Institute of Freshwater Ecology

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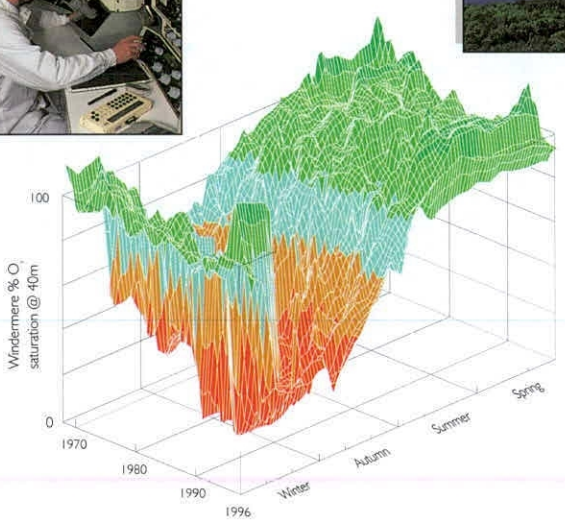
R&D PROJECT



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Progress Report

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Introduction:

There exist in many rivers populations of fish which have become isolated from populations in the main watercourse. The cause of this isolation can be geological (e.g. presence of waterfalls), biotic (e.g. coniferous forest creating conditions untenable for fish survival) or abiotic (industrial processes creating unsuitable conditions for survival). This isolation can have considerable impact on the genetic diversity and gene exchange of the populations and the possibility exists for genetically distinct populations to evolve. As well as survival implications, these genotypic differences can cause behavioural and feeding success differentiation between stocks (Dunbrack et al 1996).

Genetic differences among populations may result from selection and be adaptations to local conditions. If this is the case, populations will require specific management policies to be introduced to protect the gene pool of the population and procedures instigated to prevent genetic dilution by the introduction of fish. However, genetic differentiation may also arise from random changes in allele frequency (genetic drift) that occur in small populations with little immigration. If drift, rather than selection, is the cause of differentiation, local gene pools will not represent adaptations to local conditions. Indeed, drift can cause slightly deleterious alleles to become fixed if the population size becomes very small (a so-called 'genetic bottleneck'). Drift will also lower the total amount of genetic variation, reducing the ability of a population to respond to changing environmental conditions. Small populations may also suffer inbreeding depression: the loss of fitness that results from mating between close relatives in a species where mating is usually between unrelated individuals. In this situation, restocking may have a role in maintaining genetic variation and reducing inbreeding depression, thus preventing loss of fitness and further population decline.

Within the area of responsibility of the Midlands (Upper Severn) Environment Agency, there are several populations of brown trout (*Salmo trutta* L) that have become isolated by a combination of the conditions described above (Crisp & Beaumont 1996, 1997, R. Challis pers. com.).

The brown trout population in the upper reaches of the Severn is small and fragmented compared with that of the Wye. In the upper reaches of the Afon Hafren the upstream limit of the "normal" trout population is considered to be at Geufron (Nat. Grid Ref. SN/882855). However, despite the close proximity of the conifer plantations, a small population of trout is maintained in the Afron Hafren near the Blaenhafren falls (c.4.7 km upstream of Geufron) which is restricted to about 1700m^2 of stream. The cause of the isolation is suspected to be the presence, and associated stream chemistry changes, of large-scale afforestation in the catchment. (Crisp & Beaumont 1996, 1997, J. Hudson pers. com.)

In the Afon Tanat two populations of trout have become isolated from upstream recolonization due to the presence of high waterfalls. Below these obstructions, farmed fish have been used to augment trout populations.

The river Stour near Kidderminster has an area where brown trout population levels are very low. No stocking is thought to occur in the area and the population is believed to be sufficiently far away from stocked areas of the river to also be considered as an isolated population.

The presence of these isolated populations raises important issues for their future conservation. If the fish have survived in the given habitats due to genotypic adaptation then the potential exists for the strain to be used to recolonize areas where populations have perished. If they exist purely due to physical barriers, they could contain a repository of the original genetic make-up of the river's trout population. If the genetic make up of the sub-populations is different from that in the main populations every effort should be made to prevent stocking of non-indigenous fish and the subsequent risk of genetic dilution of the adapted strain.

Objectives:

The first objective of the study will be to establish whether the relict Hafren population is isolated genetically from others down-river, (perhaps because the Hafren population is more tolerant of acidic water) or whether the population is maintained, at least in part, by migration of fish up-river. The amount of genetic variation among populations will also be compared to examine whether drift is causing small populations to become genetically depauperate compared with larger ones.

The second objective of the study will be to determine the amount of gene introgression between farmed and native trout in the Tanat. An assessment of whether the two isolated populations are genetically similar and whether farmed fish have affected the genetic structure of the (potentially) locally adapted populations will also be made.

The final objective will be to compare genetic variation of native trout in the upper Severn with that of the small population in the Stour. An assessment of whether repeated exposure to industrial pollution causes genetic 'bottlenecks' in trout populations will be made.

Data will be published as a report to the Environment Agency and in the scientific literature as appropriate. The data will also be of wider interest as fisheries managers are increasingly concerned about the effects of farmed fish on locally adapted populations.

Methods:

Nine trout populations were sampled:

1. The upper Afon Hafren at Baenhafren Falls (NGR SN 836884). Area upstream of extensive conifer forest, the Hafren forest.

2. The Afon Hafren at Geufron (NGR SN881855) and at Old Hall (NGR SN 904847). Insufficient samples were obtained at Geufron so more were obtained from Old Hall approximately 2.5 Km downstream of Geufron.

3. Area of the main river of the Afon Tanat at Penybontfawr (NGR SJ088248). Area known to be stocked with trout.

4. A tributary of the Afon Tanat (the Afon Disgynfa at Tan-y-pistll NGR SJ070297). The site located above a waterfall impassable to fish.

5. A tributary of the Afon Tanat (the Afon Iwrch at Plas Maengwnedd NGR SJ126298). The site located above an area thought to be impassable to fish.

6. A sample of farmed fish (origin unknown) from the farm supplying the majority of the fish used to stock the Afon Tanat.

7. The upper river Teme at Felindre (NGR SO 171812).

8. Farmed fish from Burwarton fish farm located on a tributary of the river Teme.

9. The Hoo Brook a tributary of the river Stour at Duncelt farm (NGR SO 860755)

Between 15 and 62 individuals from each site / population were caught by electric fishing. Samples from the pelvic fin of each fish were removed and were stored in ethanol. Analysis of the genetic composition of each fish will be carried out using micro satellite DNA analysis.

Each fish will be genotyped at up to ten polymorphic micro satellite loci and the resulting data will be analysed to estimate:

a. the genetic diversity (amount of variation) of each population

b. the genetic distance between each pair of populations

c. gene flow (migration) between the Blaenhafren Falls and Geufron / Old Hall populations

d. the amount of introgression between farmed and native fish in the Tanat

e. whether the Stour population (and the Hafren populations if appropriate) has suffered a genetic bottleneck.

Results:

Microsatellite analysis
Allele frequencies at microsatellite loci will be used to estimate the degree of genetic differentiation among the trout populations. Microsatellites are sections of DNA where short (core) sequences, usually 2-4 bases long, are repeated one after the other. Different alleles are simply different numbers of repeat units.

Variation at a single microsatellite locus is detected as follows. DNA is extracted from the fish. Short pieces of DNA (primers), designed to bind to regions flanking the repeat sequence, are added to the DNA, along with nucleotides and a heat-stable DNA polymerase enzyme. The polymerase chain reaction is then used to amplify the DNA (including the repeats) between the primers. The amplified fragments are separated by electrophoresis through a polyacrylamide gel (fragments with a large number of repeats move slower than fragments with fewer repeats). The gel is then stained to reveal the positions of the fragments, enabling the allele size(s) for each individual to be determined.

We have tested a range of microsatellite primer pairs on trout from each of the study populations. DNA was prepared by the method of Beacham and Dempson (1997). One fin was placed in an eppendorf tube containing 0.2 ml of 5 % chelex, and autoclaved for 5 minutes at 121 °C. Next 25 µl of Tween 20 and 25 µl of proteinase K were added and the tubes were incubated overnight at 40 °C. Tubes were centrifuged at 15,000 rpm for 5 minutes and the supernatant removed and stored at –20 °C.

PCR was performed on an Omnigene Thermocycler, using a 10 µl reaction mix comprising:

DNA template	0.4 µl
NH_4 10 x buffer	1.0 µl
$MgCl_2$ (50 mM)	0.3 µl
dNTP (5 mM)	1.0 µl
Taq polymerase	0.05 µl
H_2O	7.57 µl
Forward primer	2 pmol
Reverse primer	2 pmol

PCR products were run at 60 V on 10 % non-denaturing acrylamide gels (C_{bis} 5 %) with 1 x TBE as the gel and electrode buffers. Gels were stained by ethidium bromide and photographed under UV light.

The 12 primer pairs tested so far are given in Table 1. Amplification products were obtained from all primer pairs apart from Ssosl 438, Ssosl 439. Primer pairs Ssosl 417, Ssosl 85, Ssal 97 gave good results and will be used to screen the total sample of fish. Primer pairs 20.19, F43, D30, Ssosl 444, Ogo1a may be used after further development work. Primer pairs Ssa 171, Ssa 202 gave large amplification products that could not be resolved easily.

Primer Pair	Core sequence	Origin	Reference
20.19	$(AT/TG)_n$	Atlantic salmon	Sanchez et al., 1995
F43	$(AT/TG)_n$	Atlantic salmon	Sanchez et al., 1995
D30	$(AT/TG)_n$	Atlantic salmon	Sanchez et al., 1995
Ssa197	$GT_5C(TG)_4(TG)_3(A(GTGA)_{15}$	Atlantic salmon	O'Reilly et al., 1996
Ssa171	$(TGTA)_{14}(TG)_7$	Atlantic salmon	O'Reilly et al., 1996
Ssa202	$(CA)_3(CTCA)_{17}$	Atlantic salmon	O'Reilly et al., 1996
Ssosl438	$(AC)_{26}AT(AC)_6$	Atlantic salmon	Slettan et al., 1996
Ssosl439	$(AC)_{30}$	Atlantic salmon	Slettan et al., 1996
Ssosl444	$(AC)_{41}$	Atlantic salmon	Slettan et al., 1996
Ssosl417	$(TG)_{25}$	Atlantic salmon	Slettan et al., 1995

Ssosl85	$(GT)_{22}$	Atlantic salmon	Slettan et al., 1995
Ogo1a	$(GTCT)_{26}$	Pink salmon	Olsen et al., 1998

Our present method of separating fragments on 10% non-denaturing gels and staining with ethidium bromide gives fairly low resolution; alleles that differ by a single repeat unit are difficult to distinguish. Therefore, we are developing protocols for separating fragments on 6% denaturing polyacrylamide sequencing gels and visualising the bands with silver staining. If successful, we may distinguish more alleles per locus that will give us greater power to detect genetic differentiation among the trout populations.

Data analysis

Allele frequency data from microsatellite loci will be used to calculate F_{ST} among populations. This parameter is the proportion of the total variance (σ_T^2) in allele frequencies that is due to differences in the mean allele frequency among populations. In other words

$$F_{ST} = \sigma_a^2 / \sigma_T^2$$

where σ_a^2 is the variance of the population mean allele frequencies (Weir and Cockerham, 1984). The statistical significance of F_{ST} will be tested with an exact test (Raymond & Rousset, 1995; Goudet et al., 1996) or a randomisation test (Goudet, 1995) depending on the amount of non-random mating detected within the populations.

A significant value for F_{ST} indicates differentiation among populations. In other words, the populations are not behaving as a single randomly mating group. As a rule of thumb, if F_{ST} is greater than 0.2, there is insufficient gene flow to prevent populations becoming fixed (allele frequency of 1) for different alleles. An F_{ST} value of 0.2 is roughly equivalent to one migrant into and out of each population per generation.

If F_{ST} is significant, we will test whether the amount of differentiation between populations is proportional to their geographical separation (a phenomenon called 'isolation by distance'). This is done by testing for a significant correlation between matrices of all pairwise F_{ST} values and distances. Because the pairs are not independent pieces of information, the significance of the correlation is obtained by a Mantel randomisation test (Mantel, 1967).

Conclusion:

Significant progress has been made in the refining of the procedures used for allele discrimination. These refinements, although delaying the progress of the results, will enable a far more detailed and accurate analysis of the genetic differentiation of the trout populations to be achieved.

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