WILDLIFE DNA SERVICES LIMITED

Project Report No. 05/004

Genetic analysis of the Mid-Wales red squirrel (*Sciurus vulgaris*) population

This report was prepared on 31st August 2005 by Wildlife DNA Services Limited
for the South & West Wales Wildlife Trust

Executive Summary

- A total of 32 samples of red squirrel hair were received for analysis.
- DNA was successfully extracted and amplified from seventeen samples.
- The resulting sequences revealed three different mitochondrial DNA haplotypes present in Mid Wales.
- The three haplotypes had all previously been observed in Wales, however their presence in a single locality had not been recorded before.
- The relationship of the Mid Wales squirrels to other Welsh, UK and European populations is assessed and discussed. The results further support a closely related group of genetic lineages within Wales.
- The level of genetic variation observed in Mid Wales, relative to other Welsh localities, identifies this population as being of clear importance in the conservation of red squirrels in Wales as whole.

Signed as correct on behalf of Wildlife DNA Services Ltd, on 31st August 2005

Dr Rob Ogden
(Projects Manager / Director)

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(Laboratory Manager / Director)

Details

Wildlife DNA Services Project No.	05/004
Contract description	To examine mtDNA haplotype diversity in red squirrels sampled from several forest areas in Mid Wales. To provide interpretation of the results in relation to future conservation management of the local population.
Contractor	South & West Wales Wildlife Trust
Contact	Anna Hobbs

Procedures

1. Details of samples received

Samples were received and stored by WDNAS following the general protocols described in Appendix I

Collection tubes containing 70% ethanol were provided upon request to Anna Hobbs (AH) and Huw Denman (HD) for the collection and storage of plucked hair samples.

Samples were received over an extended time period during 2005 and stored at WDNAS laboratories prior to commencement of analysis.

A total of 32 hair sample tubes were received and documented. Details of these samples are given in Table 1, below.

The sex of each sample, where given, is based on morphological examination made at the time of trapping by the collector.

Table 1: A list of the samples received for genetic analysis

Original Identifier	WDNAS Identifier	Species	Sex	Collector
W-001401	RS1BAD	S. vulgaris	M	HD
W-001402	RS2BAD	S. vulgaris	M	HD
W-001474	FRS3	S. vulgaris	M	AH
W-001475	NYHRS1	S. vulgaris	F	AH
W-001476	NYHRS2	S. vulgaris	M	AH
W-001477	NYHRS4	S. vulgaris	M	AH
W-001604	NYHRS10	S. vulgaris	M	AH
W-001605	FRS9	S. vulgaris	M	AH
W-001606	NYHRS12	S. vulgaris	M	AH
W-001607	NYHRS8	S. vulgaris	F	AH
W-001608	NYHRS11	S. vulgaris	F	AH
W-001609	NYHRS6	S. vulgaris	M	AH
W-001610	NYHRS7	S. vulgaris	?	AH
W-001772	BAD03	S. vulgaris	?	HD
W-001773	BAD04	S. vulgaris	?	HD
W-001774	BAD05	S. vulgaris	?	HD
W-001775	BAD06	S. vulgaris	?	HD
W-001776	BAD07	S. vulgaris	?	HD
W-001777	BAD08	S. vulgaris	?	HD
W-001778	BAD09	S. vulgaris	?	HD
W-001779	BAD10	S. vulgaris	?	HD
W-001780	BAD11	S. vulgaris	?	HD
W-001781	BAD12	S. vulgaris	?	HD
W-001782	BAD13	S. vulgaris	?	HD
W-001783	BAD14	S. vulgaris	?	HD
W-001784	BAD15	S. vulgaris	?	HD
W-001785	BAD16	S. vulgaris	?	HD
W-001786	BAD17	S. vulgaris	?	HD
W-001787	BAD18	S. vulgaris	?	HD
W-001788	NYHRS13	S. vulgaris	?	AH
W-001789	FRS14	S. vulgaris	?	AH
W-001790	NYHRS15	S. vulgaris	?	AH

2. DNA extraction

DNA extractions carried out by WDNAS are in line with published guidelines (1,2). For general conditions see Appendix II.

Specific Conditions:
Extraction from all samples was initially conducted using the Qiagen DNeasy extraction kit. For certain samples that yielded no apparent DNA (see Results), re-extraction was attempted using the Qiagen Micro (forensic) kit that has a higher DNA capture capability for low yield samples.

3. DNA Amplification

DNA PCR amplification follows the general protocols described in Appendix III

Amplification of population informative DNA fragment:

The utility of the mitochondrial DNA (mtDNA) control region for generating population informative haplotypes has previously been reported (3, 4). This genetic region was targeted for sequence analysis in this project, in order to allow comparison with existing studies of red squirrel populations throughout the U.K.

A summary of the PCR primers used is provided in Table 2.

Table 2: PCR amplification details for the DNA fragment sequenced.

PCR Amplification Number	Target Locus	Primer 1	Primer 2	Size (base pairs)	Reference
1	mtDNA control region	Sq070F	Sq388R	312 bp	4

4. DNA sequence resolution

DNA sequence resolution follows the general protocols described in Appendix IV

The oligonucleotide primer used for the sequencing reaction was Sq070F, which was shown, during internal validation, to be the most successful sequencing primer.

The resulting sequences were given a quality score from 1-3 to assess the use in further analysis.

5. Haplotype identification from DNA sequence

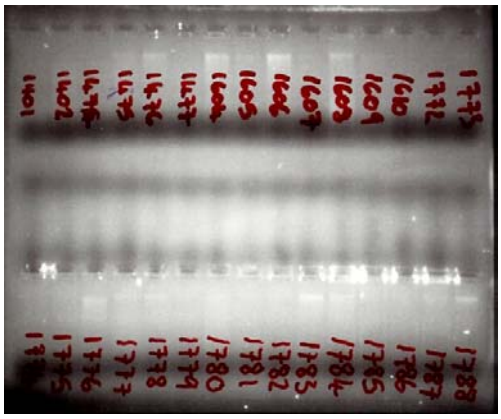
Haplotype identification was determined through comparison of the recovered sequences with sequences previously recorded in studies of European red squirrels (3, 4).

Laboratory Results

1. DNA extractions

DNA extractions were carried out using hair samples supplied. A number of the hair samples (W05/001782-1787) had been stored upside down in the tubes (i.e. root end up), which may have resulted in a lower yield of squirrel DNA. These samples were also noted to typically contain fewer individual hairs. The results of the DNA extractions are shown in Figure 1.

The DNA yield varied markedly between samples. This may a due to a number of factors including the number of hairs present, the proportion of hairs with root cells, the storage conditions and the level of contaminant DNA (indistinguishable from squirrel DNA at this stage).



2. PCR amplification

PCR amplification results were variable. The initial samples received (W05001401-1610) generally amplified quite readily, allowing them to be taken forward to the DNA sequencing stage. Subsequent samples were a lot more difficult, with PCR amplification often requiring repeated attempts and in other cases, consistently failing (Table 3).

As can be seen in Table 3, PCR success did not always relate to DNA quantity. In these cases it it is likely that samples contained squirrel DNA that was degraded due to lack of immersion in ethanol during storage, or because the extracted DNA originated from contamination rather than squirrel hair cells.

In total, 17 of 32 samples amplified sufficiently for subsequent DNA sequencing.

3. Sequencing the population informative mtDNA control region

All unknown samples that amplified successfully also sequenced successfully and were graded as sequence quality 1 following the guidelines described by EMQN (see above).

Table 3: Results of PCR amplifications from DNA extracts prior to DNA sequencing. Estimates of DNA yield are based on the photograph in Figure 1. DNA extracted is not necessarily of squirrel origin. Amplification success is indicated by an asterisk (*), the number of amplification attempts (n) is given for failed samples.

Sample id	DNA yield	Amp success	Sample id	DNA yield	Amp success
W05-001401	low	*	W05-1775	low	(2)
W05-1402	low	*	W05-1776	average	(3)
W05-1474	low	*	W05-1777	average	(3)
W05-1475	average	*	W05-1778	average	(3)
W05-1476	average	*	W05-1779	low	(2)
W05-1477	low	*	W05-1780	low	(2)
W05-1604	average	*	W05-1781	low	(2)
W05-1605	average	*	W05-1782	low	(2)
W05-1606	average	*	W05-1783	average	(3)
W05-1607	low	*	W05-1784	average	(3)
W05-1608	average	(4)	W05-1785	low	(2)
W05-1609	average	*	W05-1786	low	*
W05-1610	low	*	W05-1787	low	*
W05-1772	low	(2)	W05-1788	average	*
W05-1773	low	(2)	W05-1789	average	*
W05-1774	low	(2)	W05-1790	average	*

4. Haplotype identification from DNA sequence

The raw sequence data was aligned with existing sequences held on file from previous studies (3, 4). Sequence comparison revealed that the samples sequenced in this study were comprised of three different haplotypes, ang1, wc3 and mw1, all of which had been previously observed in Wales (Table 4). The identification of these haplotypes, allowed subsequent data analysis to be performed on the relationship of the Mid Wales red squirrels to those in the remainder of the U.K. and areas of northern Europe.

Table 4: The sequence identities (or 'haplotypes') for each sample that was successfully sequenced in this study.

WDNAS ID	WT ID	Haplotype	WDNAS ID	WT ID	Haplotype
W05-1401	RS1BAD	ang1	W05-1607	RS8	wc3
W05-1402	RS2BAD	ang1	W05-1609	RS6	mw1
W05-1474	FRS3	wc3	W05-1610	RS7	wc3
W05-1475	NYHRS1	ang1	W05-1786	BAD17	ang1
W05-1476	NYHRS2	wc3	W05-1787	BAD18	ang1
W05-1477	RS4	wc3	W05-1788	RS13	wc3
W05-1604	RS10	wc3	W05-1789	RS14	wc3
W05-1605	FRS9	wc3	W05-1790	RS15	wc3
W05-1606	RS12	wc3			

Analytical Results and Discussion

1. mtDNA Sequences and genealogy

The sequence data allows the Mid-Wales red squirrel population to be compared with other Welsh populations and the more widely distributed UK and European groups. The three haplotypes observed in Mid Wales are all very similar to each other. Genetic distances among the three haplotypes (ang1, mw1 & wc3) were smaller than their respective distances from the other haplotype currently found in Wales (wc9, in Clocaenog) and from the remainder of the previously published European haplotypes (Table 5). In particular, haplotypes mw1 and wc3 were more similar to each other (*d*=0.015), than the two haplotypes recorded from Clocaenog (wc3 and wc9, *d*=0.024). This supports the suggestion made by Ogden *et al.* (2005) that the Mid-Wales haplotypes belong to a relatively narrow lineage that may include an ancestral Welsh population, whilst the Clocaenog population is probably comprised of more widely mixed lineages.

The relationship between the existing population on Anglesey (at Pentraeth) and the Mid Wales population is very close. Previous surveys at Pentraeth found only one haplotype (ang1) present in the population (4), indicating that the Anglesey red squirrel may be a sub-population of those persisting in Mid Wales. The other Anglesey haplotype, ang2, has only been observed historically and is included for comparative purposes.

Table 5: A genetic distance matrix of the three haplotypes found in this study (ang1, mw1 & wc3) together with the other two haplotypes previously recorded in Wales and a group of the 24 remaining published UK and European haplotypes (distance to group mean used). Distance calculated, d, is the uncorrected pairwise distance.

Origin	Haplotype Code	ang1	ang2	mw1	wc3	wc9
Mid-Wales & Anglesey	ang 1					
Anglesey only (extinct?)	ang 2	0.011				
Mid-Wales	mw 1	0.007	0.019			
Mid-Wales & Clocaenog	wc 3	0.022	0.033	0.015		
Clocaenog only	wc 9	0.030	0.034	0.022	0.024	
Rest of the UK/Europe	Others	0.032	0.038	0.025	0.023	0.024

2. Phylogenetic relationships between the haplotypes

In order to reconstruct the possible evolutionary relationships between the haplotypes, a Median-joining network analysis was undertaken to examine how the haplotypes have differentiated from one another over time (Figure 2). Ang1 and mw1 form a group with ang2, the haplotype observed on Anglesey in 1980 but now considered extinct on the island. These Welsh haplotypes are also closely linked to haplotypes observed in the Channel Islands. The remaining Mid Wales haplotype, wc3, is slightly more distinct, situated four steps away from mw1 on the network. Nevertheless, the positioning of all three Mid Wales haplotypes suggests a level of geographic structure that is absent from the species in general. The widely dispersed geographic origins around the rest of the network confirm the general lack of phylogeographic structure in UK and northern European red squirrels that was originally revealed by Barratt *et al.* (1999).

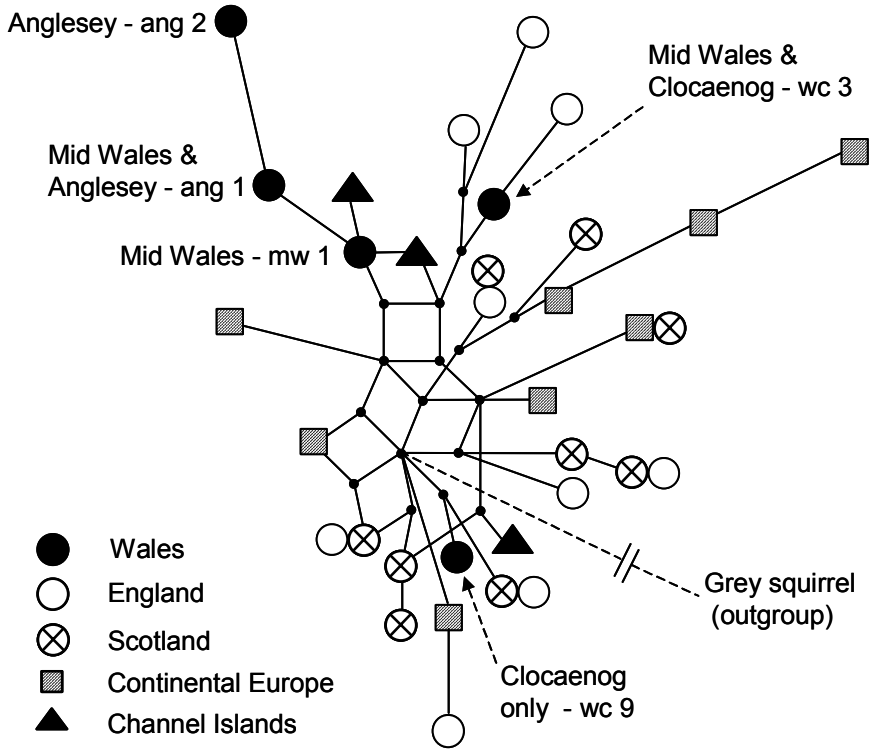


Figure 2: *A haplotype network of European red squirrels, based on observed mtDNA control region sequences. Symbols indicate sample origin for haplotypes, small black dots represent unsampled or extinct intermediate sequences. The three haplotypes observed in this study (ang 1, mw 1 and wc 3), are closely related and have been previously observed in Anglesey, Mid Wales and Clocaenog respectively.*

3. Implications for Conservation Management

Within-Species diversity

The relationships between mtDNA control region haplotypes in the UK and northern Europe carry little geographic information. This is evident from the median-joining network analysis (Figure 2) and from previous studies (3, 4). The network analysis also revealed a relatively large number of haplotypes missing from the main frame of the network. This is likely to be an artefact of historical translocations into the UK being from a subset of continental European populations, rather than an indication of the loss haplotypes from the species. The overall lack of geographic structure can also be explained by the frequent introduction of continental red squirrels into the UK during the 19th century, following native population declines (5). Such haplotype admixture has led to the suggestion that all UK populations would probably be genetically suitable sources of individuals for augmentation (3), however this is not necessarily the case in Wales.

Diversity in Wales

Results of a recent study (4) indicate a limited degree of phylogeographic structure among Welsh haplotypes. This means that most Welsh populations are more related to each other than they

are to other red squirrels in mainland Britain. This observation is important because it suggests that Welsh red squirrels represent a distinct group of populations. This has implications for both the conservation value of local populations and the way in which they should be managed. While it is important to maintain as much genetic diversity as possible in a population, it is also desirable to ensure that any squirrels brought into an area to help augment the population are not too genetically dissimilar from the original stock. In other words, with a very small isolated population, management should aim to reduce the risk of inbreeding, but also, at the other end of the scale, avoid the risks associated with outbreeding. The identification of three closely related Welsh haplotypes allows informed management of these risks and provides a possible framework for conserving Welsh populations without losing locally adapted characteristics.

Diversity in Mid Wales
Of the five mitochondrial DNA haplotypes observed in Wales, four show close genetic relationships to each other and may represent an ancestral Welsh stock. One of these four (ang2), has not been observed in a live squirrel for 25 years and. Of the remaining three haplotypes, all have been observed during this study of red squirrels in Mid Wales, compared to one extant haplotype on Anglesey and one haplotype in Clocaenog. This result indicates that the Mid Wales squirrel population is important as reservoir of genetic diversity within Welsh red squirrels as a whole. As such it identifies the Mid Wales population as a potential source of squirrels for translocation with the Pentraeth population on Anglesey, where the recent recovery project has successfully increased numbers, but also revealed a lack of genetic diversity. These findings should be taken into consideration when designing management strategies for both the red squirrels persisting in Wales and their associated habitat.

Future work
In order to more accurately assess levels of genetic diversity in Mid Wales, particularly prior to any possible reciprocal translocation events, it is recommended to undertake an analysis of nuclear genetic diversity, using microsatellites, to examine individual level relatedness and population variability.

References:

1. *Draft best practice guidelines for laboratory internal quality control*. European Molecular Genetics Quality Network. April 2003.
2. *Guidelines for Forensic Science Laboratories.* The International Laboratory Accreditation Cooperation ILAC-G19:2002.
3. Barratt EM, Gurnell J, Malarky G, Deaville R and Bruford MW (1999) Genetic structure of fragmented populations of red squirrel (Sciurus vulgaris) in the UK. *Mol. Ecol.,* **8,** S55-S63.
4. Ogden R, Shuttleworth C, McEwing R, Cesarini S (2005) Genetic management of the red squirrel, *Sciurus vulgaris*: a practical approach to regional conservation. *Conservation Genetics* (in press).
5. Shorten M (1954) *Squirrels.* Collins, UK.
6. Werle et al 1994: *Nucleic Acids Research* 22: 4354-4355
7. Atshul et al 1997: *Nucleic Acids Res*. 25:3389-3402

Appendix I Sample Storage

On entering WDNAS laboratories samples are given a project code and an individual identifier and both a paper and computer file are created for storage of all subsequent information regarding the samples. The sample storage laboratory is constantly locked when not in use, with access restricted to WDNAS personnel and building security. Files pertinent to the project are maintained within the offices of WDNAS. The paper file is locked within a filing cabinet and the computer file password protected. The office of WDNAS is constantly locked, when not occupied, and out of hours is protected by three separate door code mechanisms.

Appendix II DNA Extraction

DNA extractions are carried out in a dedicated DNA extraction laboratory, which is maintained as a clean room. All consumables and reagents are ordered solely for use in this laboratory, and where possible have been steam / pressure or ultraviolet sterilized. All bench work is undertaken within a laminar flow HEPA filtered cupboard, and all pipette tips are barrier protected.

Appendix III DNA PCR Amplification

PCR amplification takes place in our main laboratory using an MJ Research thermal cycler. A negative PCR control is included where ultrapure water replaces DNA, thus allowing the detection of contamination in any of the amplification chemistries. Target species positive controls are run alongside samples where appropriate.

Amplifications products are visualised by electrophoresis on a 2% ethidium bromide stained agarose gel. For DNA sequencing, products of the correct size are cleaned enzymatically using Shrimp Alkaline Phosphotase (SAP) and Exonuclease I (6).

Appendix IV DNA sequence resolution

The DNA sequence for each amplification product is resolved using a fluorescent dye based approach. The sequence reactions are carried out on an MJ Research thermal cycler and the DNA sequence is resolved using a Beckman-Coulter CEQ8000 automated sequencer.

The quality of each sequence recovered is assessed for peak height and spacing using the proprietary sequencing software (Beckman Coulter Inc.). In addition, all sequences are checked by eye for incorrect base calling, compressions and background noise.

The quality of each sequence is recorded following the procedure described by EMQN (1) i.e. a score of 1 is recorded when the quality of the sequence is excellent with unambiguous base calling and spacing; a score of 2 is recorded when the quality is less than perfect but reportable; and a score of 3 is recorded when a component of the sequence is not sufficiently resolved to allow a clear interpretation of the data, and is thus not reportable.

Appendix V Species determination

Determining the source species, or in actual fact the maternal source species, is accomplished by comparing the recovered mtDNA DNA sequences with homologous DNA sequences from other animal species. This is achieved using the National Centre of Biotechnology Information (NCBI) extensive DNA sequence species database. The search for the most probable match is performed using the BLASTn search algorithm (7) of all sequences held on the NCBI database. Such an approach of species identification from the mtDNA cytochrome b gene sequences has previously been validated (4).