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 20 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, July 2000.

site	*1. WH*	*2. ESMS*	*3. ES*	*4. WO*	*5. BM*	*6. EB*	*7. WS*	*8. MF*	*9. TN*	*10. NF*	*11. LM*	*12. SW*	*13. GB*	*14. HA*
1. WH														
2. ESMS	-0.0025													
3. ES	0.0428	0.0652*												
4. WO	0.0497	0.0789*	0.1166*											
5. BM	-0.0012	0.0129	0.0668*	0.0857*										
6. EB	0.0151	0.0406	0.0493	0.0746	0.0231									
7. WS	-0.0186	0.0112	0.0486	0.0654*	0.0133	0.0202								
8. MF	0.0166	0.0228	0.0623	0.0727*	0.0527*	0.0422	0.0182*							
9. TN	-0.0024	0.0274	0.1099	0.0695	0.0302	0.0809	0.0199	0.0291						
10. NF	0.0044	0.0172*	0.0992*	0.0635*	0.0229*	0.0416	0.0206	0.0394	0.0355					
11. LM	-0.0124	0.018	0.0685	0.0649*	0.0317*	0.0383	0.005	0.0371*	0.0215	0.0246				
12. SW	0.0208	0.0315*	0.1056*	0.0947*	0.0133	0.0582	0.0332*	0.0664*	0.0445	0.0405	0.0374*			
13. GB	0.03	0.0424*	0.1427*	0.0899*	0.0544*	0.0934	0.044*	0.0686*	0.0536	0.0203	0.0243	0.0533*		
14. HA	0.0382	0.0576*	0.1143	0.0939	0.0704	0.0451	0.036	0.0208	0.0934	0.0373	0.0458	0.077	0.0521	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 21 Pairwise F_{ST} for Atlantic salmon between sites in the River Frome, October 2000.

Site	*2. ESMS*	*3. ES*	*4. WO*	*5. BM*	*6. EB*	*7. WS*	*8. MF*	*10. NF*	*11. LM*	*12. SW*	*13. GB*	*14. HA*	*15. MB*
2. ESMS													
3. ES	0.0021												
4. WO	0.1027*	0.0431											
5. BM	0.0009	0.0038	0.0799*										
6. EB	0.004	-0.0092	0.0871*	0.0179									
7. WS	0.0221*	0.0027	0.0752*	0.0191	0.0098								
8. MF	0.0325*	0.0057	0.0422*	0.0335*	0.017	0.0099							
10. NF	0.0419	0.0098	0.0717	0.0228	0.0227	-0.002	0.0191						
11. LM	0.0508*	0.0189	0.0728*	0.0608*	0.0114	0.0132	0.0241	0.0153					
12. SW	0.0407*	0.0132	0.0969*	0.0465*	0.0156	0.0237*	0.0356*	0.0138	0.0173				
13. GB	0.0225	-0.0084	0.0375	0.0119	0.0036	-0.0011	-0.0017	0.011	0.0085	0.0176			
14. HA	0.0385	0.0099	0.0554	0.0494*	0.0226	0.03	0.0124	0.0464	0.0105	0.0289	-0.001		
15. MB	0.0956*	0.0388*	0.0777*	0.0736*	0.0554*	0.0663*	0.0628*	0.0331	0.0552*	0.0428*	0.051*	0.0363	

P values obtained after 105000 permutations, indicative adjusted nominal level (5%) for multiple comparisons was 0.000476.

*significant at $P = 0.05$ after Bonferroni correction.

Appendix Table 22. Mean relatedness of Atlantic salmon juveniles per site for summer 1998, 1999 and 2000.

Site	*Number of salmon July 1998*	*R*	*N July 1999*	*R*	*N July 2000*	*R*
1-WH	9	-0.0023	5	/	18	-0.001
2-ESMS	18	0.0003	14	0.0014	31	0.0071
3-ES	24	0.0028	9	-0.0002	26	0.0044
4-WO	/	/	/	/	28	0.0137
5-BM	29	0.0154	31	0.0001	31	0.0008
6-WS	52	0.0033	26	0.0029	28	0.0053
7-EB	76	0.0136	21	0.003	30	-0.0003
8-MF	58	-0.0049	49	0.0132	30	0.0075
9-TN	11	0.0006	17	0.0033	7	0.0042
10-NF	30	0.0078	53	0.0008	20	0.004
11-LM	34	0.0027	36	0.0162	30	0.0067
12-SW	/	/	16	0.0021	29	0.0009
13-GB	16	0.0016	13	0.0006	25	0.0108
14-HA	12	0.0021	20	0.0103	30	0.0118
15-MB	/	/	/	/	/	/

Appendix Table 23. Individuals related at 0.5 or over; estimates of pairwise relatedness per site.

July 1998 Site	*Number of samples*	*Number of pairwise comparisons at 0.5 or over*	*% 0.5 or over*
1-WH	8	0	0
2-ESMS	18	11	6.43
3-ES	24	21	7.00
4-WO	0	/	/
5-BM	29	23	5.29
6-WS	52	85	6.20
7-EB	76	173	5.90
8-MF	58	55	3.20
9-TN	11	1	1.51
10-NF	30	22	4.73
11-LM	34	25	4.20
12-SW	0	/	/
13-GB	16	9	6.62
14-HA	12	2	2.56
15-MB	1	/	/

July 1999 Site	*Number of samples*	*Number of pairwise at full sib >0.5*	*% 0.5 or over*
1-WH	5	/	/
2-ESMS	14	2	1.9
3-ES	9	1	2.2
4-WO	3	/	/
5-BM	31	13	2.62
6-WS	26	8	2.27
7-EB	21	10	4.33
8-MF	49	69	5.63
9-TN	17	2	1.3
10-NF	53	73	5.1
11-LM	36	23	3.45
12-SW	17	8	5.23
13-GB	13	3	3.3
14-HA	20	8	3.8
15-MB	1	/	/

July 2000 Site	*Number of samples*	*Number of pairwise at full sib >0.5*	*% 0.5 or over*
1-WH	18	5	2.90
2-ESMS	31	20	4.00
3-ES	26	14	3.99
4-WO	28	18	4.40
5-BM	31	22	4.43
6-WS	28	29	7.14
7-EB	30	16	3.44
8-MF	30	/	/
9-TN	7	2	7.14
10-NF	20	7	3.33
11-LM	30	22	4.73
12-SW	29	23	5.30
13-GB	25	17	5.23
14-HA	30	39	8.40
15-MB	1	/	/