Hystrix It. J. Mamm (n.s.) 17 (1) (2006): 47-63

LANDSCAPE GENETICS APPLIED TO A RECOVERING OTTER (*LUTRA LUTRA*) POPULATION IN THE UK: PRELIMINARY RESULTS AND POTENTIAL METHODOLOGIES

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ABSTRACT - The Eurasian otter (*Lutra lutra*) has declined significantly across its European range. In the UK, the decline was particularly severe during the late 1950's and early 1960's, and by the mid 1970's the population was largely confined to strongholds in parts of Scotland, Northern Ireland, mid and West Wales and south west England. In recent years the otter population has started to recover, with otter surveys confirming an increased distribution of otters in Wales, Scotland and England. In England, population expansion and recolonisation is believed to be occurring both through breeding and by dispersal, from the west (south west England and the Welsh borders) and from the north (Scotland). However, little is known about the degree of genetic loss due to the decline, potential barriers to recolonisation, routes of dispersal, or the contribution of reintroduction programmes to population increases. This project aims to use tissues collected since 1994 (complete with geographic location) from over 500 otters found dead on roads in Wales and England, to analyse the genetic diversity and structure of otter populations. Using molecular genetic analysis of the otter population, we will identify whether and when bottlenecks occurred, whether population decline has resulted in a loss of genetic variability, and to what degree. Preliminary analysis from 177 otters has shown that observed is generally lower than expected heterozygosity, and that the population is in Hardy Weinberg equilibrium for 11 out of the 15 loci. Spatial patterns in genetic data will be analysed, to identify clines, isolation by distance and genetic boundaries to gene flow, the contribution of released animals will also be assessed. Geographical information systems (GIS) will be used to map spatial genetic patterns and to generate hypotheses about the potential cause of genetic boundaries such as landscape or environmental features.

Key words: *Lutra lutra*, microsatellites, spatial genetic patterns, barriers to dispersal, genetic variation

RIASSUNTO – ***La genetica del paesaggio applicata allo studio di una popolazione di Lontra* (Lutra lutra) *in fase di espansione nel Regno Unito: risultati preliminari e metodologie potenziali***. Le popolazioni di Lontra (*Lutra lutra*) sono significativamente diminuite in tutto il loro areale europeo. Nel Regno Unito il declino è stato particolarmente severo nell'ultima parte degli anni '50 ed all'inizio degli anni '60, e a metà degli anni '70 la popolazione era sostanzialmente confinata in alcune aree della Scozia, nord Irlanda, Galles centrale ed occidentale, ed Inghilterra del sud-ovest. In anni più recenti la popolazio-

ne di Lontra ha iniziato a ri-espandersi, come indicato dai censimenti che hanno confermato la maggior presenza di lontre in Galles, Scozia ed Inghilterra. L'espansione e la ricolonizzazione in Inghilterra potrebbe essere sostenuta sia dalla riproduzione che dalla dispersione da ovest (dall'Inghilterra del sud-ovest e dai confini col Galles), e da nord (Scozia). Tuttavia si conosce poco circa il declino di diversità genetica dovuto alla contrazione demografica, alle potenziali barriere alla ricolonizzazione, le vie di dispersione, o il contributo dei programmi di reintroduzione all'incremento della popolazione. Scopo di questo progetto è di usare tessuti raccolti fin dal 1994 (completi di localizzazioni geografiche) da più di 500 carcasse di lontre raccolte a seguito di incidenti stradali in Galles ed Inghilterra, per analizzare la diversità genetica e la struttura delle popolazioni. Tramite analisi genetiche molecolari, ci si propone di identificare se e quando si siano stati *bottlenecks*, se il declino della popolazione abbia prodotto perdite di variabilità genetica, ed in quale misura. Analisi preliminari da 177 lontre hanno mostrato che l'eterozigosi osservata è generalmente minore dell'attesa, e che la popolazione è in equilibrio di Hardy-Weinberg per 11 su 15 loci. Si analizzeranno i *patterns* spaziali dei dati genetici, al fine di identificare clini, isolamento per distanza e flusso genico. Si identificherà anche l'eventuale contributo di lontre rilasciate. Sistemi informatici geografici (GIS) saranno usati per mappare i pattern geografici e per generare ipotesi sulle cause potenziali di barriere genetiche quali componenti ambientali o paesaggistiche.

Parole chiave: *Lutra lutra*, microsatelliti, *pattern* genetici spaziali, barriere al *dispersa*l, variazione genetica

INTRODUCTION

1. Otter distribution and declines

The Eurasian otter (*Lutra lutra*) is a member of the family Mustelidae and its vast range extends from the west coast of Ireland to Japan and from Arctic Finland to North Africa and Indonesia (Chanin, 1985). The Eurasian otter has declined significantly throughout its European range (Barbosa *et al.*, 2003) and in the UK this occurred particularly during the late 1950's and early 1960's, throughout much of Wales, England and the Scottish borders (Coxon *et al.*, 1999; Conroy and Chanin, 2000; Mason and Macdonald, 2004). By the mid 1970's the UK population was largely confined to strongholds in parts of Scotland, Northern Ireland, mid and west Wales and south west England (Jones and Jones, 2004). There are a number of reasons proposed for this decline, such as a loss of riparian habitat, hunting, water pollution, fish traps, road traffic accidents and general disturbance (Mason and Macdonald, 2004). The most likely factor, given the suddenness of the decline, was the introduction of the organochlorine group of insecticides (particularly dieldrin), and polychlorinated biphenyls (PCBs) (Conroy and Chanin, 2000; Mason and Macdonald, 2004). The suggested combination of factors has contributed to this species being listed as either vulnerable or endangered throughout much of its current range (Ruiz-Olmo *et al.*, 2001).

2. Trends in the recovery of otters

Detailed monitoring programmes have shown that since the late 1970's there has been a slow expansion of the otter population in the UK (Ruiz-Olmo and Delibes, 1998; Conroy and Chanin, 2000), which may be the result of reduced pollution. For example, Mason (1998) shows a decline in the level of PCBs found in otter tissues from England and Wales between 1983 and 1992, to a level that no longer poses a threat to otter populations and thus should no longer act as a constraint on recolonisation. In Wales, otter surveys confirm that there has been an increase in range, with recolonisation rates exceeding Biodiversity Action Plan (BAP) targets (Jones and Jones, 2004). Scotland has also shown signs of recovery, but there are still large areas, particularly of central and southern England, where the species remains absent, or is very rare. Population expansion and recolonisation is believed to be occurring in this area both through breeding and by dispersal, from the west (south west England and the Welsh borders) and from the north (Scotland) (Coxon *et al.*, 1999; Conroy and Chanin, 2000).

3. Otter population fragmentation and its genetic consequences

Little is known about otter ecology and population dynamics in the UK outside Scotland, and organisations such as the Environment Agency have channelled resources into schemes such as habitat enhancement for otter conservation with little knowledge of their long term effectiveness (Coxon *et al.*, 1999). The need for more information about otter populations and recolonisation processes has been recognised by conservation bodies such as the Joint Nature Conservation Committee (JNCC), and incorporated into the UK Otter BAP (Biodiversity Action Plan). Anthropogenic factors have caused habitat fragmentation and a reduction in total habitat area. In most species, habitat fragmentation causes a reduction in population size and increased isolation of populations (Hooftman *et al.,* 2003). Fragmentation can result in reduced migration and gene flow, which can have deleterious effects on genetic diversity, and increase the risk of inbreeding and extinction (Charlesworth and Charlesworth, 1987; Ralls *et al.*, 1988). One of the main goals of conservation should be to mitigate fragmentation of natural habitats to increase population sizes and connectivity (Hooftman *et al.*, 2003).

The JNCC Framework for Otter Conservation in the UK identified the need to assess genetic variation within and between otter populations (Coxon *et al.*, 1999). Dallas *et al.* (2002) studied the genetic structure of the British otter populations using microsatellite markers. They had two major findings, that "*populations in Scotland, regarded as continuous according to distributions of signs, were to some extent genetically subdivided and populations in mainland Scotland showed a strong pattern of isolation by distance (IBD)...*" And "*populations in southern Britain regarded as biologically equivalent to those in Scotland contained significantly reduced levels of*

microsatellite polymorphism".

Statistical assignment tests performed by Dallas *et al.*, (2002) suggest there was no gene flow between populations in Scotland, Wales and SW England at the time of study. The different levels of microsatellite polymorphism were associated mainly with the discontinuity between populations in mainland Scotland, and those in Wales and SW England. It was unclear whether the reduced microsatellite polymorphism in Wales and SW England was the result of recent or long-term population fragmentation (Dallas *et al.*, 2002). It was suspected that the reduced polymorphism reflected a long history of low effective population size rather than recent declines (Dallas *et al.*, 2002). However, assessment of the loss of variability was hampered by lack of information about the genetic composition of the same populations prior to their fragmentation and bottleneck (c.f. Pertoldi *et al.*, 2001).

Pertoldi *et al.* (2001) investigated whether the recent otter population decline in Denmark had resulted in a loss of genetic variability, using samples from the contemporary otter population, and from historical (museum) specimens collected between 1880 and 1960. The otter population in Denmark has experienced a severe population decline in the last four decades, similar to that in the UK. However, analyses of microsatellite DNA variation in the contemporary population showed surprisingly few signs of a recent bottleneck, and indicated that the extant otter population has not suffered a recent severe loss of genetic variability (Pertoldi *et al*, 2001). The study also showed that some geographical subdivision was present in historical specimens. There were indications of a drastic population decline, but this was shown to have had happened on a time scale covering hundreds or thousands of years, not during the last few decades. It was concluded that otter populations, at least those from northern Europe, generally exhibit low genetic variability. The study suggested that the variation in the Danish otters was likely to have been low even before the recent decline in otter populations and was explained either by post-glacial founder events or a decline which started *ca*. 2,000-3,000 years ago. These findings support Dallas *et al.*'s (2002) hypothesis that the low genetic variation found in the otter populations of the UK is the result of historical rather than recent population declines.

It is nonetheless important that the long-term viability of UK otter population is likely to depend upon recolonisation and the establishment of corridors for gene flow between populations. Mitigation should therefore be considered against the potentially negative effects of population fragmentation.

4. Monitoring otter populations

The UK Otter BAP identified the need to monitor populations, distribution of otters and to monitor the expansion of fringe populations to ensure the successful management and conservation of this species (Coxon *et al.*, 1999). However, in addition to its status as an endangered species, which brings with it logistical and ethical problems that

hamper data collection, otters live at low densities and are often nocturnal or crepuscular, so their study is not straightforward (Ruiz-Olmo *et al*., 2001). As a result, monitoring techniques encounter many difficulties (Ruiz-Olmo *et al*., 2001).

There have been a handful of studies in which direct, systematic visual observations have been used to gain information about European otter populations (Ruiz-Olmo *et al*., 2001, Chanin, 2003). These methods involve a large investment of time and experienced personnel, and given the secretive nature of this species, systematic watches have limited value in monitoring otter populations, especially where there is overhanging vegetation (Chanin, 2003). Direct observations using cameras are a possibility, however, the cost and difficulty in getting clear pictures renders this option impractical (Chanin, 2003). Studies have been conducted using radio-tracking, focusing mainly on space use i.e. range sizes and rates of travel (Sjoasen, 1997). This requires the trapping of individuals, which may be problematic due to the low capture rate, small population sizes, or potential for injuries caused by handling (Mills *et al*., 2000). Radio-tracking has been successful, but is more suited to monitoring introduced and translocated individuals, providing data without the risk associated with trapping wild animals (Sjoasen, 1997). Results of such a study showed that radio-tracked translocated otters spent a high proportion of their time exploring, apparently searching for a suitable area to establish their home ranges away from occupied sites (Sjoasen, 1997).

The most frequently used technique in Europe for detecting the presence, abundance or relative abundance of otters, is to search for spraints (faeces). Otters leave spraints in visible spots (e.g. stones, rocks, tree-trunks) and in predictable places (e.g. under bridges, at junctions of rivers, in basins) which facilitates survey work. This allows the possibility to differentiate between positive and negative sites and to count the number of signs (Ruiz-Olmo *et al*., 2001; Hung *et al*., 2004; Prigioni *et al*., 2005). Over the past 25 years detecting spraints has become the standard survey method and has been used on a large scale for the national surveys of Britain and Ireland (Chanin, 2003).

Mason and Macdonald (2004) tested the method of predicting abundance of otters from spraints, using river catchments where colonisation by otters was assisted by the release of a known number of captive animals. These authors showed that there was a relationship between the number of otters, the number of sprainting sites and the spraint density. Although this method cannot be used to determine the exact number of otters present, it does provides evidence that the number of positive sites and the intensity of sprainting can be used to give a broad estimation of the performance of the otter population.

5. Genetic analysis from non-invasive biological samples

DNA can be recovered from non-invasive samples such as faeces, potentially allowing genetic analysis of otter spraints. Thus the genetic identity of

individuals can be characterised, providing an abundance of information on the population (Chanin, 2003; Dallas *et al*., 2003; Hung *et al*., 2004). A positive identification provides the location of an individual at a particular point in space and time, but provides no information on whether it is resident or transient, adult or juvenile. A distinction must be made between areas of frequent use/sedentary presence, and areas through which otters move quickly (Ruiz-Olmo *et al*., 2001). A pilot study was performed by Coxon *et al.* (1999) in 1997-98. It allowed the identification of a minimum number of individuals within the study area, and repeated identification allowed the calculation of home range size for one of the individuals. To estimate the population size in elusive or rare species, a new technique of mark-recapture using non-invasive genetic sampling (i.e. faeces) has been developed by Miller *et al.*, (2005): the method is implemented through the software package *capwire.* The data generated from this sampling method differ from traditional mark–recapture data in that individuals may be captured multiple times within a session or there may only be a single sampling event. Preliminary studies of this method have shown it provides estimates with small bias and good coverage, along with high accuracy and precision, providing an improved way to estimate *N* for some DNA-based data sets (Zhan et al, in press).

There are problems associated with the use of spraints. For example, the collection of spraints involves a lot of effort, not only in the field (where it has been calculated that it can take two man hours per spraint) but also in the lab, where analysis can take ten man hours per DNA profile (Chanin, 2003). New techniques for DNA extraction from faeces are, however, reducing the time spent in the lab and improving its success (Chanin, 2003). Another limitation of this technique is the difficulty of obtaining a sufficient quantity and quality of DNA from spraints (Dallas *et al.*, 2003; Hung *et al*., 2004). If spraints are not collected fresh they may become degraded and unusable (Chanin, 2003). Also, genotyping of DNA from faeces is prone to several problems. Due to the scarcity of the template DNA, stochastic amplification of only one out of two alleles at a heterozygous locus can cause ‘allelic dropout’. Artefacts are sometimes generated during amplification to produce a ‘false allele’, and sometimes a ‘counterfeit’ or third allele is produced. Contaminant DNA can cause serious problems when the target DNA is rare and may lead to mistyping of the genotype (Huang *et al*., 2005). These errors need to be detected and resolved and this can mean repeating the DNA amplification independently several times in order to obtain reliable genotypes (Taberlet *et al.,* 1997; Dallas *et al.*, 2003; Hung *et al.*, 2004).

6. Genetic analysis from otter tissue

With an increasing otter population in Britain, the likelihood of an encounter with humans increases. Unfortunately in the last 15 – 20 years, mortality due to road traffic accidents has increased, and has become one of the most important causes of death of otters in most

European countries (Hauer *et al.*, 2002; Philcox *et al.*, 1999). Although unfortunate, where carcasses are collected they provide an ideal source of samples for genetic analysis, because the extraction of DNA from tissue samples is much more reliable than from faeces.

The collection of genetic data from many individuals of known geographic origin, in combination with recently developed statistical tools, potentially allows the identification of spatial genetic patterns (Manel *et al.*, 2003). This approach enables the spatial mapping of allele frequencies and potential correlation with landscape or environmental features. This 'landscape genetic approach' combines landscape ecology with population genetics, allowing the examination of biogeography at a fine spatial and temporal scale. This provides information on the interaction between environmental or landscape features, and microevolutionary processes such as genetic drift, gene flow and selection (Manel *et al.*, 2003; Berthier, 2005). Geographical information systems (GIS) can be used in conjunction with statistical tests to visualise spatial genetic patterns, by overlaying landscape variables and genetic data (Manel *et al.* 2003). An important feature of this approach is that it aids in the identification of cryptic genetic discontinuities (barriers to gene flow) across populations which have no obvious cause and can identify secondary contact between previously isolated populations. Spatial delineation of genetic discontinuities within a species can also allow for the formation of operational units, important for management purposes (Manel *et al.*, 2003).

7. Molecular approaches

Microsatellites consist of tandemly repeated units, generally less than 5bp (base pairs) in length such as (TG)n or (ATT)n (Bruford and Wayne, 1993). These repeat units are often highly polymorphic with many different alleles segregating in a population. Due to their attributes they have been used in many different areas of study ranging from ancient and forensic DNA studies, to population genetics and conservation/management of biological resources (Jarne and Lagoda, 1996; Zhivotosky and Feldman, 1995; Zane *et al.*, 2002). Locus-specific PCR primers are designed to recognise sequences flanking the tandem repeats (Bruford *et al.*, 1996).

8. Background and aims of study

The otter population in England and Wales is known to be growing (Coxon *et al.*, 1999; Conroy and Chanin 2000; Jones and Jones, 2004) but little is known about the dynamics of recolonisation events associated with this expansion. Using genetic data available from otter carcasses found and collected in this area since 1994, the genetic structure of remnant and newly established populations will be investigated. This information can be used to analyse the origin, rate and direction of recolonisation into formerly vacant regions using spatial genetic analysis and population assignment tests (e.g. Piry *et al.*, 2004).

In a 'source-sink' situation such as recolonisation into a vacant habitat, where otters are expected to spread

from stronghold populations, a correlation between genetic and geographic distance from the source can be expected (Bertorelle and Barbujani, 1995), with a continuous increase of genetic distance with geographic distance (isolation by distance). The identification of spatial genetic patterns will show both the degree and direction of spread of the otter population from strongholds to adjacent unpopulated areas, and demonstrate the success and spread of any otters introduced. GIS will be used to visualise spatial genetic patterns and to generate hypotheses about the cause and consequence of genetic boundaries, which can then be explicitly tested.

Our study will concentrate initially on the genetic structure of the Welsh otter population, to identify if genetic differences exist at local and regional levels. If sub-structures do exist, GIS will be used to identify whether genetic boundaries are associated with physical obstacles such as roads and other landscape features. Later in the study, we aim to include English otter populations, again to investigate the genetic structure but also to assess the relative contribution of source populations in Wales, SW England and Scotland. We also aim to use spatial genetic patterns to identify the degree, direction and routes of dispersal as well as identify barriers. Genotype mapping will also demonstrate the origin and success of otters that have been introduced.

PRELIMINARY ANALYSIS

As a first step in this study we have analysed samples from Wales and bordering catchments to establish molecular methodologies, and to examine the genetic structure of the Welsh otter populations.

METHODS

1. Sampling

Over the past two decades in the UK, the Environment Agency along with other regional organisations have recorded the geographical location and collected otter road casualties (over 500 individuals) throughout England and Wales. Muscle samples have been removed from otters and stored in ethanol at -20°C. Of these, 177 samples have been selected from Wales and bordering catchments (Fig. 1), for use in this preliminary analysis.

2. DNA extraction

DNA was extracted from muscle tissue, using the QIAGEN DNeasy tissue kit following the ‘isolation of total DNA from animal tissues’ protocol (QIAGEN, #65906).

3. Primers

Using primers that have been designed for the Eurasian otter, we identified the genotypes of individuals for 21 loci. The microsatellite loci used comprise lut 435, 453, 457, 604, 615, 701, 715, 717, 782, 818, 832, 833 (Dallas and Piertney, 1998) lut 902 (Dallas *et al.*, 1999) and 04OT02, 04OT04, 04OT05, 04OT07, 04OT14, 04OT17, 04OT19 and 04OT22 (Huang *et al.*, 2005). (Following preliminary analyses, the number of loci will be reduced using rarefaction analysis, see below).

4. Multiplex design

For more efficient analysis, four PCR mul-

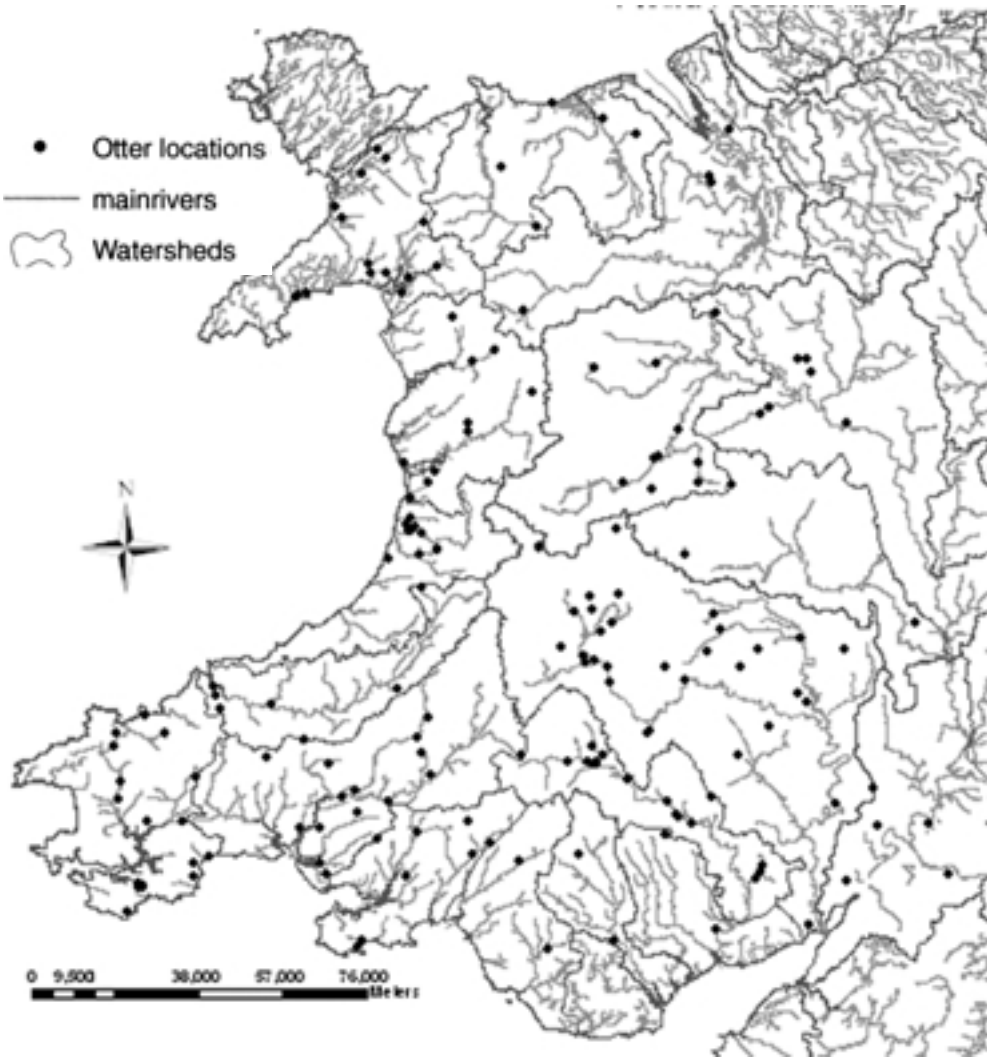

Figure 1 - Map of Wales and Borders showing major rivers, watersheds and otter location.

tiplex groups were designed and optimised. The Forward primers of each primer pair were labelled with a fluorescent dye (Ned, Hex or Fam). The dye used to label each primer was chosen as part of the design of the multiplex group which also took into account the allele size, to ensure that each locus was distinct. Two multiplex groups contained five primer pairs and two contained six. PCR reactions were conducted with a QIAGEN Multiplex PCR kit following the 'amplification of microsatellite loci using multiplex PCR' protocol (QIAGEN, #206143). Amplification of DNA extracts was performed using a GeneAmp® PCR system 9700 (Applied Biosystems) in 6.5 µl reactions containing DNA template, 1x QIAGEN Multiplex PCR Master Mix (containing HotStarTaq® DNA polymerase, Multiplex PCR buffer (contains 3 mM $MgCl_2$) and dNTP Mix), 10x Primer Mix (0.2 µM of each primer) and sterile water.

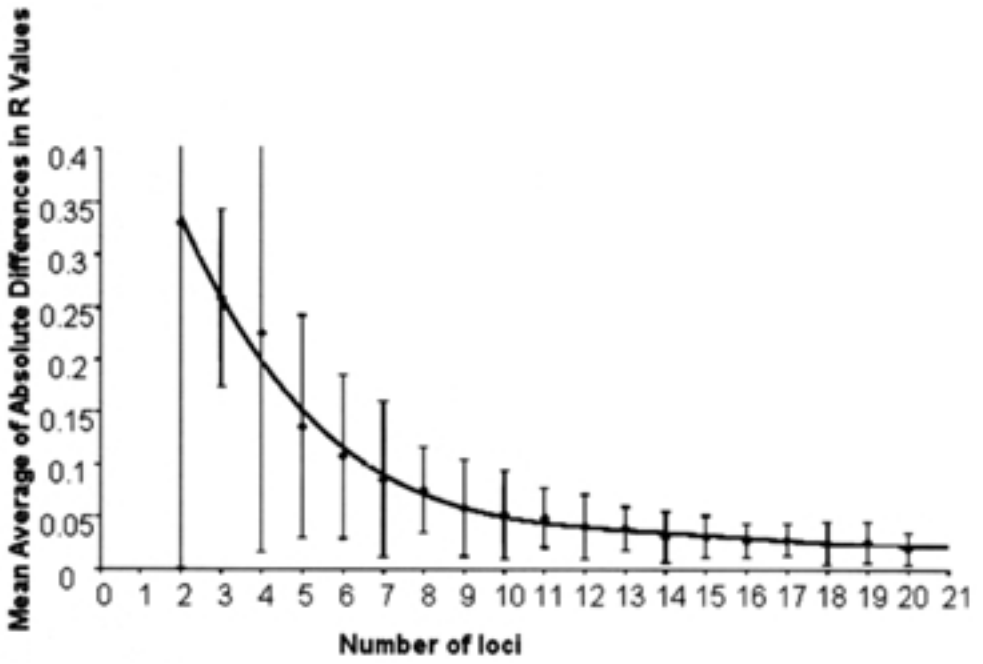

Figure 2 - The decrease in the mean difference between consecutive relatedness estimates as a function of the number of microsatellite loci analysed.

The PCR profile was identical for each multiplex and included an initial denaturation step of 95 ºC for 15 min, 29 cycles with 94 ºC for 30 s, 58 ºC for 90 s and 72 ºC for 1 min and a final extension of 60 ºC for 30 minutes.

PCR products were analysed using an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) and gel analysis was performed using the software Genescan v 3.7 and Genotyper version 3.6 (Applied Biosystems).

5. Rarefaction analysis

A random sample of 100 otters from the Wales and Borders region were genotyped for all 21 loci using the methods described above. These genotypes were input into the program POPASSIGN version 4.3a (http://www.darwinfox..org/fulvipes/EnHome.htm) to conduct rarefaction analysis. Rarefaction analysis aims to identify the combination of loci which most efficiently recover data, enabling accurate relatedness and genetic diversity estimation (Kays *et al*., 2000; Smith *et al.*, 1997; Altmann *et al*, 1996). In POPASSIGN, relatedness is assessed by simulating first order relative datasets based on the observed allele frequencies, estimating 'Queller and Goodnight (1989) relatedness' (*R*) using the simulated data, and repeating the process for all possible combinations of loci to be used. Standard errors are generated by permuting loci without replacement. The number of loci was increased by addition without replacement until all 21 loci were selected (Girman *et al.*, 1997; Kays *et al.*, 2000). This procedure was repeated 1000 times. The mean difference in relatedness estimate *R* for different numbers of loci and jackknifed standard errors were calculated as the average of absolute differ-

ences in *R* values calculated between steps (Altmann *et al*., 1996).

6. Genetic variability

Genotyping using 15 loci (the optimal combination identified by rarefaction analysis; lut435, lut453, lut717, lut604, lut733, lut615, lut902, lut782, lut701, lut833, lut818, lut715, lut832 (Dallas and Piertney, 1998), 04OT05, 04OT22 (Huang *et al.*, 2005)) was conducted for 177 individuals. POPASSIGN was used to identify the allelic diversity and the observed (*Ho*) and expected (*He*) heterozygosity of the loci. Significant deviations from Hardy-Weinberg equilibrium (HWE) for each locus in the population were tested using the software GENEPOP Version 3.3 (Raymond and Rousett, 1995).

RESULTS

1. Rarefaction analysis

The difference between consecutive sampling in the outcome of *R* was expressed as a function of the total number of loci drawn, and showed that mean and variance estimates of relatedness *(R)* stabilised after 15 loci (Fig. 2). Therefore 15 loci can be used to provide consistent measures of relatedness.

2. Genetic variability

The microsatellite loci for Wales and Borders otters are polymorphic with an average of 5.1 alleles per locus (min-max: 3-7). Comparison with the results of other studies of the European otter (Table 1) shows that the larger sampling area of the European population studied by Randi *et al*, (2003) had a higher average number of alleles per locus of 7.8. The smaller island populations of Kinmen (China) and Sealand (Denmark) showed fewer alleles per locus averaging 0.35-0.39 and 3.6 alleles per locus respectively.

The Wales and Borders otter population had an average expected heterozygosity (*He*) of 0.53 over the 15 loci. This was somewhat lower than the European average *He* = 0.74 (Randi *et al*, 2003), and also lower than the island population of Kinmen *He* = 0.61, 0.70 (Hung *et al.*, 2004, Huang *et al*, 2005). The *He* of the Wales and Borders population was however, similar to Sealand in Denmark, *He* = 0.51 (Pertoldi *et al.*, 2001) despite having 40 % more alleles on average per locus.

The pooled European samples (Randi *et al*, 2003) showed significant deviation from HWE, with significantly positive *Fis* values for 9 out of 11 loci. In contrast, they found that most local populations were actually in HWE (over all loci) when analysed separately. However, French and German samples still showed significant deviations from HWE which Randi *et al.*, (2003) suggested could be due to the Wahlund effect.(artifactual deviation due to a sample that is composed of sub-samples from separate populations; Hartl and Clark, 1997). Pertoldi *et al.*, (2001), Hung *et al.*, (2004) and Huang *et al.*, (2005) studied populations over smaller areas than Randi *et al.*, (2003) and found little evidence for deviations from HWE.

In this study the Wales and Borders samples show that the observed were generally lower than the expected heterozygosities. Significant deviations from HWE were observed at four out of

Table 1 - Summary of observed (Ho) and expected (He) heterozygosity and observed allele number (n alleles) for the 15 loci chosen by rarefaction analysis over five studies of European otters (* p<0.05, ***p<0.001 significant difference between Ho & He).

	Hobbs *et al.*, unpublished		Hung *et al.*, 2004		Huang *et al.*, 2006		Randi *et al.*, 2003		Pertoldi *et al.*, 2001	
	Wales and Borders		Kinmen (island), China		Kinmen (island), China		Europe		Sealand, Denmark	
Locus	H_o *(in alleles)*	H_e	H_o *(in alleles)*	H_e	H_o *(in alleles)*	H_e	H_o *(in alleles)*	H_e	H_o *(in alleles)*	H_e
lut435	0.44 (5)	0.47					0.61 (12)*	0.83	0.33 (5)	0.60
lut453	0.27 (5)	0.31					0.69 (9)*	0.82		
lut604	0.54 (4)	0.63					0.43 (9)*	0.75		
lut615	0.55 (6)	0.63					0.63 (1)*	0.83		
lut701	0.46 (3)	0.42	0.61 (5)	0.56			0.58 (8)*	0.76	0.50 (3)	0.42
lut715	0.55 (6)*	0.57	0.89 (6)	0.76			0.46 (6)*	0.64		
lut717	0.35 (5)	0.41	0.71 (3)	0.52					0.56 (2)	0.55
lut733	0.47 (5)	0.46	0.89 (4)	0.69			0.57 (8)	0.69	0.39 (4)	0.46
lut782	0.46 (4)	0.47	0.79 (2)	0.5			0.54 (8)	0.55	0.33 (3)	0.38
lut818	0.64 (7)	0.67					0.49 (6)*	0.76	0.69 (4)	
lut733	0.47 (5)	0.46	0.89 (4)	0.69			0.57 (8)	0.69	0.69 (4)	0.62
lut832	0.26 (5)**	0.35	0.66 (3)	0.55			0.48 (6)*	0.69	0.56 (4)	0.49
lut833	0.71 (5)	0.71	0.74 (4)	0.7			0.54 (6)*	0.78		
lut902	0.55 (7)***	0.65							0.60 (4)	0.57
04OT05	0.63 (6)	0.67			0.83 (4)	0.72				
04OT22	0.50 (4)	0.53			0.59 (3)	0.68				
Mean	0.49 (5.1)	0.53	0.76 (3.9)	0.61	0.71 (3.5)	0.70	0.55 (7.8)	0.74	0.50 (3.6)	0.51

teen loci (see Table 1), with loci lut733 standing out as the only locus having significantly more observed heterozygotes than expected. The three other loci showed a significant deficit in observed heterozygotes with lut832 and lut902 showing highly significant deviations. This could be due to a number of reasons, such as allelic dropout or DNA degradation, however, neither of these seem likely given the quality and quantity of DNA extracted from muscle tissue. Randi *et al.* (2003) suggested that significant deviations from HWE in their samples from France and Germany could be due to the Wahlund effect as a result of differentiation at a lower geographical scale. If this was the case it would be expected to see more loci showing significant deviation from HWE. This was the case when nine English samples were added to the analysis of the Wales and Borders population (results not presented here), when eleven loci showed deviations from HWE. Likewise if inbreeding was a cause more loci would be expected to show significant deviations from HWE.

FUTURE DIRECTIONS

Future work will identify the reasons for the anomalies for these two loci, using the suggestions made by Wondji *et al.* (2002), for example focusing on locus-specific constraints such as null alleles (Callen *et al*., 1993), limited allelic range (Epplen *et al*., 1993) or preferential amplification of one allele in heterozygotes (Wattier *et al*., 1998), rather than population substructure or inbreeding (Wondji *et al.*, 2002).

In addition, further analyses will be undertaken. Using the perspectives of landscape genetics, spatial genetic patterns will be assessed at an individual level without defining populations in advance (Manel *et al.*, 2003).

Methods that can be used for analysis of the results include Mantel's test, to identify the presence of an isolation-by-distance pattern between individuals using genetic differentiation and geographical distance (Manel *et al.*, 2003). Multivariate analysis and synthesis maps, using principal component analysis (PCA) vectors can also be used. PCA summarises all the variation for many loci in the study area, and can accommodate individuals as the operational units. The interpolation of the major principal components derived from the PCA leads to a synthesis map (Manel *et al*., 2003).

There are specific methods to infer genetic boundaries from allele frequency spatial distributions. Monmonier's algorithm visualises data contained on a genetic distance matrix on a geographical map. A Womble approach locates boundaries across a surface for an interpolated variable (i.e. allele frequency surface) by searching for regions in which the absolute value of the surface slope is large (Manel *et al.*, 2003). Delaunay triangulations and Voronoi diagrams can be used for surface modelling by using a finite set of points scattered over a surface to construct a three-dimensional model (Attali and Boissonnat, 2004).

Once the genetic pattern is identified it must be correlated with environmental and landscape variables. In parallel to statistical tests, GIS will be used to visualise spatial genetic patterns and

also generate hypotheses about the cause of genetic boundaries because it allows landscape variables to be overlaid onto genetic data.

ACKNOWLEDGEMENTS

I (H.G.I.) would like to thank my supervisors for their guidance, Vic Simpson for otter samples for the south west of England and the Environment Agency, for their funding of the Cardiff University Otter Project. We would also like to thank the Environment Agency, CCW and UK Wildlife Trusts for their collection of otter carcasses.

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