6. DISCUSSION

6.1 The Project Objectives

6.1.1 Analysis of Carcasses

Objective: The collection and DNA fingerprinting of tissue samples from about 100 carcasses from southern England to provide essential data on levels of genetic diversity in the feasibility study area.

The target was exceeded with 168 different tissue samples analysed. Analysis using 9 microsatellite loci indicated that the south west population has sufficient genetic diversity for study using DNA fingerprinting. There were insufficient samples from carcasses originating from Hampshire to allow statistical analysis of the genetic variability of that population. It was concluded that the south west population has sufficient genetic variability for DNA fingerprinting of spraint to be feasible. However, only 6 loci proved reliable for analysis of DNA extracted from spraint. With 6 loci only there is not enough genetic variation to determine relatedness, even with 1st-order relatives.

All individuals from the Itchen population proved to be identical at locus 701 so that it was not possible to differentiate between same sex parent/offspring and full siblings. This is discussed further in Section 6.2.

6.1.2 Spraint Collected

Objective: The collection and DNA fingerprinting of about 500 spraint from a transect across high to low density otter populations in southern England.

The original estimate of 500 spraint was exceeded. However, this estimate was for spraint on the Torridge (200), Brue (200) and Itchen (100). The proportions reflected the estimated density of otters and likely number of spraint to be found. The actual numbers collected from the Torridge and Brue (89 and 97 respectively) fell below the estimated total partly due to the delayed start to surveys. Also, on the Torridge there were several months when spraint sites were washed out. Additional samples were collected on the Itchen (261 in total) as the overall total for all the catchments was below target. An additional 200 samples had been allowed for the PhD study in Somerset. The inclusion of samples collected from the Tone catchment (175) brought the overall total up to well over 600 spraint analysed.

6.1.3 Progress Reviews

Objective: To report and review progress regularly during the project.

Progress was reviewed at regular Project Board meetings, which resulted in some changes to the sampling strategies. These included additional surveys of the Brue and Itchen and splitting the surveys on the Torridge to cover several days to allow for reduced numbers of surveyors. More detailed reviews of survey strategies were hampered as the results of analyses were only received immediately prior to the quarterly Project Board meetings. Ideally samples from each monthly survey should be analysed and reviewed prior to the next survey on the watercourse.

It was recognised that feedback to the surveyors on a frequent basis was important to maintain motivation. Identification of new otters within a volunteers survey area was always received with great excitement and helped to increase surveyors' identification and ownership of the project's objectives. Otters identified became 'their otters', their success. This type of feedback provides very important motivation at 5am on a Sunday morning when it is pouring with rain. Providing feedback on the project as a whole emphasises just how important everyone's role is in the study. Where stretches of river were consistently negative it was found helpful to swap survey areas so that surveyors did not lose interest or become despondent because they had not found any fresh spraint for months.

Minutes of the Project Board Meetings and Progress Reports are available on request to the Environment Agency.

6.1.4 Feasibility of a Long Term Study

Objective: To produce guidance and recommendations on the feasibility of a long term study into factors limiting otter recovery in the UK.

This project has shown that it is possible to survey and monitor otter populations on a catchment basis using DNA fingerprinting where there is sufficient genetic variability within the population. However, in southern England some populations have levels of variability below that needed to reliably discriminate between individuals (River Itchen) whereas others are close to the borderline (in the south west).

Although the proportion of spraints successfully typed in the feasibility study is greater than in some early trials, where only one in seven could be typed, there would be considerable benefit in developing techniques or protocols which would lead to a higher success rate.

A useful baseline data set has been developed which has provided an introduction to the populations of the four catchments. However, there is insufficient information at present to make very reliable comparisons between these populations and published data from other studies. The data have also raised new questions.

Identification of individual DNA profiles in the intermediate and fragmented populations has shown that they contain more otters than expected. These numbers represent minima for the populations since a) in at least one case two otters had the same profile; b) some of the partial fingerprints appeared to differ from the full profiles; c) some otters may not have been recorded, either because their spraints were not collected or their spraint may not have been successfully typed. Further studies are needed to establish sampling procedures that can provide reliable estimates of the numbers of otters present at one time.

The distribution and movements of individuals within the Brue (intermediate) and the Itchen (fragmented) populations are very different. The data for the Itchen identifies at least five resident otters over 40 km of watercourse, higher than expected, while there was no evidence of resident females on the Brue. Longer term studies are needed to determine whether these observations are typical of these rivers or result from different environmental conditions or stages of recolonisation.

For all catchments there is a high percentage of animals found only once. Is there a substantial population of non-residents, which could provide an important source for recolonisation?

The project has demonstrated that surveying and monitoring otter populations using DNA analysis of spraint is feasible and has the potential to answer questions such as these. However, in order to fully exploit this technique, there is a need to identify more polymorphic loci for use in fingerprinting. In addition increasing the proportion of spraints that are successfully typed would greatly enhance its value. Detailed recommendations for a long term study are set out in Section 8.3.

6.1.5 Resource Requirements

Objective: To identify the resource needs, in terms of costs and time, and a robust protocol which could be repeated by anyone in the future if the method is considered feasible.

A protocol for DNA extraction and typing is presented in Appendix D. The field method and sample survey sheets are presented in Appendix E. Possible improvements on the laboratory protocol are recommended in Section 8.2. The costs of the project in terms of resources and mileage are summarised below.

Table 6.1 Resource Requirements per Sampling Event

Catchment	Itchen	Brue	Torridge	Tone
Average number of sites checked	71	28	9	59
Total miles (all surveyors)	167	450	57	120
Total number of hours (all surveyors)	25	28	4	18

The costs, based on mileage and time requirements, are dependent on the catchment characteristics. These may also change with any future improvements to sampling methods and success rate of analysis.

The feasibility study has produced a protocol for field sampling together with Health and Safety guidelines (see Appendix E) and background information on DNA Fingerprinting (see Appendix B).

6.2 Problems Encountered

Several problems were experienced during the feasibility study and these are discussed below. Recommendations to resolve these problems are presented in Section 8.0.

6.2.1 DNA Typing

Many of the spraint samples only gave partial DNA fingerprints. Not all loci developed and those loci that did develop may not have been reliable if the DNA extraction was incomplete. For example, 02 06 could actually have been 04 06. Where four out of the six loci developed it is probable that the results for those four loci were accurate. However, to ensure accuracy only those profiles with five or six loci developed have been included in the analysis of results.

Anal jelly was found to give a significantly higher success rate compared to spraint during analysis (Table 6.2) and should always be collected.

Table 6.2 Comparison of Success of Analysis of Spraint Compared to Anal Jelly

	Sample Size	Percent Identified
Jelly	61	34%
Spraint	269	17%

Table 6.3 Analysis of Effect of Time of Spraint Collection Against Success of Analysis

Time	Sample Size	Percent Identified
Before 07.00	31	13%
07.00-08.00	44	20%
08.00-09.00	74	27%
09.00-10.00	85	21%
10.00-11.00	57	18%
After 11.00	44	14%
Total	335	20%

There appears to be no significant difference between these time periods. It should be noted that there was high variability between the success rate of analysis between each batch of samples. Sample batches were too small to test whether there was any significant differences due to the time spraint was collected in the field within any given batch of samples. Insufficient data was collected to be able to look at the effects of temperature, rainfall and substrate under spraint when collected, on the success rate of analysis.

6.2.2 Identifying Individual Otters

For the Itchen catchment there is uncertainty as to whether any one DNA profile is specific to an individual otter. On 23 April 1998 a juvenile female (2.7 kg and 82cm total length) was found dead on the middle Itchen at SU 4772 2743. There is no reliable database correlating body weight or body length to age for wild animals. The age of this animal is therefore unknown but it is unlikely to have been more than 6 months old. Tissue samples from the dead animal generated the same DNA profile that had been developed from spraint collected during September 1997 and in June and July 1998, after the animal's death. The initial conclusion was that a mother and cub shared an identical genetic profile for the 6 loci analysed.

Following the analysis of the 168 tissue samples it was concluded that there was just enough genetic variability within the south west population for DNA extraction from spraint to provide reliable indications of individual otters based on the 6 loci typed. The Itchen population was not included in this initial analysis as there were too few tissue sample DNA profiles to be included in the statistical analysis. All spraint samples collected from the Itchen catchment have been identical at one locus, leaving only 5 loci for identification of individuals. This would appear to bring the genetic variability below the level at which individual otters can be reliably identified on the Itchen. Statistical analysis of the DNA profiles from spraint collected in Devon and Somerset indicate that individual profiles may be shared by full siblings but not parent/offspring or unrelated individuals using the current

version of the DNA typing system. However, two assumed siblings found in Devon gave two very distinct fingerprints (see Section 5.4).

6.2.3 Project Initiation

A longer than anticipated set up time for two of the volunteer groups was experienced because of the large number of individuals involved. For the River Brue catchment volunteers were asked to reorganise an existing survey programme and routine.

6.2.4 Training Volunteers

Finding and training enough volunteers where there is no existing group to carry out the monthly DNA spraint collections proved difficult in Devon. Loss of volunteers due to job changes and house moves over a long term study will always be a problem. There were also months when some volunteers had other commitments. This was particularly true during school and national holidays.

6.2.5 Fluctuating River Levels

For some rivers, the regular spraint sites were frequently under water or washed-out, preventing collection of spraint samples. For example the Torridge responds rapidly to rainfall with a rise in river levels and high flood peaks which meant that monitoring sites were washed out quite frequently. This resulted in a smaller data set for this catchment than anticipated.

7. CONCLUSIONS

The key conclusions from the project are summarised below:

- The Feasibility Study was an outstanding success. It answered many of the questions asked, achieved its objectives and identified ways in which the DNA Fingerprinting technique needed improving. The Study has provided a unique insight into otter biology in southern England.

- The 6 loci available for spraint analysis were not sufficiently variable to permit identification of individual otters on the Itchen where the genetic diversity of the population is low. The south west population appears to be on the borderline of variability required to successfully identify individuals.

- More loci need to be developed to confidently identify individual otters in populations with low genetic diversity. This will require renewed research effort and resources. The number of loci required will depend on the levels of polymorphism they exhibit but a total of fifteen would be sufficient at the levels found at the loci already used.

- Within the Itchen population two otters, assumed to be closely related, were found to share the same DNA profile for the 6 loci analysed. This implies that the total number of otters identified, at least on the Itchen, is a minimum. This also means that the estimates of home range may be over estimated being based perhaps on more than one individual. There was no evidence of similar duplication within the Brue, Tone or Torridge populations.

- Otter DNA was recovered from 20% of the spraints collected. A consistently higher success rate would greatly improve the effectiveness of this survey method. Staff at the University of Exeter are currently investigating this.

- The level of genetic variability in the UK otter population is such that it is probably not possible to determine the relatedness of individual otters using existing techniques.

- The genetic variability of the population to be surveyed should be checked by analysis of tissue samples prior to collection of spraint. DNA profiles of at least 10 otters are required to determine the suitability of a population for applying the technique to spraints.

- To be cost and resource effective the survey method requires the use of highly committed and motivated volunteers with individual training needs. A sampling protocol and proper equipment is necessary. Health and Safety is of paramount importance.

- The success rate of analysis was greater from anal jelly than spraint. Anal jelly should be collected as well as spraint wherever possible.

- Rapid analysis of spraint is required to enable a continuous review of any survey structure and allow the feedback of results to the volunteers.

- The physical characteristics of the rivers to be surveyed will influence the planning and frequency of surveys.

- The method is appropriate to long term monitoring but could also be used for an intensive survey/sampling programme to assess a population over a short time scale.

- Increasing the density of survey sites, as on the Itchen, does not provide significantly more information on otter movements, known home ranges etc directly. However, the associated increase in the number of spraint found does improve the database. Increasing the number of survey days should have the same effect.

- The period covered by survey should be extended to be able to confirm the information gained so far on individual otters known home ranges and the estimated total number of otters within each catchment. However, preliminary findings indicate very different distributions between the Brue, Tone and Torridge. The Itchen results are difficult to interpret due to the duplication of DNA profiles.

- Addressing the problems identified in the Feasibility Study will require new resources and research effort. Improvements to the technique will not only facilitate a longer term study but should also permit its development as a reliable standard tool for monitoring otter populations.

8. RECOMMENDATIONS

8.1 Project Planning and Management

8.1.1 Project Initiation

Prior to any decision to use DNA fingerprinting of spraint to survey a population it is advisable to establish the DNA baseline of that population through tissue analysis. This will be dependent on the availability of suitable material. DNA profiles from at least 10 carcasses are required to statistically confirm the suitability of the population.

8.1.2 Training Volunteers

Future studies will need to acknowledge the long set up period required for volunteer recruitment, training and deployment and to recognise the organisational time needed for new volunteers throughout the project. Two or three months is a realistic time scale from project start to commencement of fieldwork.

It is recommended to train 'surplus' surveyors, who can stand in, either on a short or long term basis when regular surveyors are unable to cover their usual sites. Where there is a shortage of volunteers, surveys of a river could be completed over several days. However, this puts a lot of pressure on individuals if they need to go out several days a month. Each volunteer's responsibilities should never be so onerous that they lose interest in the project. It is recommended that any individual's programme is readily achievable. The number of sites can always be increased as experience and enthusiasm grows.

In addition to training in field skills to identify and collect fresh otter spraint, volunteers should be provided with adequate health and safety instructions (Appendix E) and offered background information on DNA and the fingerprinting technique (Appendix B).

Surveyors should always be encouraged to work in pairs, for safety reasons and also for support. Covering expenses such as mileage and providing basic equipment such as boots or waders may enable volunteers with spare time but not financial resources to get involved in the project.

8.1.3 Fluctuating River Levels

Programme flexibility will be more important for some rivers than others and should be a consideration in the planning stage of a study. This can be difficult if volunteers are only available at the weekend. It is easier for people to fit surveys in with their other commitments if a specific weekend is chosen each month; for example, the Brue catchment was surveyed the second Sunday each month. The second Sunday avoided most Bank Holidays but coincided with high tides some months and the possible loss of some spraint in the tidal reaches of the Rivers. In some months no samples were collected due to high water levels following heavy rainfall. Reorganising survey dates at short notice to suit weather conditions may not be practical although it may be possible to identify those volunteers with flexibility at the beginning of the study. If monitoring sites are washed out on the specified survey day, these 'reserves' may be willing to go out as soon as water levels drop. These issues should be discussed with individual volunteers as they are introduced to the project.

8.1.4 Feedback of Results

Any survey or project of this type is impossible without highly motivated volunteers. It must be recognised that volunteers are being asked to make a big commitment in terms of time and effort. Early starts, in often far from ideal weather conditions is a lot to ask anyone. Frequent feedback of results is very important in maintaining interest.

The use of a regular newsletter with results and contributions from volunteers is recommended to maintain a regular feedback of results to maintain volunteer interest and involvement. Annual or more frequent meetings of a more social nature will strengthen the team allowing people to share stories and even complaints. These can be very informal social gatherings, a thank you to everyone for their hard work, and can usefully incorporate presentations of the results showing everyone what their hard work has achieved on a wider scale. For example, the Hampshire team had a rounders match followed by a BBQ for the volunteers. The Somerset results were presented to the SOG at their AGM followed by an informal discussion during a buffet supper. One request from the volunteers at these meetings was that they would like the opportunity to go out on the surveys of the other catchments.

8.1.5 Publicity

Publicity was also found very useful in gaining external support for the project and in maintaining the enthusiasm of those involved. A press release prepared and issued by the Project Board in April 1998 raised strong interest in the project with good coverage in local and national papers and several news items on television and radio.

To a certain extent this level of coverage reflects the universal appeal of the otter but it also acknowledges the importance of the application of a new method of surveying otters. Currently there is high level of interest in the use of DNA for surveying different species, and the potential of the application of DNA fingerprinting to otter conservation is no exception. The project was well represented by a feature on BBC TV Tomorrow's World in September 1998. As a result of the publicity, a number of enquiries have been received about the Project from students at various UK universities and from other Government organisations interested in learning more about the DNA fingerprinting technique.

There has generally been good support for the otter surveys from the public. Frequent questions are asked when out surveying. Most landowners approached for permission for access have given enthusiastic support. Any concerns or suspicion as to what you are up to typically changes to support once otters are mentioned, together with requests for information as the project progresses. It may be helpful if a small pamphlet/news sheet on the project was produced for distribution to volunteers, landowners and general public during the next phase of the project. This may also help to encourage sponsorship of the project.

8.2 DNA Typing

8.2.1 Spraint Collection Methods

There is a significant difference between the success rate of DNA extraction and typing from anal jelly compared to spraint and jelly should therefore always be collected for analysis.

It is recommend that, once the success rate of laboratory analysis becomes more consistent, the effect of time of collection of spraint should be assessed. It may be appropriate to record

time spraint collected after sunset previous day (assuming otters only active after dark) to see if there is any correlation with success of analysis. The effects of overnight temperatures and weather conditions should also be assessed.

8.2.2 Success Rates of DNA Profile Development

The following recommendations have been made to improve the success rate of profile development where incomplete DNA Profiles develop.

The detection method used for DNA typing of microsatellites and SRY (sex chromosome) should be changed from radioactivity and ethidium bromide, respectively, to fluorescence detection on an ABI automated DNA sequencer. This should increase the detection rate of low amounts of PCR product undetectable using radioactivity. If this was used in conjunction with multiplex PCR, this change will also lead to labour savings and more efficient use of limited amounts of spraint DNA.

The number of replicate typings per microsatellite locus/sample should be increased to eight in cases where four replicates have given a homozygous genotype. This will increase the confidence in such designations, because they can also arise from the dropout on one allele in a true heterozygote. Four replicates are sufficient for the SRY marker.

Improving the success rate of DNA typing of samples and simplifying the collection and extraction methods would reduce the costs and increase the effectiveness of the technique.

8.2.3 Differentiating Between First Order Relatives

Increasing the number of loci assessed will increase the reliability of identification of individual otters. It has been estimated that, at the levels of polymorphism found in south west England, 15 loci would be required to reach the same level of confidence in identification as has been found for otters in Scotland (J. Dallas, unpublished data). If new loci have a greater degree of polymorphism, fewer will suffice. Fewer loci are required where there is high genetic variability within a population. In inbred populations with very low genetic diversity derived from captive releases it may not prove possible to use this method. Staff at the University of Exeter are addressing this problem by investigating alternative microsatellite sequences.

8.3 Further Research

8.3.1 Proposal

A detailed proposal is presented in Appendix F. To maintain continuity in the data set, in anticipation of the long-term study, the four catchments are still being surveyed, with spraint samples now being stored at the University of Exeter using the protocol developed by John Dallas.

8.3.2 The Otter BAP

The Otter BAP identified two 'Objectives and Targets':

- Maintain and expand existing otter populations.

- By 2010, restore breeding otters to all catchments and coast areas where they have been recorded since 1960.

In order to achieve these, it recommended a series of proposed actions under the headings: Policy and legislation, Site safeguard and management, Species management and protection, Advisory, Future research and monitoring, Communications and publicity.

The proposals for a further three year study mainly addresses issues identified under Future research and monitoring, Site safeguard and management and Species management and protection.

The general aims of the proposed three year study are:

- To develop the technique of DNA fingerprinting otter spraint so that it can become a standard tool for monitoring and studying otter populations.

- To extend the successful pilot project into a further three year study in order to provide information required to ensure that the Otter BAP Objectives are achieved, particularly in terms of successful monitoring and management of otter populations.

- To improve our understanding of otter ecology and provide data for modelling and/or a PHVA (Population and Habitat Viability Analysis) in order to assess the likelihood of the BAP objectives being achieved by natural recolonisation.

- To work closely with other projects where DNA fingerprinting would contribute significantly to the management and conservation of otter populations.

8.3.3 Specific Objectives

Note: the bracketed references (BAP: n.n.n) following each objective below refer to the relevant paragraph(s) in the Biodiversity Action Plan for Otters which is attached as Appendix A.

8.3.3.1 Development of the technique

- To improve the reliability of the technique by increasing the number of genetic loci which can be fingerprinted, thereby reducing the risk of misidentifying otters. A target of 15 polymorphic loci is proposed.

- To improve techniques for collecting and extracting otter DNA from spraints in order to simplify field collection, reduce the costs of extraction and storage and, if possible, increase the number of spraints which can be successfully typed. A target of typing 33% of very fresh spraints is proposed.

8.3.3.2 Monitoring and Modelling

- To determine whether a relationship exists between the standard otter survey method (Strachan *et al.*, 1990) and the results of DNA fingerprinting (monitoring at the national, catchment or county level). (BAP: 5.5.4; 5.5.6)

- To devise a protocol for using DNA fingerprinting as a means of assessing the size and nature of local otter populations (monitoring at the local level). (BAP: 5.5.6)

- To provide information on otter movements, home ranges, population structure for modelling populations. (BAP: 5.5.4)

8.3.3.3 Factors Affecting Recolonisation

- To assess the relationship between habitat quality (including food supply) and otter populations (BAP: 5.2.1, 5.2.2, 5.5.1)
- To assess the impact of disturbance on otter populations (BAP: 5.5.3)
- To assess the impact of riparian management (BAP: 5.2.1, 5.2.2)

8.3.3.4 Relationships with Other Projects

To collaborate with other projects on otters in southern Britain where information on otter identity and genetics would be of value, including:

- the release of otters from captivity;
- proposed studies on breeding;
- the collection of road casualties and other otter corpses.

8.3.4 Approach

It is proposed to develop the technique and extend the programme for a further three years using the same basic approach as in the pilot study but the work will be co-ordinated by the University of Exeter. Work on development of the technique and the extraction and fingerprinting techniques will be undertaken in the laboratory of Professor John Bryant by a full time research assistant and technician. The field work will be undertaken by a second research assistant, also based in the School of Biological Sciences at the University of Exeter, under the supervision of Dr Paul Chanin. The network of volunteers will be maintained and extended and it will be part of the research assistant's responsibility to recruit, train and co-ordinate the efforts of volunteers.

The existing study sites will be maintained if possible to enable us to follow the otter populations in them over a period of at least four years. It is intended to recruit and train more volunteers to work on the Torridge and set up one or more additional sites.

The detailed proposal on how these objectives will be met is set out in Appendix F.

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10. GLOSSARY

Allele	Common shortening of the term allelomorph. One of two or more forms of a gene that arise by mutation and occupy the same locus on homologous chromosomes.
Chromosome	A DNA-histone protein thread occurring in the nucleus of the cell. Chromosomes occur in pairs that associate in a particular way during cell division.
DNA	Deoxyribonucleic acid (see Appendix B)
Fingerprint	Genetic profile specific to individual otter derived from DNA extracted from the spraint or tissue sample.
Gene	The fundamental unit of inheritance, comprising a segment of DNA that codes for one or several functions and occupies a fixed position (locus) on a chromosome
Genetic diversity	Differences between genetic material between individuals and within population in general
Habitat	Place with particular kind of environment inhabited by organism(s)
Holt	Enclosed Otter den or resting site
Homologous chromosomes	Chromosomes which contain identical sets of loci. Can be applied to parts of chromosomes
Home range	The area within which an animal normally lives
Locus	The specific place on a chromosome where a gene is located (pl loci)
Microsatellite	Sequence of repeats of base pairs of amino acids
Polymorphism	Having several different forms
Primer	Sequence of DNA on either side of the micro-satellite. Each satellite has its own, characteristic primer sequence
Ramsar	Wetlands designated under the Convention of Wetlands of International Importance, especially as Wildfowl Habitat (Ramsar, Iran) 1971
Resident/non-resident	Otter profiles only found once are assumed to indicate non-resident individuals for the purposes of this study
Spraint	Otter faeces
Transient	Non-resident animal
Type/typing	Specific identification of fingerprint produced by DNA analysis

APPENDIX A: THE BIODIVERSITY ACTION PLAN FOR OTTERS EXTRACTED FROM BIODIVERSITY: THE UK STEERING GROUP REPORT

OTTER (*Lutra lutra*)

1. CURRENT STATUS

1.1 Formerly widespread throughout the UK, the otter underwent a rapid decline in numbers from the 1950s to 1970s and was effectively lost from midland and south-eastern counties of England by the 1980s. Populations remain in Wales, south-west England and much of Scotland, where sea loch and coastal colonies comprise one of the largest populations in Europe. There is also a significant population of otters in Northern Ireland. The decline now appears to have halted and sightings are being reported in former habitats.

1.2 The otter is listed on Appendix I of CITES. Appendix 11 of the Bern Convention and Annexes 11 and IV of the Habitats Directive. It is protected under Schedule 5 of the WCA 1981 and Schedule 2 of the Conservation (Natural Habitats, etc.) Regulations, 1994 (Regulation 38).The European sub-species is also listed as globally threatened on the IUCN/WCMC RDL.

2. CURRENT FACTORS CAUSING LOSS OR DECLINE

2.1 Pollution of watercourses, especially by PCBs.

2.2 Insufficient prey associated with poor water quality.

2.3 Impoverished bankside habitat features needed for breeding and resting.

2.4 Incidental mortality, primarily by road deaths and drowning in eel traps.

3. CURRENT ACTION

3.1 The JNCC has prepared a Framework for Otter Conservation in the UK 1995-2000.

3.2 National surveys have been conducted every five to seven years. Local surveys by Wildlife Trusts and other organisations have established the present distribution and potential for future spread in many areas.

3.3 Research is in progress on the implications of heavy metal and PCB contamination in fish and ecosystems.

3.4 Conservation management (for example creating log piles and artificial holts, and designation of "otter havens") has proved successful in many river catchments.

3.5 The Habitat Scheme Water Fringe Option administered by MAFF is being used to manage waterside habitat in six pilot areas. MAFF also provides advice on creating otter havens on set-aside.

3.6 FA and FE promote sensitive woodland management and expansion to favour otters, through preparation and implementation of their Forest and Water Guidelines, e.g.

managing riparian areas with deciduous trees and shrubs mixed with open grassland and wetland habitat, and the prevention of sediments and other pollution.

3.7 Two SACs have been proposed for this species under the EC Habitats Directive.

4. ACTION PLAN OBJECTIVES AND TARGETS

4.1 Maintain and expand existing otter populations.

4.2 By 2010, restore breeding otters to all catchments and coast areas where they have been recorded since 1960.

5. PROPOSED ACTION WITH LEAD AGENCIES

5. 1 Policy and legislation

5.1.1 Seek to secure agreement on the UK Framework for Otter Conservation. (ACTION: JNCC)

5.1.2 Seek to ensure management agreements and incentive schemes (e.g.: ESAs, Countryside Stewardship and Tir Cymen) take account of the requirements of otters in occupied areas. (ACTION: DANI, MAFF, SOAEFD, WOAD)

5.1.3 Seek to determine by 2000 Statutory Water Quality Objectives for standing and running waters in Britain which will sustain otters. (ACTION: DoE, EA, OFWAT, SEPA, SOAEFD, Water Services Association, WO)

5.1.4 Review the protection afforded to otters by current legislation and investigate the usefulness and appropriateness of licensing to control release of otters. (ACTION: CCW, DoE, EN, JNCC, SNH)

5.1.5 Identify and resolve problems with existing legislation. Seek to clarify the definition of "trap" in the WCA 1981 and resolve inconsistencies over the use of otter guards on fish traps. (ACTION: CCW, DoE, EN, JNCC, SNH)

5.2 Site safeguard and management

5.2.1 Seek to include action for otters in Catchment Management Plans for all rivers containing otter populations by 2000, including "otter havens" in relevant areas. (ACTION: DANI, EA, SEPA, MAFF, WOAD)

5.2.2 Continue to secure appropriate management of riparian habitats and catchments in woodlands to maintain or enhance otter populations. (ACTION: FA)

5.3 Species management and protection

5.3.1 Seek to establish an "Otter Forum" to co-ordinate conservation, information exchange, publicity and research. (ACTION: JNCC)

5.3.2 Ensure otter releases are carried out only under the guidelines set out in the Framework for Otter Conservation. (ACTION: CCW, DoE(NI), EN, SNH, JNCC)

5.3.3 Attempt to limit accidental killing or injury (for example by provision of road underpasses and fyke net guards), particularly on key catchments. (ACTION: DoE(NI), DOT, LAs, EA, SEPA).

5.4 Advisory

5.4.1 Ensure the provision of information on otter requirements to key groups, to include land owners, through the publication of posters and guidelines. (ACTION: CCW, DoE(NI), EN, FA, SNH)

5.5 Future research and monitoring

5.5.1 Collate information on prey productivity, biomass and pollution in occupied and re-colonisation areas (ACTION: DANI, DoE(NI), ITE, JNCC, EA, SEPA, SOAEFD, WOAD)

5.5.2 Develop a standard methodology to analyse the level of pollution accumulation in otters. (ACTION: DANI, DoE(NI), JNCC, EA, SEPA, SOAEFD, WOAD)

5.5.3 Investigate the effects of disturbance on otter populations. (ACTION: DoE(NI), JNCC, EA, SEPA)

5.5.4 Develop and implement means to estimate otter numbers and permit population modelling. (ACTION: DoE(NI), JNCC, EA, SEPA)

5.5.5 Monitor populations and distribution of otters throughout the UK, including local survey to monitor the expansion of fringe populations. (ACTION: JNCC)

5.5.6 Pass information gathered during survey and monitoring of this species to JNCC in order that it can be incorporated in a national database and contribute to the maintenance of an up-to-date Red List. (ACTION: CCW, DoE(NI), EN, SNH)

5.6 Communications and publicity

5.6.1 Use this popular species to publicise the importance of water quality and riparian habitats to biodiversity. (ACTION: CCW, DoE(NI), EN, FA, JNCC, SNH, EA, SEPA)

APPENDIX B: INTRODUCTION TO DNA AND OTTER DNA FINGERPRINTING

or 'How a spraint in the hand...' **Dr Kathy Sykes**

B 1.1 The Importance Of Otter Spraints

Spraints are very useful to otters, they help them to 'communicate' with each other. The faecal matter carries chemicals that actually give signals to other otters. For example, an otter might be able to find out whose territory he or she is on just by the smell of a spraint. A frisky male otter may be able to work out whether a female is ready to mate. But otter spraints can be very useful to us too. Indeed, for the last 20 or so years, people have been collecting them to find out more about otter populations. The majority of what we know about otters, their local and national distribution and status, has been gleaned by surveys searching for otter spraint. The finding of a spraint is often the first indication that an otter is around, sometimes after absences of 20 years.

You may ask why use spraint to try to find out about otter populations? Otters are by nature nocturnal and secretive which makes them notoriously hard to study. Smaller animals can easily be trapped and either marked or given a radio tag. But it's expensive doing this with otters, and trapping them is difficult and may change their behaviour. Collecting spraints is cheap and effective, and it doesn't involve interfering with the animals.

However, until recently, a spraint has only been able to tell us a few bits of information: that an otter has been in a particular area; its diet. But in fact, a fresh spraint in your hand holds a whole gold-mine of information about the otter, for example, its gender and clues about its 'identity'. The information is tightly locked up, and needs some clever scientific tricks to unleash it. But we've only learned how to do this recently, with the advent of **'DNA fingerprinting'**. In order to find out how it works, we need to dive into an otter spraint, and take a *much* closer look.

But before we do that, you may be wondering '*why* do we want to know so much about otters?' Well, the more we can learn about otter populations and understand their behaviour, the better position we are in to help them to re-colonise areas and to make sure that otter populations are healthy. In section **B 1.6** there is more explanation about what we can find out by doing otter DNA fingerprinting.

In this appendix, after you've 'dived' into an otter spraint, section **B1.3** will explain the things you found there: the chromosomes, genes and DNA. The following section **B1.4** explains how you can do fingerprinting and how to use it for otters. Section **B1.5** describes the lab and field techniques and how you can interpret the results, so you'll be able to understand all the long lists of numbers in the tables of the report. Finally, the last section explains why we're doing this project and what the feasibility study has shown. It also takes a sneak view at what we'd like to do next.

B 1.2 Taking a dive into an otter spraint

Imagine a fresh otter spraint in your hand. Take a sniff. It has a sweet musky odour, a little reminiscent of jasmine tea or hay, with a dash of fish. Now imagine you're the size of your fingernail. Get ready: you're about to dive into the spraint…

You start your journey by plunging into some soft squelchy stuff. It's hard to tell what it is, but you quickly feel something hard and sharp jutting out. It's like a skeleton. You realise that it *is* part of a skeleton: it's a pile of broken fish bones. You also find some giant shield-like things almost as big as you: they are undigested fish scales. You realise that the soft stuff is actually some mucus from the otter, maybe it's carrying some of those chemicals that aid communication with other otters? You wonder what messages it might be carrying.

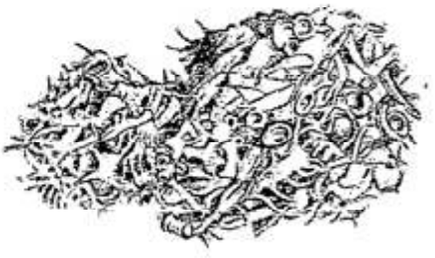


Figure 1 An otter spraint

Now it's time to shrink down again. This time you're about the size of the full stop at the end of this sentence. You're surrounded by blobs, like balloons filled with liquid. You grab some and find that they are cells, and that there are a great variety of them. Some are bacterial cells, presumably from the inside of the otter's gut. Others are fish cells, remaining after not being quite fully digested. Otter spraint are a rich broth of fish remains and bacteria. But there are some cells from the otter itself, probably ripped off the otter's gut wall by some of those sharp fish-bones. You also find that the squelchy mucus seems to contain lots of the otter cells.

Now, it's time to zoom into one of those cells, so you'll need to shrink again. You're already the size of a full stop, but imagine having to shrink down by the same amount *again*. Now you're over a million times smaller than you are as you sit reading these words, and you're small enough to plunge into a cell.

You just have to wriggle a little to get through the cell's outer membrane, which feels a little oily as you slide through. Once inside, the most striking thing is the nucleus, right at the centre. The nucleus has its own membrane wall to wriggle through. Once inside, you can feel very fine strands of material, that seem to be in a mad tangle, rather like a bowl of spaghetti. It's too fine to be able to see, but if you could, you'd realise that each is a spiral. Each strand is a *chromosome*, and the stuff in your hand is *DNA*, the 'Spiral of Life'.

In every nucleus of every cell, **all** the information needed to create a particular animal is locked up. The DNA, existing in long tangled strands, holds a 'recipe' with instructions about how to make up the creature. Even a single nucleus from a single cell from the otter's intestine holds information about all the other parts of the body; for instance, it holds the information that an eye cell would have needed to become an eye. It's utterly mind-boggling. This is what makes it possible to 'clone' a new creature from just a single cell of the 'parent'. And all this information is held in these lovely fine strands of material in your hand.

So, by diving into an otter's spraint you have found handfuls of cells from the otter itself which contain nuclei which have these silky spiral strands of DNA. These should enable you to unlock masses of information about the otter. All you'd have to do is:

(1) increase the quantity of DNA from the cells (since the DNA from a handful of cells is barely enough to detect);
(2) find some way of unlocking the information in the cells to be able to differentiate between otters. It should be possible, shouldn't it, as every creature's DNA is unique?

The two tasks posed above will be dealt with in the next two sections. But first we'll have a closer look at chromosomes, genes, and DNA.

B 1.3 Chromosomes, Genes, and DNA

Chromosomes

Almost all human cells and otter cells have nuclei which contain chromosomes. Within any species, the nuclei from different animals all contain the same number of chromosomes. It is possible to identify and number each chromosome. So each species of plant and animal has a characteristic number of chromosomes. In humans, there are 23 pairs of chromosomes in each nucleus, making up 46 in total. Each one is a strand of DNA.

When chromosomes are preparing to reproduce themselves, they untangle from each other and ravel up on themselves, spiraling on top of the existing spirals, to form the neat shapes that are usually shown in pictures (e.g. see opposite). When in their natural state, all tangled up, they are very difficult to see under a microscope.

Figure 2 Human chromosomes

Genes

'Genes' are what we usually refer to when we talk about how we inherit traits from our parents. A gene is just a region of DNA that is able to specify the structure of one protein molecule. Each chromosome contains many genes and each gene is found in a characteristic place on a particular chromosome.

DNA

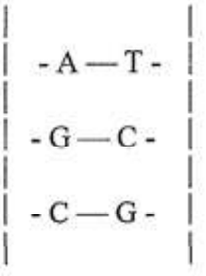
The initials DNA stand for **D**e-oxy-ribo-**N**ucleic **A**cid. Outside the cell, it looks like a tangled mass of string. Our own cells each contain about 2 meters of DNA, specially packaged to fit inside. It can be bent, wrapped, looped, twisted and even tied in knots. But in spite of its apparent flexibility, it has a very rigid way of storing information. The same information content must pass from generation to generation with little change. And it stores enormous amounts of information: all the 'instructions' needed to make an organism.

The incredible thing is that just about **all** life on Earth is built using the information contained in DNA. From lichens in the Arctic tundra to blue whales in the Pacific, from bacteria in the gut of

an elephant to the flowers in your garden: all of it is based on DNA. Us included. And all DNA exists in the same helical structure, based on just four simple building blocks.

Think of DNA as a ladder laid out on the ground. The 'legs' of the ladder consist of a long chain of molecules (including a sugar) which form a 'backbone' which supports the ladder's rungs and spaces them out. The rungs are the important bits and they consist of molecules called *bases*'.

There are just four bases in DNA and they come in pairs: one type of rung consists of the two bases *Adenosine* and *Thymine* linked together, and the other type of rung has the two bases *Cytosine* and *Guanine* linked together. Each rung is called a *base pair*. Usually they are shown as their initials A & T and C & G. It turns out that A can only ever link to T, and C only links to G, but they can link in any order, either C-G or G-C. The ladder is an incredibly long one: millions of base pairs rungs make up a strand of DNA.



Note: A, T, C and G are quite simple molecules, with about 15 atoms (including atoms of carbon, oxygen, nitrogen, and hydrogen).

DNA is normally coiled up and forms a 'Double Helix'. Imagine taking a small flexible ladder in your hands and then twisting the ends in opposite directions. It would coil up into a simple helix like a two stranded piece of rope or string - the double helix:

So, DNA codes the genetic information which makes up our genes and it is usually organised into chromosome structures, described in the sections above. It's just staggering that such a simple system can carry all the information need to make a living thing. Even more staggering is to wonder how it could possibly have formed in the first place. Was it by chance, accidentally forming from the slush of molecules in a primordial soup? Or from outer space, riding in on a meteorite? Scientists speculate, but we really don't know.

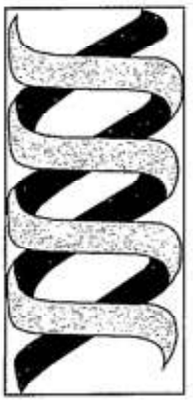


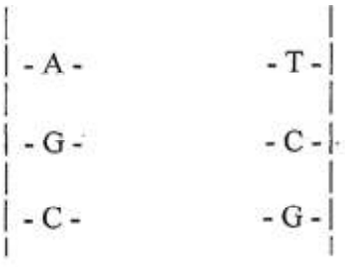
Figure 3 Simplified piece of DNA molecule with two spiralled strands but no 'rungs'

In 1953 in Cambridge James Watson and Francis Crick worked out how the base pairs of DNA could carry instructions. In a marvelous bit of understatement they 'mentioned' in their Nobel prize-winning paper, 'It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material'.

The other crucial thing about DNA, in addition to it being able to store so much information, is that it can copy information with amazing accuracy so that it can be passed on to another generation. The next section describes how this copying happens.

B1.4 Copying DNA - 'How to get lots of otter DNA from the tiny amount in spraints'

We can look at how nature copies DNA and learn how to mimic the process to increase the amounts of DNA we can get from otter spraints. Imagine taking the twisted ladder, standing on the top rung and jumping hard. Imagine that the top rung, and every consecutive rung, breaks in the middle. You would be left with two separate legs of the ladder, each with half rungs sticking out. In fact, this is like the way that DNA splits into two strands. The strands then each consist of the backbone and one half of each base pair sticking out. Each broken rung is attractive to particular bases e.g. T will be attractive to the A rung. So all you need to do to 'grow' more DNA is to add a mixture of all the 4 possible bases (each with a sugar attached), and wait for them to do the work of attaching to the right broken rung of the ladder. The bases will link to the two half strands of DNA, one by one, and rebuild them into two complete double strands. Because A only binds with T, and C with G, the final result is two identical copies of the original DNA. This is how, in nature, one cell divides into two daughter cells, each of which has an identical copy parental DNA.



Incredibly, the DNA copying process that occurs in cells, we are now able to mimic - in test tubes. The ladder can be ripped apart by heating. A mixture of bases, each with a sugar attached, needs to be added along with an enzyme (*DNA polymerase)*. This procedure increases the amount of DNA by making copies. It is usually repeated time after time, doubling the amount of DNA every cycle, until there are considerable quantities.

This process is called *'Polymerase Chain Reaction'*, or PCR for short. And it is the perfect tool for getting our tiny amount of nucleic material that we found in the otter spraint into a manageable amount that we can use for other things... such as 'fingerprinting'.

B1.5 How do we use DNA for 'Fingerprinting'? And how does it work for otters?

In the early 1980s Professor Alec Jeffreys at Leicester University discovered a bizarre thing that eventually gave us the key to how to use DNA to distinguish between different animals. While studying a particular gene, Jeffreys found sequences of bases that made sense, but in amongst the sequences, there were lengths that seemed to have no meaning at all.

Imagine having a conversation with someone, and they suddenly started saying 'baaa baaa baaa baaa baaa baaa', then went back to talking to you normally. It was a bit like that.

Alec Jeffreys found that there were sequences of 10-15 base pairs that just repeated several times over: gobble-de-gook, that would not produce anything recognisable. He described these bizarre sequences as *'mini-satellites'*. It soon became clear that other genes also had mini-satellites. The

exact nature of the satellite changed from gene to gene. Later people found that that there are even shorter sequences of repeats, known as *'micro-satellites'*, which have less than ten repeats. Here is an example of a mini satellite:

TC TACGT***CACACACACACACACACACACA***CTC GTACG
AGATGCA***GTGTGTGTGTGTGTGTGTGTGT***GAGCATGC

Each mini or micro-satellite is found at a different place on a particular chromosome and is described as being at a particular locus (plural - loci).

But why should there be sequences of complete nonsense in DNA? No one yet really understands what they are for, or how they arose. But even though we don't understand mini- and micro-satellites, we're able to make use of them: they have a feature which gives us the key to DNA fingerprinting.

From one person to another, or from one otter to another, the number of repeats of the sequence in any one satellite can vary. In fact, it varies from one chromosome to another. Just as you might have a gene for blood group A from your father and O from your mother, you might have 10 repeats of ***CA*** in the micro-satellite in a particular gene from your mother and 8 repeats in the corresponding gene from your father.

These variants are called *alleles*. You need to have a reasonable quantity of alleles to be able to distinguish individuals. Most satellites have relatively small numbers of alleles and so a single satellite can not be used to distinguish individuals. But if you can examine several satellites, it is possible to discriminate between individuals with more certainty. Even matching up parents and offspring. However, it is generally easier to be certain that an otter is *not* the parent of an offspring, than to be sure that it *is* the parent.

It may seem ironic that, with the huge amounts of information being held in chromosomes, it is the lengths of chromosome that appear to be nonsense that lets us find a way to distinguish between animals. But the lengths of sequence that *do* make sense are very complex and are still being identified (eg you may have heard of the Human Genome project in the media, which is trying to map all the genes for humans). It is the very simplicity of the strings of repeats that makes them easy to identify and 'measure'.

How do we do DNA Fingerprinting on Otter Spraints?
It's necessary to do some detective work with the mini and micro-satellites. Scientists need to find ways of using them to distinguish between animals.

The first step in carrying out DNA fingerprinting is to find suitable satellites for analysis that can help with the detective work. The micro-satellites in the otter DNA need to be quite different from the satellites in any other DNA that may be present (from say the fish cells, or bacterial cells). John Dallas of Aberdeen University has worked with nine micro-satellites found in otter chromosomes that have not been found in any of the other chromosomes. However, only six of these have proved reliable in studies of spraint DNA. These micro-satellites have a number of

alleles. John Dallas has also found a micro-satellite which only occurs in males (it is found on the otter's Y chromosome). This is important: it means that the otter's gender can be established.

Another important factor to check is whether the otters in the population of interest show sufficient diversity in the satellites. If the satellites are similar in a population, the fingerprints of different otters may look too similar to be distinguishable. This was one of the aspects that needed assessing in the feasibility study.

B1.6 Field and Laboratory Techniques

Field Techniques

Spraints need to be collected as fresh as possible. That means, as early in the morning as possible. Ideally they should be less than 12 hours old. On summer mornings, if it starts to get hot, you should try to collect them even earlier. Why? DNA degrades very easily. Many things, including heat, light, enzymes and bacteria, can break it down. DNA in otter spraints has already survived some of the journey through the otter's gut, where it will have been attacked by various enzymes and bacteria. Once deposited in the environment, the degradation continues: it is subjected to atmospheric conditions, including ultra-violet light, more bacteria, fungi, heat and also dehydration.

As time goes on and breakdown proceeds, fewer and fewer micro-satellites can be detected. Sometimes incomplete DNA profiles are still useful, but it's usually only possible to show that an animal is *not* a particular individual, rather than show that it *is* one. Fortunately, the breakdown of DNA can be slowed down once the spraint has been collected, by storing it in a freezer and using chemicals to preserve it.

We have also found that spraints with lots of sticky mucus ('anal jelly') are much more likely to provide useable DNA than those without. The success rate for identifying the otter is 34% when anal jelly is present, compared with 17% when it is not there. This might be because the mucus protects the cells and DNA from breakdown. So collect as much anal jelly as you can!

Figure 5 Anal jelly find

Laboratory Techniques.

The first problem back in the laboratory, is that the spraint not only contains otter DNA but also that of other organisms such as prey and gut bacteria as well as a mass of other components. Standard techniques are used to separate DNA from other material and these are used to extract all the DNA from the spraint sample.

It is now necessary to isolate the otter DNA from all the other DNA. This is achieved by gently heating the mixture so that the DNA unwinds and separates into two strands (this is described in **B1.3**), then adding 'primers' to the mixture. Primers are short bits of DNA which have been identified as binding to each end of a particular satellite. They will only bind to appropriate sites on the otter's DNA, not to bacterial or prey DNA. The primers thus mark out the relevant

satellites. Developing these primers specifically suitable for otter DNA is difficult and expensive but it is the key to making the technique viable.

The next stage is to greatly increase the quantity of otter DNA. From section **B1.3** we know that we can just use Polymerase Chain Reaction (PCR) to do this.

At this stage the DNA solution is placed on a jelly-like substance (a gel called 'agar' which is actually made from seaweed) which has an electric current running through it. Because DNA has a negative charge it will tend to move towards the positively charged electrode.
Since larger fragments of DNA move more slowly than small ones, after a period of time, different micro-satellite alleles (which by definition are of different sizes) will move different distances through the gel. The small fragments will cover greater distances than larger ones. By measuring the distance travelled by each fragment and comparing it with distances travelled by known alleles, you can work out which alleles are found in each animal. The method is known as *Electrophoresis*.

Interpretation
The end result of this process is a picture with a series of bands representing the alleles of the micro-satellites being investigated. Three examples are shown. Appropriately, they look a little like bar-codes, and after all, DNA is rather like a 'bar-code' for life.

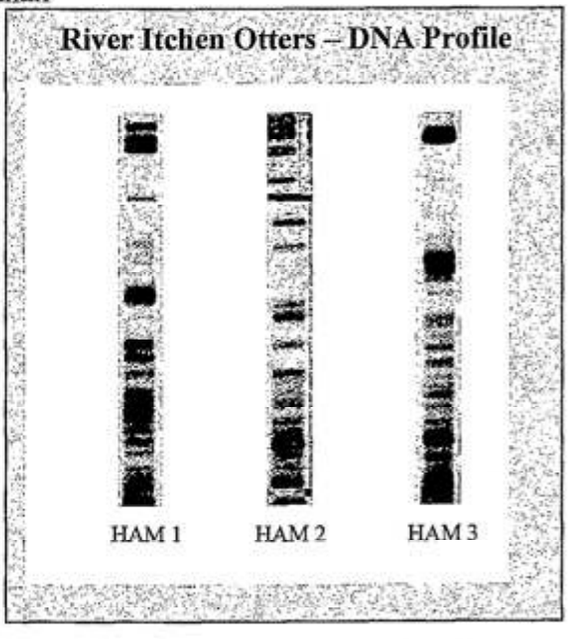


Figure 6 Three electrophoresis gels. Each black bar shows the distance that one of the micro-satellites has travelled.

The following table shows how results are recorded. Results are fed into a computer program which compares new samples with all the previous ones.

Otter No.	701	715	717	832	833	902	Sex
H01M	04 05	04 04	06 06	02 03	02 04	01 07	M
D07F	04 04	04 06	01 05	05 05	02 05	02 02	F
S12F	04 04	06 07	05 05	05 05	05 05	02 06	F
S16M	04 04	04 07	05 05	05 06	02 02	02 02	M
S20F	X	04 06	01 05	05 05	02 05	02 06	F

Most of these are real animals and the codes show that one comes from Hampshire (H01M), one from Devon and three from Somerset. The figures in the top row refer to the loci being investigated and the numbers in the body of the table show which two alleles at each locus were found in each otter. Note that there are always two numbers given as there are two alleles at each

locus (one from each of the otter's parents). Also note that the X for the Somerset female, number 20, at locus 701, means that the bands on the electrophoresis gel were not clear, and it wasn't possible to measure the position. This might be due to the spraint not being quite fresh enough.

From this table we can see that H01M and D07F cannot be closely related (parent and offspring or siblings) because they have no alleles in common for loci 717, 832 or 902. On the other hand, D07F and S20F are very similar, differing in only one allele, at only one locus, 902. They could be sisters or mother and daughter, but with a relatively small number of loci and alleles, and with locus 701 missing completely for one animal, this wouldn't be safe to assume (especially as they come from different counties).

B1.7 So why are we doing otter fingerprinting?

The information that might be gleaned from DNA fingerprinting is of three types:

a) the nature of the population e.g. sex ratios;
b) behaviour e.g. movements, extent of range overlap;
c) genetic e.g. how genetically similar otters in a population are.

As mentioned at the start of this appendix, the more we can learn about otter populations and understand their behaviour (a and b), the better position we are in to help them to re-colonise areas and to make sure that otter populations don't decline again. For example, it is useful to know what sort of otters first recolonise as they move back into river catchments which they formerly occupied. Do males arrive before females and do they behave differently? Do they establish home ranges or travel extensively? If they establish ranges to what extent do they overlap? Where otters do become resident, how long do they stay? We would also like to know how many otters there are in a particular area and hope to obtain information on this by, over a period of time, identifying all the resident otters in each study area. We may be able to find all this information by using DNA fingerprinting.

Surprisingly little is known about otter ecology in southern England. Scottish sea-based populations have been studied, but as they haven't undergone large-scale re-colonisation, much of this information is not relevant.

One reason for studying otters in three different counties is so that we can compare the populations. They are interesting because of their differences: otters in Devon have been well-established for some time; the Somerset population seems to be the current 'front' of recolonisation; and Hampshire has a small population of otters, which seem to be almost in isolation from otters in the south-west.

The genetic information is also useful in its own right. When the otter population declined in the 1960s there was a 'genetic bottleneck' in England. A 'genetic bottleneck' is where a population has been derived from so few individuals that they are all very closely related and genetically very similar. In evolutionary terms this is not ideal: genetic diversity is desirable for the long-term survival of the species. So, it is useful to be able to establish and compare the diversity of the otter populations in England and Scotland. Indeed, various otter conservation strategies in the UK state the need to assess the genetic variation within and between otter populations in different parts of the UK (eg JNCC 1996).

What have we learned from the present study?
The project was a feasibility study, with over fifty volunteers working closely with professional biologists, helping to collect the spraints on a regional basis. The aim was to:

- test whether DNA fingerprinting could be used to identify otters using spraint;
- see if it was practical for volunteers to collect otter spraints of suitable freshness and quantity to make further studies viable;
- set up standard techniques that could be used in future studies.

The project has been a great success. We have demonstrated that we can use the technique in the field, and that although the otter populations are in some cases rather similar, they can in many cases be distinguished. The volunteers have done a fabulous job, and have shown that a larger scale project is viable. Standard techniques have been set up, and some improvements in approach identified. And we have already discovered some useful things about the otter populations; for example, we have learned that the River Brue population seemed to have eight males and only three females during the study period.

As far as we know, there are only a handful of other projects around the World that are using fingerprinting techniques using faeces to study animal populations including: wild baboons; bonobos; sea otters in Alaska; and Brown Bears in Italy, Slovenia, Croatia and Bosnia. The information gained and techniques learned in our study have already been shared with the Alaskan researchers in their studies of otters.

Close to the end of the project, something happened that surprised everyone. The team knew there were problems with some of the populations being so genetically similar it was hard to distinguish the animals (i.e. the 'polymorphism was low'). But they still believed that the technique was sensitive enough, and that individual otters were being identified. The project manager, Tim Sykes, found a dead otter cub on the River Itchen. A post mortem showed that she'd died of pneumonia at less than six months old. The DNA fingerprinting showed that she was H06F, whose spraints had been found seven months before and one month after the discovery of the corpse. Clearly, this wasn't possible! Seven months beforehand she hadn't been born, and one month beforehand, she was already dead.

John Dallas checked the literature, and realised that the technique just wasn't sensitive enough to distinguish between very closely related individuals, in populations with such low polymorphism. The same thing can happen in genetic fingerprinting when used by the police. Twins, for instance, can be hard to distinguish.

While this is a significant drawback, it is important that it has been realised. It is just the kind of thing that the feasibility study was intended to establish. More research is now needed to refine the fingerprinting technique, so that individual otters can be identified with certainty. And it's possible that fingerprinting studies of animals in others countries may have the same problem. Now we have identified it here, we need to share the information.

Another thing to remember is, however sad it may be to find a dead otter, it may show some incredibly useful things about the population, as illustrated by the above example. If you ever find a dead otter, let the Environment Agency know immediately, so that they can collect the corpse whilst it is still fresh.

What next?

Now, thanks to the help of volunteers, we have shown that the technique can work. Protocols have been established that may now be used by anyone wanting to do this kind of work.

Future studies could aim to find out more about the behaviour and ecology of otter populations in southern England. For example establishing otter movements, home ranges and population structure so that populations can be modelled. We are also interested in finding out how the population is affected by the following: habitat quality; disturbance; and management of riverbanks.

However the first priority is to develop DNA fingerprinting further so that we can distinguish between members of the same family. This will involve identifying and producing more primers to refine and improve the technique. We believe that if 15 loci can be developed, it should be possible to differentiate between parents and offspring in populations with low genetic diversity.

And now, while otters swim and play undisturbed at night, we can go ahead by day collecting their spraints. Using our detective work of fingerprinting, we can try to answer some questions that we hope will help to ensure that otter populations continue to recover and eventually recolonise all the UK's wetlands, rivers and coasts.

Further reading

Genetics for Beginners, Steve Jones and Borin Van Loon, Icon Books, 1993

The Fifth Miracle, the search for the Origin of Life, Paul Davies, Penguin, 1998

The Selfish Gene, Richard Dawkins, Oxford University Press, 1976

Otters, Chanin, P.R.F. 1993.. Whittet British Natural History Series.

Facts from faeces revisited, Michael H Kohn, Robert K Wayne (1997) Trends Ecol. Evol. vol. 12 no. 6, 223-227, June 6

Wild Otters: predation and populations, Kruuk, H., 1995. Oxford University Press, Oxford.

APPENDIX C1: THE SUITABILITY OF THE SOUTH WEST POPULATION FOR DNA TYPING

APPENDIX C2: DNA EXTRACTION FROM VERTEBRATE TISSUE – SALT-CHLOROFORM METHOD

APPENDIX C3: DNA EXTRACTION FROM OTTER SPRAINTS – CTAB/GITC/DIATOM/ VECTASPIN METHOD

APPENDIX C4: IDENTIFICATION OF INDIVIDUALS

APPENDIX C5: STATISTICAL ANALYSIS

APPENDIX C6: RATIO TESTS FOR INDIVIDUALS

APPENDIX C1 SUITABILITY OF THE SOUTH WEST OTTER POPULATION FOR DNA TYPING

C1.1 Background

The kind of information that DNA typing of wild populations is expected to yield depends on two factors: the levels of genetic polymorphism in the population, and the number of polymorphic loci available to assay. Three kinds of information are typically required: evidence for population subdivision, and the identification of individuals and pairs of relatives. Table C1.1 shows that it is feasible to obtain the first two kinds of information across a wide range of polymorphism levels and numbers of loci, whereas to identify 1st–order relatives requires high values of both polymorphism levels and numbers of loci. It is only feasible to identify pairs of more distant relatives when 40 – 50 highly polymorphic loci are available, and this many loci could not be assayed on the limited amount of DNA in otter spraints.

Fortunately, the levels of genetic polymorphism in the otter population in SW England can be estimated accurately thanks to the availability of a large collection frozen of tissue from carcasses collected in this region over the past 22 years. The number of polymorphic loci available for otter is 14, (13 published, 1 unpublished), and only six of these were found to be both polymorphic in SW England and suitable for DNA typing by the methods employed in this study.

Table C1.1 Factors that influence the yield of information from DNA typing

FACTORS		YIELD OF INFORMATION		
Level of polymorphism	**Number of loci**	**Population subdivision**	**Individual identification**	**1st order relatives**
High – 10 alleles per locus	10 – 15	Yes	Yes	Yes
High	5 – 10	Yes	Yes	Possibly
Intermediate – 5 alleles per locus	10 – 15	Yes	Yes	Possibly
Intermediate	5 – 10	Yes	Yes	No
Low – 2 alleles per locus	10 – 15	Yes	Possibly	No
Low	5 – 10	Yes	Yes	No

C1.2 Methods

C1.2.1 Otter tissue samples

Tissue samples of 162 otter carcasses were obtained from five collections of frozen tissue (Table C1.2). These represent Cornwall, Devon, Somerset and Hampshire, and mainly cover the period from 1986 to 1998. Ninety-five percent of samples yielded sufficient DNA for reliable typing. This is a very high rate considering that the original carcass may have remained at ambient temperature for several days before collection. Of the samples suitable for DNA analysis, 86% had location details such as OS grid references. Only the 133 samples of known location were used for statistical analyses.

Table C1.2 Otter carcass samples from SW England						
	N	**N DNA**	**OS ref.**	**No OS ref.**	**Source**	**Period**
	74	68	60	8	Mr M. Rule, EA Bodmin	1993-97
	37	37	25	12	Mr V. Simpson, VIU Truro	1996-98
	27	27	27		Dr D. Jefferies, JNCC retired	1976, 1986-92
	21	19	19		Dr C. Mason, University of Essex	1988-92
	3	3	2	1	Mrs R. Green, VWT and Mr J. Conroy, ITE-Banchory	1995 and 1997
Total	162	154	133	21		

C1.2.2 DNA typing methods for otter tissue

DNA was extracted from otter tissue using standard methods (Appendix C2). Kidney and gonad appeared to yield more DNA than muscle or liver. Nine microsatellite loci were typed for each individual to generate a DNA profile consisting of 18 numbers: two numbers (03 05) per locus. The loci used were 701, 715, 717, 733, 782, 818, 832, 833 and 902. The PCR primers and conditions for the first eight loci are published (JF Dallas & SB Piertney 1998, Molecular Ecology 7, 1248-1251). The details of locus 902 are not given because these were obtained prior to publication from Professor R.K. Wayne, Department of Biology, UCLA. Please contact Professor John Bryant, University of Exeter, UK, for further details. The remaining five loci published by Dallas & Piertney were not typed because they were considered unsuitable for subsequent typing in spraint DNA, due either to large PCR product size, or to excess production of artefactual stutter bands during the PCR assay.

C1.3 Results

C1.3.1 Levels of polymorphism in the otter population in SW England

The frequencies of the alleles detected at nine microsatellite loci in the sample of 133 carcasses that yielded DNA and had OS references are shown in Table C1.3. The individual genotypes of the 105 carcasses from the Bodmin archive and from VIU Truro that yielded DNA are given in Appendix D4. The samples were separated into two regional groups, ESW and WSE, which correspond roughly to Devon/Somerset and Cornwall, respectively. The justification for doing so is described in the following section. The numbers of alleles detected at each locus fell consistently in the range from 3 to 5, so the otter population in SW England falls between the "Low" and "Intermediate" categories of polymorphism (Table C6.1). Locus 782 has one allele at high frequency, so is of no use for individual identification because most individuals share the genotype 08 08 (Appendix D). These results imply that it will be feasible to study population subdivision and individual identity in SW England, but not to identify pairs of 1st-order relatives.

C1.3.2 Subdivision in the otter population in SW England

It is necessary to identify any subdivision within the study area because many of the calculations involved in the identification of individuals and relatives assume that alleles occur in the same individual by chance, i.e., that there are no statistical associations among alleles either within or between loci. Such associations can arise if two genetically different populations are unknowingly combined. Subdivision of wild populations can arise at the regional scale from barriers to dispersal, and at the local scale from relatives choosing to breed close to their place of birth. As nothing is known about dispersal and breeding patterns

in otter populations in the UK, evidence for subdivision is of great biological interest in its own right, quite apart from its technical relevance to the feasibility of DNA typing.

Evidence for regional subdivision was sought by comparison of allele frequencies between two groups of samples designated WSW and ESW. The WSW samples were all those located west of a straight line running from OS 1 km grid 200 100 to 300 050, and the ESW samples were all those located east of this line. Two samples from Dorset and three samples from the Itchen catchment were excluded as being too spatially separate to be included in the main cluster of samples, and too few to be analysed as separate groups. The rationale for choosing to site the line between WSW and ESW was the correspondance with the areas of Bodmin moor, Dartmoor and Plymouth, which were devoid of samples. These areas may function as barriers to otter dispersal because of lower fish biomass at higher altitudes, and because of the presumed difficulty of crossing an urban area. Allele frequencies were tested for significant differences between groups using the test for genic differentiation in the package GENEPOP 3.1a, option 3.1 (M. Raymond & F. Rousset, 1995, Journal of Heredity, 86, 248-249).

Table C1.4 Genic differentiation between WSW and ESW samples

Locus	All samples	females	males	1986 1992	1993 1995	1996 1998
701	***	***	**	NS	**	**
715	NS	NS	NS	NS	NS	NS
717	NS	NS	NS	NS	NS	NS
733	NS	NS	NS	NS	*	NS
782	NS	NS	NS	NS	NS	NS
818	***	***	***	***	***	***
832	***	*	***	***	***	NS
833	***	***	***	*	**	***
902	***	***	***	**	***	***
All loci	HS	HS	HS	HS	HS	HS

Notes: *** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, NS $p > 0.05$, HS highly significant.

The results show that there are highly significant differences in allele frequency at five out of the nine loci between the groups WSW and ESW (Table C1.4, column 2). These differences are reasonably consistent when the data were divided into subsets consisting of separate sexes and three different time periods (columns 3-7).

Evidence for further spatial subdivision within the areas WSW and ESW was then sought. The above test of genic differentiation was not used because there were no obvious spatial criteria for division of WSW and ESW into smaller groups. However, if ESW and WSW are further subdivided, there should be detectable associations between alleles: either at the same locus or at different loci. Two types of test were performed to detect such associations. Alleles at the same loci were tested for Hardy-Weinberg equilibrium versus heterozygote deficit (GENEPOP 3.1a, options 1.1 and 1.4). "Equilibrium" means that there are no statistical associations between alleles, whereas heterozygote deficit means that the same allele is more likely to occur in the same individual than expected by chance. WSW shows a deficit of heterozygotes at three loci, which is sufficient to produce a significant overall deficit. ESW shows a deficit of heterozygotes at one locus only, which is not sufficient to give

an overall departure from Hardy-Weinberg equilibrium (Table C1.5). The linkage equilibrium test (GENEPOP 3.1a, option 2.1) test for statistical associations between alleles at different loci was then performed. This showed that alleles at five pairs of loci are significantly associated in WSW and ESW (Table C1.6). The sequential Bonferroni correction for non-independent tests was applied to the probability values generated by the GENEPOP test (a = 0.05, k = 36, cutoff p = 0.0014) because of the large number of tests performed. Only those cases with a p value lower than the cutoff are shown. Finally, tests for genic differentiation within WSW and ESW, between sexes and between the three time periods listed in Table C1.2, provided no evidence for any sexual or temporal differentiation (not shown).

Table C1.5 Hardy-Weinberg disequilibrium in the WSW and ESW samples

Locus	WSW	ESW	WSW females	WSW males	ESW females	ESW males
701	*	NS	NS	NS	NS	NS
715	NS	NS	NS	NS	NS	NS
717	NS	NS	NS	NS	NS	NS
733	NS	NS	*	NS	NS	NS
782	NS	NS	NS	NS	-	NS
818	NS	NS	NS	NS	*	NS
832	**	*	*	NS	NS	*
833	NS	NS	*	NS	NS	NS
902	*	NS	NS	NS	NS	NS
All loci	**	NS	**	NS	NS	NS

Notes: *** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, NS $p > 0.05$, - not possible to test.

Table C1.6 Cases of linkage disequilibrium in the WSW and ESW samples

Locus 1	Locus 2	WSW	ESW
701	902	+	
733	832	+	
701	818		+
717	818		+
832	902		+

The results on subdivision do not pose problems for the feasibility of individual identification in SW England. However, it will be necessary to use separate reference allele frequencies for the areas WSW and ESW, should such references be required for intensive local studies. The study sites in the Torridge and the Somerset levels reported on here are all located within the area ESW. In addition, only one of the cases of linkage disequilibrium involves loci that are also suitable for typing of spraints. Finally, it is only the calculation of relatedness values which makes the assumption of linkage equilibrium, and this is unlikely to be attainable due to a lack of polymorphism.

C1.3.3 Identification of individuals in the otter population in SW England

The level of polymorphism in ESW and WSW corresponds to the borderline of feasibility for individual identification (Tables C1.1 and C1.3). Furthermore, only six of the nine loci were found suitable for DNA typing of spraints. Two types of analysis were therefore performed to

verify whether or not individual identification by spraint DNA typing is likely to be feasible in these regions. In the first analysis, values of the probability of identity (PI) were calculated from the allele frequencies for each locus (Table 6.3, last column). PI is the probability that two individuals chosen at random will match by chance. This analysis assumes the statistical independence of alleles within and between loci. The overall PI values for the six loci used for spraint typing are 10-fold lower than those for the nine loci due to the loss of three loci from the individual genotypes. Nonetheless, the six-locus PI values are sufficiently low that chance matches are expected only when more than 50 otters are present in the same area. This is because there are only 1225 possible pairs of individuals present, so only 1225 opportunities for a chance match to occur, and the inverse of 1225 is greater than the six-locus PI values.

In the second analysis, the number of chance matches between pairs of actual genotypes in the EWS and WSW samples was calculated. Where mismatches occurred, the numbers of mismatched loci was calculated in order to assess the reliability of DNA typing for individual identification. The identification of individuals will be much more reliable if the majority of loci show mismatches than if one or two loci do so. This analysis does not depend on any assumptions about the statistical independence of alleles because the units of comparison are multilocus genotypes. The distributions of mismatched loci are shown for both the nine loci used for tissue typing and the six loci used for spraint DNA typing (Figures C1 and C2). In the case of nine loci, fewer than 5% of all the possible pairs of individuals mismatch at only one or two loci, most pairs mismatch at between five and seven loci, and none show a complete match. In the case of six loci, around 10% of pairs mismatch at only one or two loci, most pairs mismatch at either four or five loci, and five pairs show a complete match (two in ESW and three in WSW).

The two analyses suggest that the majority of DNA profiles of otters in SW England will be individual-specific provided that all six of the loci used for spraint DNA typing are assayed. The loss of any loci due to typing failure would have the effect of removing the left-hand end of the distribution in Figure C2, thus decreasing the numbers of mismatched loci for a given point on the distribution. Thus, 25-30% of five-locus profiles are expected to match completely or to mismatch at only one or two loci, and around 50% of four-locus profiles are expected to do so. In addition, typing failure appears to be nearly random with respect to locus. In consequence, most pairs of profiles lacking one locus each will be comparable at only four loci, not five, because a different locus will be missing from each profile. Lastly, the degree of DNA profile mismatches at the local spatial scale and over short time periods is what will determine the reliability of individual identification. This is the situation in which close relatives are likely to be detected. Such mismatches may have been underestimated by the analysis of carcass samples due to the absence of close relatives, because carcasses were collected from large areas and over long periods of time.

Table C1.3 Allele frequencies and probabilities of identity for each locus in WSW and ESW

		Alleles numbered from 01 to 08					Probability of Identity
		02	03	04	05		
Locus 701-S	WSW	0	0.01	0.50	0.49		0.33
	ESW	0.01	0.03	0.76	0.20		0.43
Locus 715-S		01	04	06	07		
	WSW	0	0.47	0.43	0.10		0.26
	ESW	0.01	0.42	0.41	0.16		0.21
Locus 717-S		01	04	05	06	07	
	WSW	0.17	0.02	0.80	0.01	0	0.49
	ESW	0.22	0	0.76	0.01	0.01	0.45
Locus 733		03	04	05	06		
	WSW	0	0.83	0.06	0.11		0.52
	ESW	0.01	0.76	0.14	0.09		0.40
Locus 782		01	02	07	08		
	WSW	0.03	0.02	0	0.95		0.82
	ESW	0.03	0.01	0.03	0.93		0.75
Locus 818		02	06	07	08		
	WSW	0.22	0.35	0.10	0.33		0.13
	ESW	0.33	0.03	0.45	0.19		0.19
Locus 832-S		02	03	04	05	06	
	WSW	0	0.08	0.02	0.63	0.27	0.29
	ESW	0.01	0.19	0.15	0.61	0.04	0.23
Locus 833-S		02	03	04	05	07	
	WSW	0.26	0.17	0	0.57	0	0.23
	ESW	0.59	0.07	0.01	0.32	0.01	0.29
Locus 902-S		02	03	05	06	07	
	WSW	0.44	0	0.38	0.11	0.07	0.16
	ESW	0.59	0.01	0.03	0.29	0.08	0.24
Nine loci	WSW						2.5×10^{-5}
	ESW						3.8×10^{-5}
Six -S loci	WSW						4.5×10^{-4}
	ESW						6.6×10^{-4}

Note: only the loci marked as –S were used for DNA typing of spraints

The feasibility of distinguishing first-order relatives from unrelated individuals in the otter population in SW England was assessed by simulation. The allele frequencies of the six loci used for spraint typing in ESW were used to simulate the genotypes of 1000 pairs of first-order relatives and 1000 pairs of unrelated individuals. The values of relatedness between pairs were calculated, then the two distributions of relatedness values were superimposed. These calculations were performed using the packages "Kinship" and Excel. If the two distributions do not overlap, i.e., if the first-order relatives have consistently higher R values than the unrelated individuals, then it will be feasible to identify pairs of first-order relatives on the basis of R values. Alternatively, if the two distributions overlap to a large extent, then the identification of relatives will not be feasible. Figure C3 shows that the latter is the case. This confirms the initial conclusion reached above on the basis of the levels of polymorphism. Unpublished data from red grouse suggest that there must be available around 15 loci, each possessing 10 alleles, for the two distributions not to overlap (S.B. Piertney, personal communication). It is most unlikely that such highly polymorphic loci occur in the otter population in SW England, for two reasons. Firstly, the stable otter population in Scotland does not possess such high levels of polymorphism for any of the nine loci analysed here (J. Dallas, unpublished data). Secondly, the population in SW England will have lost some of its original polymorphism as a result of the acute decline that occurred during the late 1950s and 1960s.

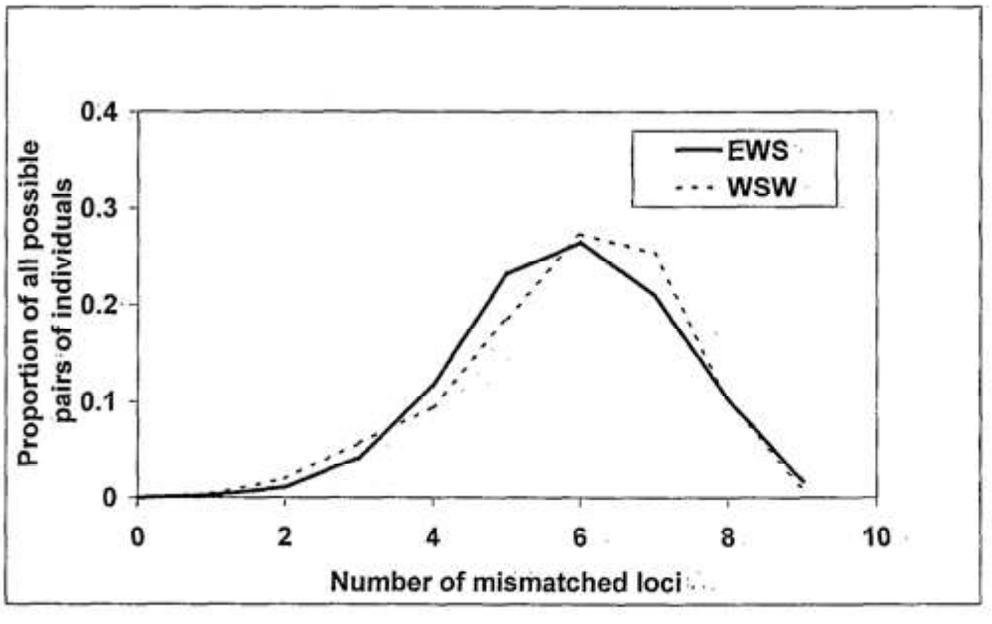


Figure C1 Distribution of Mismatches at the Nine Loci Used for Tissue Typing

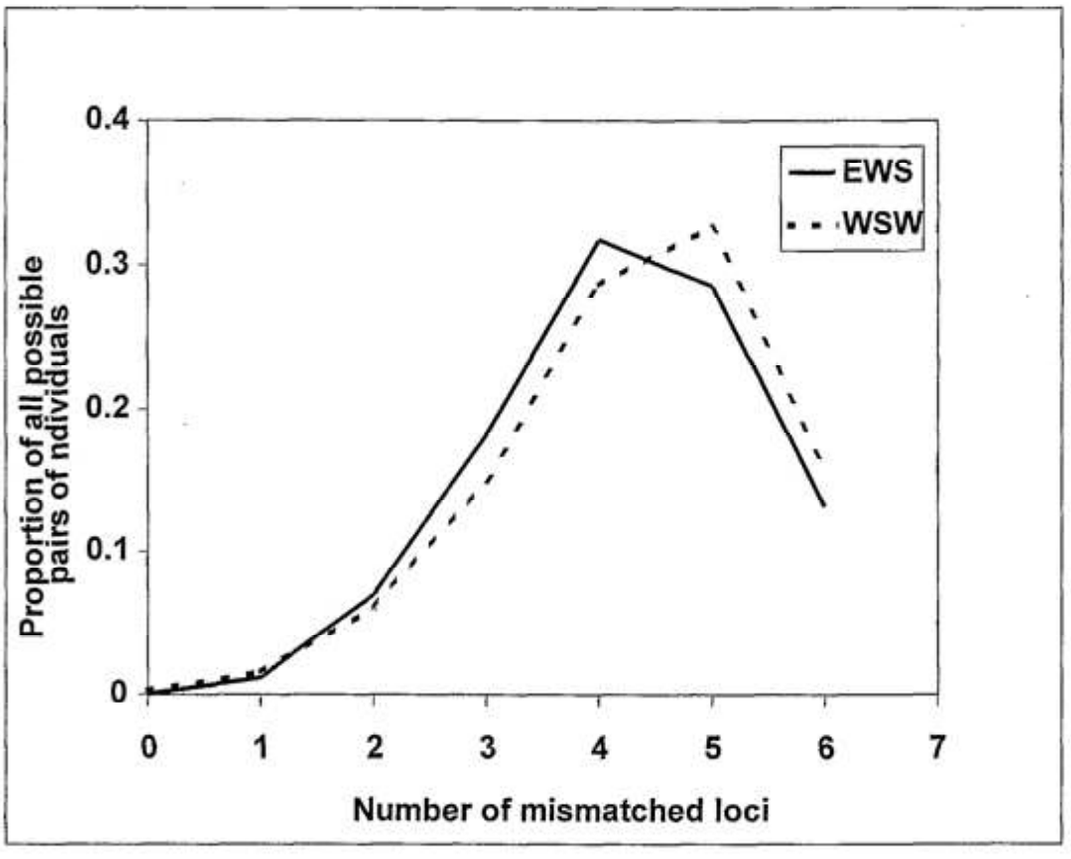


Figure C2 Distribution of Mismatches at the Six Loci Used for Spraint Typing

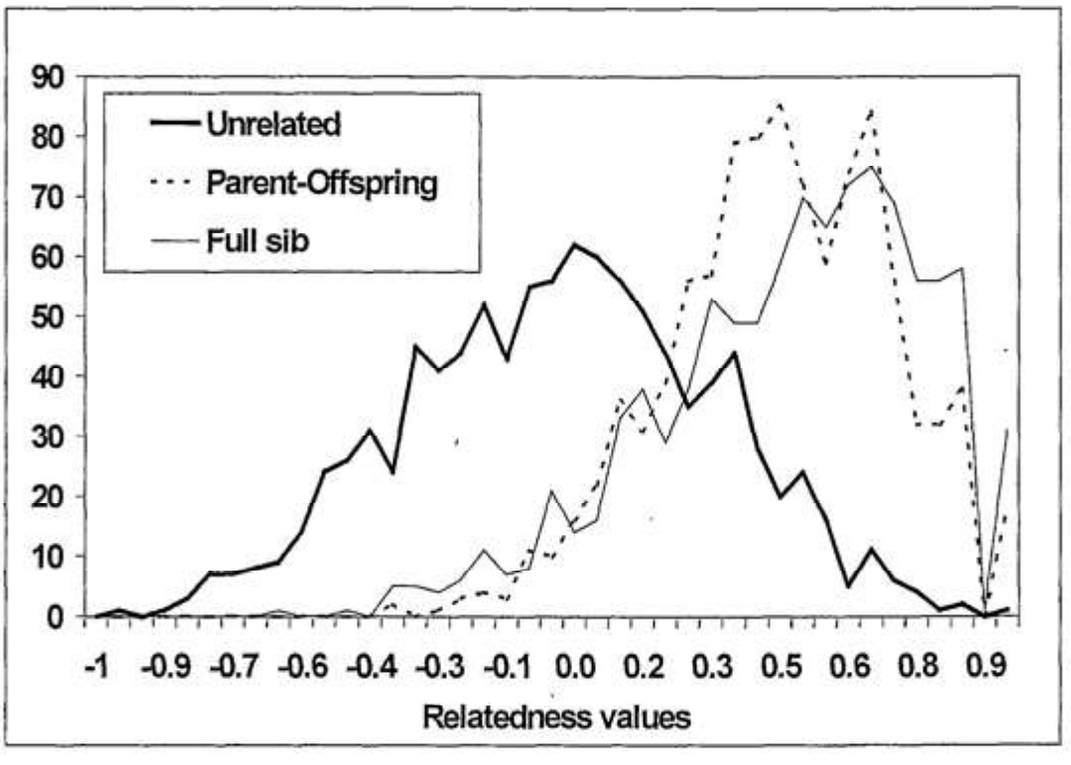


Figure C3 Simulated Values of Pairwise Relatedness Based on the Six Spraint Loci: Unrelated Versus 1st-Order Relatives in ESW

APPENDIX C2 DNA EXTRACTION FROM VERTEBRATE TISSUE: SALT-CHLOROFORM METHOD

Bruford et al. 1992, Molecular Genetic Analysis of Populations, pp. 227-9
Müllenbach et al., 1989, Trends in Genetics 5, 391.

1. Add **800 µl of Extraction Buffer** containing **Proteinase K** and **RNase A** to a 1.5 ml tube. Cut 3 x 1 mm slices (total 50 mm^3 or 50 mg) of tissue using a scalpel and add these to the tube. Place tube in heating block at 55°C immediately. Rinse and wipe with a tissue the scalpel between each sample.

2. Incubate at 55°C overnight, vortexing a few times to disperse the tissue in solution.

3. Spin tube at 13000 rpm / 5 min. to pellet undigested debris, and transfer the supernatant to a **2.0 ml** tube. Be careful not to transfer floating or pelleted debris.

4. Add **350 µl of 5M NaCl**, and **750 µl of chloroform** then vortex 5 sec. until the mixture appears milky. Put the tube on the rotator for 30 min.

5. Spin tube at 13000 rpm / 5 min. The DNA solution is the upper phase, the chloroform is the lower phase, and the precipitated debris is the layer between the two phases. Transfer the upper phase to a new **2.0 ml tube**. Be careful not to transfer debris.

6. Add **750 µl of isopropanol**, and mix by inversion x 25. A clump of DNA fibres may form, if not don't worry.

7. Spin tube 13,000 rpm / 5 min. A pellet should be visible, if not the extraction hasn't worked.

8. Remove supernatant with a 1 ml micropipette. Give the tube a pulse spin to get all remaining liquid to the bottom of the tube, and remove it with a smaller micropipette. Change the tip between each sample for both uses of the micropipettes.

9. Add **1 ml of 70% EtOH**, close and put the tube on the rotator for 15 min. Alternatively, the tube can be left overnight at 4°C at this stage if there is not enough time to finish.

10. Spin tube 13,000 rpm / 1 min. Remove supernatant with a 1 ml micropipette. Give the tube a pulse spin to get all remaining liquid to the bottom of the tube, and remove it with a smaller micropipette. Change the tip between each sample for both uses of the micropipettes. Be very careful that the white DNA pellet remains in the tube.

11. Leave tube open 10 min. at 55°C to dry the DNA pellet. Do not overdry as this makes the pellet difficult to resuspend.

12. Add **200 - 500 µl of TE**, and leave the pellet to resuspend 1 hour at 55°C (or overnight at 4°C). Vortex the tube at low speed to ensure that the DNA solution is homogeneous.

13. Spin tube 13,000 rpm / 5 min. to pellet any debris. Transfer the DNA solution to a 1.5 ml tube, write the sample number on the tube, then store it in a - 20°C freezer.

Plasticware, *etc.*

Always wear gloves, and always include an extraction control in each days-worth of extractions, *i.e.*, a tube containing extraction buffer but no added tissue.
Use only disposable pipettes and blue-cap 50 ml tubes for preparing working solutions.
For large series, it is convenient to work in multiples of 24 tubes, and to do inversions, *etc.* of 24 or 48 tubes simultaneously in an 8 x 12 rack with another rack on top.

Proteinase K solution (20 mg / ml)

Dissolve 100 mg of Proteinase K in 5 ml of AR water. Make 10 x 500 µl aliquots in 1.5 ml tubes and store at -20°C.

DNAse-free RNase A solution (10 mg / ml)

Buy from Sigma, no need to boil before use.

Extraction Buffer

0.1 M Tris.HCl, 0.1 M NaCl, 5 mM EDTA, 0.5% SDS, pH 8.0.
Add Proteinase K to 200 µg/ml and RNase A to 20 µg/ml just before use.

70% EtOH

Mix 35 ml of absolute ethanol and 15 ml of AR water in a 50 ml blue-cap tube.

TE (10 mM Tris.HCl, 0.1 mM EDTA, pH 8)

Mix the following in a 50 ml blue-cap tube:
49.5 ml AR water
0.5 ml 1 M Tris.HCl, pH 8
10 µl 0.5 M EDTA, pH 8

APPENDIX C3 DNA EXTRACTION FROM OTTER SPRAINTS: CTAB / GITC / DIATOM / VECTASPIN METHOD

CAUTION: ALWAYS WORK IN A FUME HOOD, AND WEAR EYE PROTECTION AND GLOVES. GITC PARTICLES CAN BURN YOUR SKIN AND EYES, AND CAN PRODUCE HYDROGEN CYANIDE IF ADDED TO ACIDIC SOLUTIONS. Dispose of all GITC solutions by addition of NaOH pellets, then treat as hazardous waste.

RATIONALE: The first extraction with CTAB removes polysaccharides that otherwise are co-purified with DNA in the second GITC/diatom extraction. Process up to 24 spraints at a time. Use only disposable tubes and pipettes for preparing solutions. Use the same glass bottle from new for any large-volume solutions.

1. Arrange the tubes containing spraints in ascending numerical order. Make a note of the tube numbers in a lab book. Use a temporary extraction code (say 1-24) for labelling during the extraction.

2. Take each tube in turn. First, check that there is no more than 1 ml of spraint in the bottom of the tube. If there is more, remove the excess using a spatula. Retain the fine material and get rid of the excess of solids. Before dealing with the next tube, rinse the spatula in deionised water, wipe it dry, and flame it in a Bunsen.

3. Spin the 24 tubes in a benchtop centrifuge at 4K RPM/24°C for 5 min. to pellet spraint. Tip the ethanol out of the tubes and dispose.

4. Add **2 ml of 2 x CTAB buffer** to each tube, replace the cap, and vortex to resuspend the spraint. Tape the tubes securely in a rack, attach the rack to a rotator, then rotate for 15 minutes.

5. Spin the tubes in a benchtop centrifuge at 4K RPM for 5 min. to pellet spraint debris. Meanwhile, label 24 x 2 ml tubes with the extraction codes in a yellow flipper rack.

6. Transfer 2 ml of CTAB lysis supernatant to a 2 ml tube, taking care not to transfer debris. Spin the 2 ml tube at 13 K RPM for 5 min. in the high-speed microcentrifuge.

7. Transfer 1.5 ml of CTAB lysis supernatant to a new 2 ml tube. Add 0.5 ml of chloroform, vortex at high speed for 10 sec., then put tubes on a rotator for 10 min.

8. Spin the CTAB / chloroform mix at 13 K RPM for 5 min. in the high-speed microcentrifuge. Transfer supernatant to a new 2 ml tube, then repeat the chloroform extraction and spin.

9. Transfer CTAB lysis supernatant to a new 2 ml tube. Add 0.67 ml of isopropanol, mix by inversion, then spin at 13 K RPM for 5 min.

10. Tip out liquid, pulse spin to get all traces of liquid to the bottom of the tube, then remove them with a yellow tip.

11. Add 1.8 ml of GITC buffer to the pellet. Resuspend the DNA pellet by vortexing, and use a blue tip if necessary, then put the 2 ml tube on a rotator for 10 min.

12. Add 100 µl of just-vortexed diatom suspension. Close the tube, and put it on a rotator for 10 min. to bind the DNA. Spin the tubes at 13K RPM for 1 min. to pellet diatoms. Pour off the supernatant and dispose.

13. Label a series of 24 x **10 µm mesh VectaSpin tubes** (Whatman, # 6838 0002) on the caps with the extraction codes: one VS tube per spraint.

14. Add **650 µl of GITC buffer** to each 2 ml tube, resuspend the diatom pellet using a blue tip, then transfer the diatoms plus buffer to the inserts of the VS tubes.

15. Spin VS tubes 10 K RPM for 30 sec. in the high-speed microcentrifuge to spin through the wash buffer. Remove the insert from the VS tube, tip out the wash buffer and replace the insert.

16. Add **700 µl of 70% EtOH** to the insert of the VS tube, close the cap, then vortex the top of the tube at low speed for 5 sec. to resuspend diatoms. DO NOT OVERDO THIS: VORTEX JUST ENOUGH TO RESUSPEND THE DIATOMS.

17. Spin tube 10K rpm / 30 sec. to spin through the EtOH. Remove the insert from the VS tube, tip out the EtOH and replace the insert. Don't worry about the small amount of diatoms that come through the insert.

18. Add **700 µl of absolute EtOH** to the insert of the VS tube, then spin tube 10K rpm / 30 sec. to spin through the EtOH. Remove the insert from the VS tube, tip out the EtOH and replace the insert. Finally, spin the empty VS tube plus insert 10,000 rpm / 30 sec. to spin out residual EtOH.

19. Prepare a series of 2.0 ml tubes labelled with the extraction codes and with the caps cut off, Cut the numbered cap off the VS tube and keep it safe, transfer the inserts of the VS tubes to the same-numbered 2.0 ml tubes. Place inserts + 2.0 ml tubes without VS caps in the 55°C oven for 1 hour to dry off remaining EtOH from the diatoms.

20. Add **130 µl of TE** to the diatoms in the insert and replace the numbered VS cap. Shake the rack containing all the tubes gently back and forth until the diatoms are visibly resuspended in the TE. Place the rack in the 55°C oven for 30 min., shaking intermittently, to complete the elution of DNA from the diatoms.

21. Spin tubes 10,000 rpm / 1 min. to spin through the TE containing the eluted DNA. Remove the VS insert and dispose.

22. Transfer the TE to a 1.5 ml MC tube, spin at 13 K RPM for 1 min., then transfer 100 µl of the TE to a 200 µl PCR tube. Avoid transferring any of the small diatom pellet.

23. Store the TE/DNA tubes in a green 8 x 12 PCR rack labelled with the spraint series and with a taped-on lid in the - 80°C freezer.

SOLUTIONS

2 x CTAB buffer (100 mM Tris.HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB)
Make up by dilution of stock solutions. From B.G. Milligan, chapter 3 in the IRL Press book "Molecular Genetic Analysis of Populations" (1992, ed. A.R. Hoezel)

Diatom suspension (10 ml)

1. Add 3 g diatomaceous earth and 50 ml AR water to a 50 ml blue-cap tube.

2. Mix 25 times by inversion then spin 500 rpm / 1 min in benchtop centrifuge to pellet the largest particles.

3. Pour off the cloudy liquid above visible solid-liquid interface, then add AR water to 50 ml.

4. Repeat three times the resuspension in water, spin, and removal of cloudy liquid.

5. Add AR water to a final volume of 12 ml.

6. Store suspension in the dark at room temperature.

GITC buffer (5M GITC, 0.1 M Tris.HCl pH 6.4)

Work in a fume hood. This procedure can generate hydrogen cyanide, a highly toxic gas.

1. Add 300 g GITC (Guanidine thiocyanate, Fluka, 50990), 225 ml AR water, 50 ml 1 M Tris.HCl pH 6.4, to a 500 ml blue-cap glass bottle.

2. Place capped tube in hot water in a beaker, and dissolve with intermittent mixing. Store dark at RT.

TE (10 mM Tris.HCl, 0.5 mM EDTA)

Mix in a 50 ml blue-cap tube: 49.5 ml AR water, 0.5 ml 1 M Tris.HCl pH 8, 50 µl 0.5 M EDTA, pH 8

APPENDIX C4 IDENTIFICATION OF INDIVIDUALS IN THE OTTER POPULATION IN SW ENGLAND

The most important concept relevant to this section is that the identification of individuals always involves the comparison of at least two scenarios, or hypotheses. There is no such thing as the simple identification of one individual from one sample. In this study, DNA profiles detected in spraint samples were the potential means to define individual otters. This section describes how useful such profiles appeared to be for this purpose. It was assumed initially that there were no errors of tube mislabelling, and that the multiple PCR assays described above were sufficient to exclude any experimental errors. Any mismatches between profiles were therefore ascribed to genetic differences between individuals, not to experimental artefacts. Only matches within the R. Torridge, Somerset levels, and R. Itchen sites were analysed. Matches between these sites were ignored on the grounds that no individual otter could have travelled between sites during the study period. The profiles obtained were either complete (seven loci consisting of six microsatellites plus SRY) or incomplete (fewer than six microsatellites plus SRY).

Prior to biological interpretation, the profiles of all spraints typed successfully were divided into three categories:

(a) Single (S): a profile from one spraint only that mismatched at one or more of the seven loci with all the other profiles. Single profiles were both complete and incomplete.

(b) Group (P): profiles that formed groups within which profiles matched and between which profiles mismatched. At least one multiple profile in each group was complete. P was used to avoid confusion with G, which is used for another purpose below.

(c) Ambiguities (A): profiles that matched those in more than one of the S or P categories. All A profiles were incomplete, and those for which sufficient DNA remained were set aside for further typing.

The biological interpretation of single and multiple profiles was carried out using two logical steps. The first step was to consider all the possible pairs of profiles, then to consider two alternative hypotheses.

H1.1. that the two profiles in each pair represent the same individual
H1.2. that they represent two different individuals.

No statistical calculations were required for this step. Simple categorisation was sufficient to assign all S profiles to different individuals, and to assign all the P profiles into groups, each of which represents at least one individual. The A profiles were not analysed. The complete list of the S and P profiles is given in Appendix C5.

The second step was to analyse pairs of P profiles within each group by considering the four alternative hypotheses that the profiles represent:
H2.1. the same individual
H2.2. two unrelated individuals
H2.3. parent and offspring
H2.4. two full siblings

It is only when H2.2-4 can be rejected with confidence that P profiles can be attributed to the same individual. The statistical method used for this step was the calculation of likelihood ratios. This method allows two alternative hypotheses to be compared using the value of the statistic "G". For example, the value of G calculated for the comparison of H2.1 versus H2.2 indicates how much more likely it is that the same individual is responsible for a match than two unrelated individuals. The values of G for two pairs of alternative hypotheses (H2.1 vs. H2.2, and H2.1 vs. H2.3) are given in Appendix C5 for "best-case" and "worst-case" groups in each of the three study sites. The best-case groups contain the fewest profiles, most of which are complete. The worst-case groups contain the most profiles, some of which are incomplete. The hypotheses H2.1 vs. H2.4, were not tested except for illustrative purposes for the group S01M because the existing "product-rule" method for calculation of G is know to be biased towards rejection of H2.4 (Donnelly, 1995; P. Taberlet, personal communication). An unpublished computer package that may provide improved calculations has been identified, and has been requested by JD. A description of the calculation and assessment of statistical significance of G values is given in Appendix C6.

There are four factors evident in Appendix C5 that reduce the values of G, and hence reduce the statistical power to exclude the presence of extra individuals within groups. Firstly, it is increasingly difficult to exclude extra individuals in the order: unrelated individuals, parents-offspring, and full siblings (e.g., group S01M). Secondly, incomplete profiles have lower values of G (group D01F, samples 25 vs. 2 vs. 19). Thirdly, the G values tend to be higher in the Somerset levels, where all six microsatellites are polymorphic, that in the R. Torridge (701 monomorphic) and the R. Itchen (715 monomorphic). Lastly, higher values of G are required for larger groups of profiles, due to the application of the Bonferroni correction for multiple tests (Appendix C6). The G values were ranked according to biological inference. Rank 1 has been reserved for the results of improved tests of H2.4.

Rank 3 (rejection of H2.2): unrelated individuals excluded, parents/offspring or full siblings may be present.

Rank 2 (rejection of H2.3): unrelated individuals and parents/offspring excluded, full siblings may be present.

The overall conclusion of this analysis is that when DNA profiles are complete one can be reasonably sure that groups of matching profiles do not arise from the presence of either unrelated individuals or parents and offspring. An important exception is the group H01M, which contains by far the largest number of profiles. In this case, the largest G values are not sufficiently high after Bonferroni correction to reject H2.3. This result strongly suggests that any future DNA typing studies in which large groups are required, e.g., for estimation of individual home ranges, should aim for higher G values. It is worth noting that the analyses employed here do not estimate the number of additional individuals that may be present, but only suggest that their presence cannot be excluded.

It is not clear at this point in time whether DNA typing as performed in this study provides truly individual-specific tags, i.e., has the capacity to exclude full siblings. Two measures should be taken to increase such capacity. Firstly, an improved method for the likelihood ratio test should be employed. Secondly, additional loci should be included in the DNA profile. Simulations using the improved test will indicate how many additional loci having levels of polymorphism typical of the study sites will be required. On a more positive note, DNA typing is clearly able to provide minimum numbers of individuals present in given sites over specified intervals. The utility of such numbers for monitoring population trends remains to

be assessed. This is likely to involve the use of demographically and spatially explicit models, which goes beyond the scope of the present study.

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