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Genetic management of the red squirrel, *Sciurus vulgaris*: a practical approach to regional conservation

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Abstract

The progressive decline in red squirrel (*Sciurus vulgaris*) numbers in Wales has led to conservation and reintroduction projects being established on the island of Anglesey. The recovery of the island's remnant wild population was initially successful, however concern remained over potential loss of genetic diversity resulting from an observed demographic bottleneck. We used mitochondrial DNA (mtDNA) control region sequences and six microsatellite loci to assess current levels of genetic variation in the population. Samples were monomorphic for control region sequences and a historic specimen from the same area carrying a different haplotype demonstrated a loss of mtDNA diversity during the last 20 years. Inclusion of other Welsh haplotypes indicated phylogeographic structure in the region, in contrast to previous UK studies. Genotyping results showed allelic diversity and heterozygosity to be less than 50% of that recorded in other UK populations, with strong evidence for a recent genetic bottleneck. A parallel reintroduction programme on Anglesey included genetic analysis of individuals during the selection of captive breeding pairs. We present analysis of sequence and microsatellite data, and subsequent management decisions taken to maximise diversity in the founder and F1 generations. Population and Habitat Viability Analysis applied to both populations modelled future levels of heterozygosity and allelic diversity. Supplementation of the remnant and reintroduced populations with translocated squirrels was simulated as a potential management tool; results support use of this strategy to reduce loss of diversity and increase survival. The limitations of applying conservation genetic theory within small-scale management projects are discussed.

Introduction

The red squirrel, *Sciurus vulgaris*, is widespread across much of mainland Europe, but has suffered a dramatic decline in the United Kingdom over the last 50 years. The majority of populations in England and Wales have been lost due to a combination of habitat loss, resource competition from the introduced North American grey squirrel, *Sciurus carolinensis* (Skelcher 1997; Wauters *et al.* 2000; Gunnell *et al.* 2004) and the spread of the 'red squirrel parapox' virus (Sainsbury and Ward 1996; Sainsbury *et al.* 2000; Rushton *et al.* 2000; Tompkins *et al.* 2002). The current distribution of red squirrels in England and Wales is highly fragmented, with small populations vulnerable to extirpation. Previous genetic studies based on mitochondrial DNA (mtDNA) control region sequences revealed no evolutionary divergence between UK and northern European populations,

and a marked lack of phylogeographic structure among the nineteen UK haplotypes recorded (Barratt *et al.* 1999). These findings support the principle of augmentation of small isolated populations from larger stable groups as a potential conservation strategy.

In Wales, the need for active red squirrel conservation is particularly urgent with only three viable red squirrel populations remaining (Shuttleworth 2003). Of these, only the Pentraeth population on the island of Anglesey can be effectively isolated from grey squirrels (Figure 1). A conservation programme began in 1998 aimed at increasing the size of the Pentraeth population (Shuttleworth *et al.* 2002). In accordance with UK conservation guidelines (JNCC 1996; Pepper and Patterson 1998), the project focused on systematic control of grey squirrels on Anglesey, which have been implicated in the transmission of parapox (Tompkins *et al.* 2002) as well as resource competition. As a direct result of this strategy, red squirrel numbers at Pentraeth increased from fewer than 40 adults (1998) to 95 adults by the spring of 2002, corresponding to the effective eradication of grey squirrels from the area during the same period (Shuttleworth 2003). The red squirrel population has probably now reached carrying capacity at Pentraeth with juvenile recruitment falling rapidly since the initial population recovery. A second phase of the project is now underway, aimed at establishing a new population of red squirrels at Newborough, on the west coast of Anglesey (Figure 1). This involves the reintroduction of breeding pairs from captive populations into a woodland habitat known to have supported red squirrels prior to grey squirrel introduction.

The use of genetic management in conserving threatened populations is widely recognised, both for accurately assessing contemporary and historic diversity and for developing individual and population level strategies for the future (Frankham *et al.* 2002). Both the recovery programme at Pentraeth and the reintroduction programme at Newborough have incorporated genetic screening to examine current levels of diversity. This paper describes the results of mitochondrial sequencing and microsatellite genotyping, and their immediate use in constructing an applied conservation strategy for red squirrels on Anglesey.

Pentraeth population

The initial success of the Pentraeth recovery project has now been followed by an assessment of population genetic diversity in this recovering group. Here, we present mtDNA control region data to examine population structure and assess the nucleotide diversity remaining in the population. The gene was chosen as a rapidly evolving marker suitable for population studies and to allow incorporation of the results into the wider UK dataset (Barratt *et al.* 1999). Our study also includes Mid-Wales and historical Anglesey specimens, enabling a broader assessment of population diversity in Welsh red squirrels than is currently available.

Large, sustained reductions in population size or non-random mating may lead to bottleneck events, which can increase extinction risk within

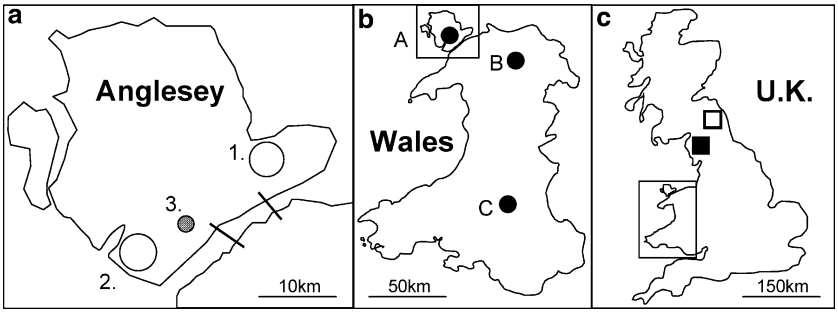

Figure 1. Geographic origins of the relevant red squirrel populations: (a) the study populations on Anglesey (1 = Pentraeth, 2 = Newborough, 3 = origin of the 20-year old historical specimen); (b) the three remaining viable populations in Wales (A = Pentraeth, B = Clocaenog, C = Mid-Wales); (c) comparative UK populations (closed symbol = north-west England, open symbol = north-east England).

populations (Newman and Pilson 1997). Given the recent population history at Pentraeth, combined with the hierarchical mating system of the red squirrel, mitochondrial and nuclear markers are employed to test for evidence of bottlenecks. Reduced genetic diversity has been associated with reduced fitness (Reed and Frankham 2001), but estimating the extent of genetic loss without baseline data requires the use of control populations (Bouzat 2001). In the absence of comparative historic datasets, the current study uses microsatellite loci previously applied to red squirrel populations in northern England (Hale *et al.* 2001b) enabling the levels of nuclear diversity observed at Pentraeth to be interpreted.

Newborough reintroduction

Reintroducing captive animals into the wild is fraught with difficulties. From a genetic viewpoint, the negative consequences of deleterious allele fixation in captivity (Lynch and O'Hely 2001), relaxed selection in captivity (Bryant and Reed 1999) and selective adaptation to captivity (Frankham and Loebel 1992) are well-documented problems. Levels of success vary widely in reintroductions, but certain trends have become evident. Higher success rates are often observed when wild-caught vs. captive-bred individuals are used (Wolf *et al.* 1996), when reintroductions occur in core historical habitat, and where larger numbers are introduced over longer timescales (Beck *et al.* 1994), Despite the continued development in applied conservation genetic strategy, optimal genetic management often remains an under-recognized criterion in conservation, competing for finite resources. The scope of genetic work undertaken prior to the reintroduction at Newborough has consequently been restricted. Nevertheless, through genetic analysis of founder individuals, we attempt to promote genetic management 'best practice' within the framework of the reintroduction project.

The reintroduction programme at Newborough began in 2003 with the construction of large pens (120 m^3) within the forest to house breeding pairs. The management strategy is to release F1 progeny, but retain adult pairs in the pens and exchange them for new founders in subsequent years. The availability of red squirrels for such reintroductions is limited to captive bred animals or those captured under license. Non-genetic criteria such as individual squirrel behaviour and regional politics also limit the number of candidate squirrels for reintroduction. However, within such restrictions, genetic selection of founders has been applied as a project management tool at Newborough.

The genetic aims of population reintroduction are to maximize the diversity of the founders and to minimize the loss of heterozygosity (Frankham *et al.* 2002) and allelic diversity (Grativol 2001) in the F1 generation. This can initially be achieved through careful selection of breeding pairs. Here, we present genetic data for founder individuals and describe subsequent breeding pair strategies designed to maintain genetic diversity in this new population.

Population viability analysis

In addition to historical demographic data and contemporary genetic data, the development of effective management strategies on Anglesey required an indication of the future viability of the two populations. Population Viability Analysis (PVA) is widely used to model extinction risk and test potential management strategies in small populations (Beissinger 2002). Although its use has been cautioned (Coulson *et al.* 2001), it is still supported as the most useful tool for accurately predicting future demographic change (Brook *et al.* 2000, 2002). Population dynamic modeling has been used to examine the spatial distribution of red squirrels under the effects of different forest management strategies (Lurz *et al.* 2003) and the presence of grey squirrels (Rushton *et al.* 1997) in the UK. This work demonstrated the need to incorporate information on habitat structure to accurately model red squirrel populations, therefore Population and Habitat Viability Analysis (PHVA) was implemented in the current study.

Despite only occasional inclusion in published PVAs (Groom and Pascual 1998) the importance of genetic considerations in population viability modeling is widely recognized, especially where small populations are reproductively isolated (Allendorf and Ryman 2002). The application of PHVA described here is directed towards evaluating the effect of demographic predictions on levels of heterozygosity and allelic diversity.

Simulations are run under current management conditions and include genetic augmentation via translocation of individuals to help maintain genetic diversity and direct a reduction in the long-term extinction risk.

Materials and methods

Sampling

A total of 62 red squirrels were included from the four sample groups. 48 individuals were sampled from the Pentraeth population in the east of Anglesey. One extant individual from Mid-Wales and one individual recovered in 1983 as a road kill on Anglesey were also included as Welsh origin squirrels. In addition, 12 candidate individuals for the Newborough reintroduction programme were screened. The origin of these animals is split between captive bred squirrels from unknown geographic provenance and wild-caught (under licence) individuals from Cumbria, in northern England.

Plucked hairs were used as a source of DNA. Between 10 and 20 hairs were recovered from each individual during licensed mark/recapture studies (Pentraeth and Mid-Wales) or from caged individuals prior to reintroduction (Newborough). The 20-year-old Anglesey specimen had been frozen whole since 1983. Hairs were stored in 80% ethanol at −20 °C prior to extraction.

DNA was extracted within a dedicated laboratory from hair root cells (Pentraeth, Mid-Wales & Newborough) or whisker root cells (20-year-old specimen) using the Genelute™ Mammalian DNA Extraction kit (Sigma-Aldrich). Multiple contamination controls were run alongside all DNA extractions.

PCR and Sequencing

Sequencing was undertaken on 25 of the Pentraeth individuals and all the available samples from the other sample groups. Novel mtDNA control region primers were designed (Sq070F: 5′–ATG CCT GTC AAA GAG CAT AG & Sq388R: 5′–TAG AAC ATA TCA TGT TTA AT) based on the published haplotypes (Barratt *et al.* 1999), which amplified only 280 bp, but incorporated 100% of the sequence variation previously observed. Reactions were carried out in 20 μl volumes containing 2 μl of Taq buffer (0.67 M Tris–Cl, pH 8.8, 0.02 M $MgC1_2$, 0.166 M NH_2SO_4, 0.1 Mβ mercaptoethanol), 8 μl dGATC-mix (0.5 μM per nucleotide), 2 μl of each primer (10 μM), 5 μl of ddH_2O, 0.10 Jumpstart Taq DNA polymerase (Sigma) and 1 μl of extracted genomic DNA (approximate concentration 20 ng μl^{-1}). PCR reactions were carried out using a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 2.5 min at 94 °C, 31 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 30 s, then 7 min at 72 °C. PCR product was cleaned using both undiluted Shrimp Alkaline Phosphotase (1 unit) and Exonuclease I (2 units) and incubated at 37 °C for 45 min. PCR products were sequenced using BIG DYE v3.1 chemistries (ABI) and the forward primer (Sq070F) under standard conditions and then resolved on an ABI 377 automated sequencer.

Microsatellites

Microsatellite screening was carried out on 48 individuals from Pentraeth to examine levels of nuclear diversity and 12 individuals from the Newborough reintroduction programme to assist in the choice of breeding pairs. Six previously published microsatellite loci (Scv3, Scv8, Scv9, Scv10 (Hale *et al.* 2001a) & Rsu4, Rsu5 Todd 2000) were chosen for their variability and previous use in analogous studies (Hale *et al.* 2001b). 10 μl PCR reaction volumes were used consisting of 1× PCR buffer (50 mM KC1, 20 mM Tris–HCl pH8.4), each dNTP (120 μM), each primer (1 μM), Taq DNA polymerase (0.75 U) (Sigma), magnesium chloride (1.5 mM) and 10 ng of DNA. PCR conditions followed those published for each locus. PCR products were subsequently separated on a denaturing polyacrylamide gel, detected using an ABI 377 automated sequencer and scored using GENESCAN and GENOTYPER software (ABI).

Genetic analysis

Sequences were edited by eye using CHROMAS vl.6 and then incorporated into the data set containing mtDNA control region haplotypes from around the UK and northern Europe (Barratt *et al.* 1999). This included two haplotypes (wc3

and wc9) sampled from Clogaenog in north-east Wales (Figure 1). Previously unobserved sequences were deposited in GenBank, under accession numbers AY372270–AY372272 and AY534120–AY534121.

Gene diversity was examined by estimating pairwise nucleotide distances (uncorrect p-distance) using the software MEGA v.2.1 (Kumar *et al.* 2001). Genealogical reconstructions were performed using maximum parsimony (MP), maximum likelihood (ML) (neighbour-joining, K2P) and genetic distance (GD) (neighbour-joining, K2P) approaches with PAUP* (Swofford 1998). Bootstrap support for each node was calculated (MP and ML, 100 reps; GD, 10,000 reps). Given the low resolution found during reconstruction of the original data set (Barratt *et al.* 1999), sequence data were also analysed using the median-joining network analysis programme, NETWORK v.4.1 (http://www.fluxus-engineering.com). This approach is designed to investigate intraspecific phylogenies and displays multiple evolutionary paths between haplotypes that would reduce to polytomies in a tree reconstruction. The resulting network also allows inference of unsampled or extinct sequences within the network (Bandelt *et al.* 1999).

For the Pentraeth population, the possibility of having missed haplotypes due to insufficient sampling was investigated using the programme GenesAmp (Sjogren and Wyoni 1994). Microsatellite data were examined for departures from Hardy–Weinburg equilibrium and null alleles using GENEPOP v 3.3 (Raymond and Rousset 1995), before calculating allelic diversity and effective allele numbers. Evidence of a recent bottleneck event was investigated using the programme BOTTLENECK (Cornuet and Luikart 1996; Piry *et al.* 1997).

PHVA

Modelling was performed using VORTEX 9.161 (Lacy 2001), with simulations run under a range of conditions to assess different management criteria. Although the PHVA usually examines 100-year demographics, a time period of 20 years was used here to realistically simulate the duration of management and habitat conditions on Anglesey. The short generation time of the red squirrel (1 year) allows a meaningful number of generations to be modelled in this time period. Each model was run 10,000 times, using the species and population parameters shown in Table 1. Initial demographic parameters were based on studies of other UK populations (see Table 1) that had previously been subjected to sensistivity analysis and validated against historical datasets (Rushton *et al.* 1997).

Table 1. A summary of the input parameters used in PHVA simulations at Pentraeth and Newborough, estimated from referenced historical data. Asterisk (*) denotes estimated effect of supplemental feeding

Parameter	Value		Ref.
Inbreeding depression	No		Wauters et al. (1994)
Catastrophe (parapox virus), effect on Survival and Reproduction	S: 0.25 R: 1.00		Tompkins et al. (2002)
Mating system	Polygynous		Gunnell (1987), (1991)
Female breeding age	1 year		Gunnell (1987), (1991)
Male breeding age	1 year		Gunnell (1987), (1991)
Max. breeding age	6 years		Gunnell (1987), (1991)
Sex ratio at birth	1:1		Gunnell (1987), (1991)
Max litter size (1–2 litters per year)	7		Holm (1989)
% Adult females breeding	70	73*	Shuttleworth et al. (2002)
% Adult males breeding	80	83*	Shuttleworth et al. (2002)
% Mortality in year 1	65	60*	Rushton et al. (1997), Wauters (2000)
% Mortality adult	45	40*	Rushton et al. (1997), Wauters (2000)
Adult mortality SD	20	15*	Shuttleworth et al. (2002)
Carrying capacity (Pentraeth)	110		Shuttleworth et al. (2002)
(Newborough)	200		Estimate based on number of adult grey squirrels removed

Table 2. Mitochondrial control region haplotypes generated in this study, showing sample origin and number, with prior publication codes and sources, where relevant

Sample Group	Haplotype	*n*	Previously recorded
Pentraeth	ang1	25	No
Anglesey 20 ya	ang2	1	No
Mid-Wales	mw1	1	No
	wmz1	2	No
	wmz2	2	No
Newborough	ah163*	1	
Reintroduction	ah178*	2	Scotland/England
	cl7*	3	Border region
	s6*	2	

*Haplotype codes follow Barratt et al. (1999)

Data from monitoring the Pentraeth population during the first 5 years of the conservation project were also included (Shuttleworth *et al.* 2002). Carrying capacities were estimated within the geographical boundaries of the Pentraeth (200 ha) and Newborough (550 ha) mature coniferous woodlands. These are minimum estimates, as adjacent broad-leaved habitats are likely to be colonised following population expansion. Catastrophes were limited to outbreaks of parapox virus, controllable through the continued eradication of grey squirrels. Supplemental feeding reportedly increases individual survival and breeding success in red squirrels (Magris and Gurnell 2002). Quantification of this effect has not been previously published, but is estimated here (Table 1) for use as a potential management tool.

For the Newborough population, a founder group of four females and four males was used with subsequent augmentation initially set to one female and one male annually for 9 years. This simulates the founder group with a realistic level of future founder availability to the project. Other population parameters remained the same as those used to model the Pentraeth population.

Selection of breeding pairs

There are many published recommendations and management strategies aimed at minimising loss of diversity in captive populations through selective breeding (see Fernandez and Caballero 2001). However, these all assume some knowledge of the ancestral relationships between candidate individuals. In this study, we had no pedigree information, but knew the mtDNA haplotype and the nDNA multi-locus genotype. We therefore devised a two-stage process, firstly selecting eight individuals (4 male, 4 female) from the 12 candidates and secondly selecting 4 breeding pairs from these 8. Criteria used at the first stage were, (i) selection of wild-caught in preference to captive-bred squirrels, (ii) maximisation of allelic diversity in the population, (iii) maximisation of heterozygosity in the population. The second-stage selection (breeding pairs) was based on minimising relatedness between pairings. This was undertaken using the programme RELATEDNESS (Goodnight Software 2001) that produces a matrix of relatedness coefficients (r) for pairs of individuals based on allele frequencies (Queller and Goodnight 1989). The first pair selected was comprised of the lowest pairwise r-value, these two individuals were then removed and the next lowest r-value was used to select the next breeding pair. This process was repeated until only one pair remained.

Results

mtDNA sequences and genealogy

The three Welsh sample groups (Pentraeth, Mid-Wales and Anglesey-historical) each displayed different, previously unpublished, haplotypes (Table 1). The Pentraeth population was monomorphic for the 25 individuals sequenced with a sample size sufficient to detect haplotypes at a frequency of $>0.1 (P<0.05)$. The twelve squirrels for reintroduction at Newborough displayed six haplotypes, two of which were previously unpublished with four others being previously recorded in northern England and southern Scotland.

Genetic distances among the three new Welsh haplotypes (ang1, ang2 and mwl) were smaller than their respective distances from the Newborough reintroduction haplotypes (wmzl and wmz2) and the remainder of the previously published European haplotypes (Table 3). The two haplotypes recorded from Clocaenog (wc3 and wc9) were more distant from each other ($d=0.024$) than wc3 was from the mid-Wales haplotype ($d=0.015$), indicating that the Clocaenog population is comprised of mixed lineages that may include an ancestral Welsh population.

Table 3. A genetic distance matrix of the five new haplotypes found in this study, the two haplotypes recorded at Clocaenog (north-east 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. Overall mean for ingroup samples $d = 0.027$

Origin	Haplotype Code	ang1	ang2	mw1	wmz1	wmz2	wc3	wc9
Anglesey	ang1							
	ang2	0.011						
Mid-Wales	mw 1	0.007	0.019					
Newborough	wmz 1	0.027	0.035	0.020				
reintroduction	wmz 2	0.038	0.046	0.030	0.027			
Clocaenog, NE	wc 3	0.022	0.033	0.015	0.027	0.030		
Wales	wc 9	0.030	0.034	0.022	0.015	0.019	0.024	
UK/Europe	Others	0.032	0.038	0.025	0.027	0.033	0.023	0.024

Genealogical reconstruction combining the five new haplotypes with the 26 previously published (Barratt *et al*. 1999) yielded polytomic trees with little internal structure that were topologically identical under MP, ML or GD approaches (Figure 2a, MP strict concensus tree shown). However, a low level of bootstrap support was returned for the mid-Wales and Anglesey populations, together with the Channel Island haplotypes, suggesting a degree of phylogeographic structure in Wales that is not found in the rest of the UK (Figure 2a). These Welsh populations did not include the two haplotypes sampled from Clocaenog or the Newborough reintroduction, which remained unresolved among the other European haplotypes.

Median-joining network analysis supported the grouping of mid-Wales and Anglesey populations and highlights their similarity to the Channel Island haplotypes (Figure 2b). The network also confirmed the general lack of phylogeographic structure in UK and northern European red squirrels shown in the MP tree and originally revealed by Barratt *et al*. (1999). The two Clocaenog haplotypes were positioned on opposite sides of the network, with wc3 placed relatively close to the mid-Wales sample. The main frame of the network included eleven inferred haplotypes representing sequences that were either unsampled or have become extinct. The haplotypes present in the reintroduction programme at Newborough, including the two new haplotypes (wmz 1 and wmz 2), were distributed around the network but were separated from the Anglesey/mid-Wales group by at least four intermediate sequences.

Population analysis at Pentraeth

Results of the microsatellite screening at Pentraeth revealed very low levels of genetic diversity (Table 4). Three of the six loci were found to be monomorphic, two loci displayed two alleles and the remaining locus three alleles. The allele frequencies at this last locus meant that the effective allele number was considerably lower than three ($n_e = 2.2$). The average number of alleles per locus, or allelic diversity, was $A = 1.5$. This value is markedly lower than levels of allelic diversity found in two populations in northern England from smaller sample sizes (Hale *et al*. 2001b, Table 4). Similarly, expected heterozygosity was less than half that observed in the two comparable populations. No significant departures from Hardy–Weinberg equilibrium were recorded ($P = 0.01$). Bottleneck analysis was limited by the fact that only three loci were polymorphic. This result in itself suggests that genetic diversity has fallen significantly, however three loci are too few for the programme BOTTLENECK to be implemented (minimum loci, $n = 4$). Qualitative analysis was therefore undertaken to examine the allele frequency distribution. Recently, bottlenecked populations are expected to show a mode-shift in this distribution due to the pronounced loss of alleles at low frequency (Luikart *et al*. 1998). The shift from an L-shaped curve expected under equilibrium conditions to the

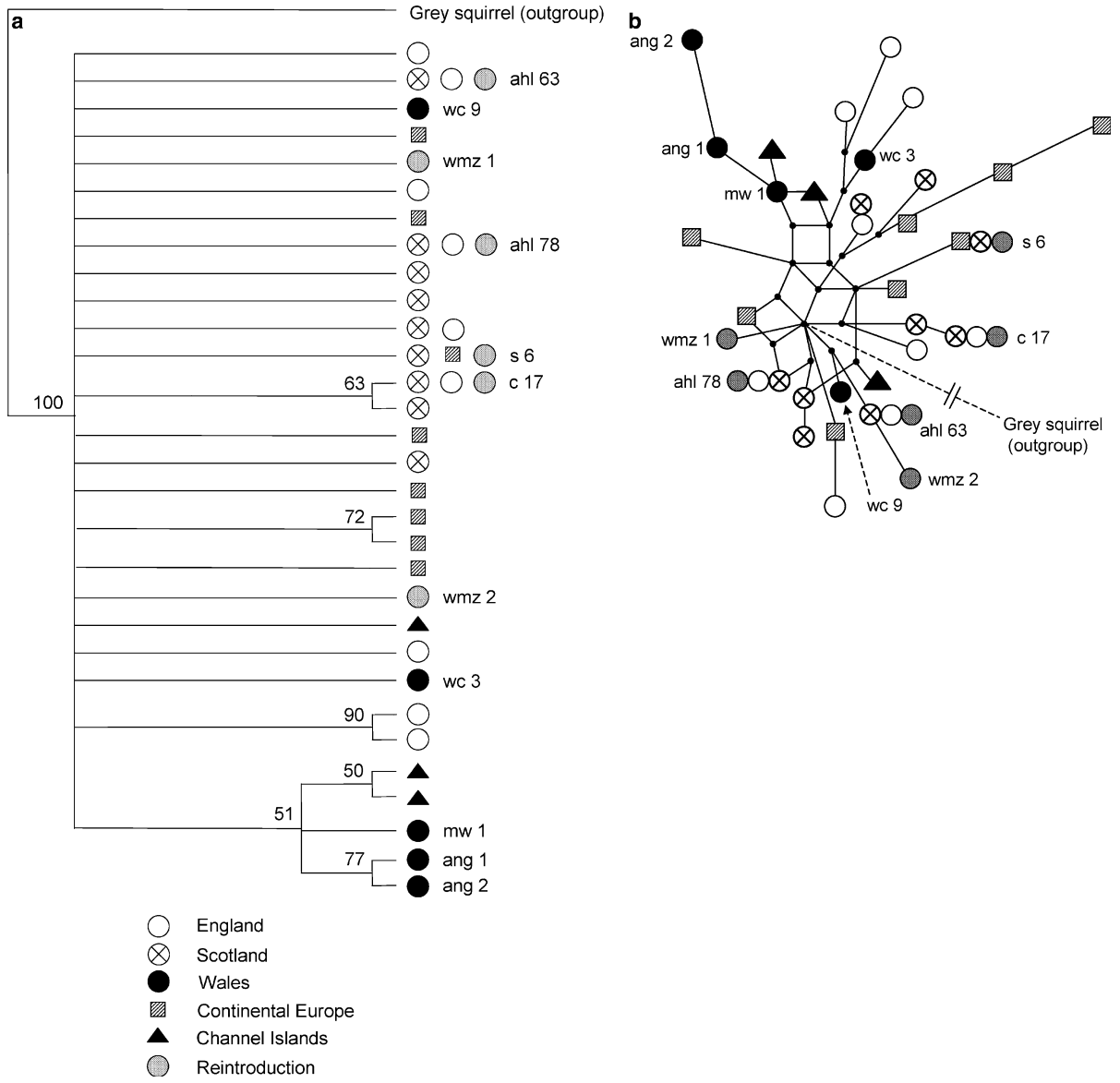

Figure 2. (a) Maximum parsimony (strict consensus) tree of UK and northern European mtDNA control region haplotypes. Multiple symbols indicate that haplotypes were observed in more than one region. Low bootstrap support is concordant with previous work (Barratt et al. 1999), however some phylogeographic structure is evident for Welsh populations. Newborough reintroduction individuals distributed throughout the tree include two new haplotypes (wmz1 & wmz2). The positions of previously observed haplotypes from Clocaenog (wc3 and wc9) are unresolved. (b) Median-joining network of the observed mtDNA control region sequences. Symbols indicate sample origin for haplotypes (key follows Figure 2a, haplotype codes follow Table 2), black dots represent unsampled or extinct intermediate sequences.

distribution pattern found at Pentraeth is indicative of a recent bottleneck event in this population (Figure 3).

The PHVA for Pentraeth indicated that under current management conditions, including continued grey squirrel exclusion and appropriate tree planting schemes, the probability of population survival was 0.82 with the average size falling to 51 squirrels in extant populations over 20 years (Table 7). Heterozygosity would be expected to fall by 19% over the same period, with 11 alleles remaining. Subsequent simulations were run to include additional feeding and allowing for the genetic supplementation (reciprocal translocation)

Table 4. Microsatellite results from Pentraeth showing allele frequencies, expected heterozygosity (H_E), effective allele number (n_e) and allelic diversity (A) for each locus. Expected heterozygosity and allelic diversity are markedly low relative to other UK populations. No deviation from Hardy–Weinberg equilibrium was observed

Locus	Allele			n_e	n	H_E	A, allelic diversity
	1	2	3				
Scv3	1.000			1.000	46	0.224	1.50
Scv8	0.471	0.529		1.993	35	Scv loci	Scv loci
Scv9	0.739	0.261		1.628	46		
Scv10	1.000			1.000	46	0.242	1.67
Rsu4	1.000			1.000	46	all loci	all loci
Rsu5	0.122	0.600	0.278	2.212	45		
North East England population (Scv loci only)*					30	0.527	4.50
North West England population (Scv loci only)*					21	0.555	4.00

*Data for other populations only available for Scv loci.

of adult females each year. Under this management strategy the probability of survival increased to 0.97, estimated heterozygosity increased to 0.89 and the number of extant alleles almost doubled ($n = 21$).

Population analysis at Newborough

Selection of eight founder individuals from the 12 candidate squirrels screened for the Newborough reintroduction was performed based on individual genotypes (Table 5). The two wild-caught individuals from Cumbria (R3&R4) were selected by default because, although possibly related, they were not liable to the negative effects of captive breeding. The remaining six animals were chosen on the basis of the number of alleles they possessed that were not present in the two wild-caught squirrels, and the number of heterozygous loci that they displayed. Unfortunately two squirrels died between genetic screening and the commencement of reintroduction, further reducing the pool of candidate individuals.

The eight individual squirrels selected for the reintroduction programme at Newborough displayed markedly more allelic diversity ($A = 4.50$) than at Pentraeth ($A = 1.5$) (Table 6). Levels of heterozygosity were also higher at all loci. Pairwise relatedness coefficients allowed kinship to be minimised between individuals in successive pairings (Table 6). This resulted in pairing individuals R2&R6, followed by Rl&R4, R5&R9 and lastly R3&R7. These pairings were then recommended for the Newborough reintroduction study.

The PHVA model for the reintroduction at Newborough was strongly influenced by the annual addition of a new breeding pair for the first 10 years. Under this level of supplementation, 20-year simulations resulted in a 25% loss in heterozygosity and a 25% risk of extinction (Table 7). Without this level of genetic augmen-

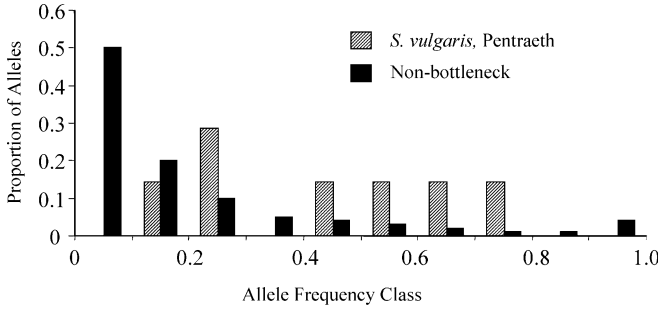

Figure 3. Allele frequency distribution for the six microsatellite loci screened in the Pentraeth population ($n = 46$), compared to an idealized non-bottleneck population (Luikart et al. 1998). The shift away from an L-shaped distribution suggests a recent bottleneck event.

Table 5. Results of mitochondrial sequencing and microsatellite genotyping for the 12 candidate squirrels in the reintroduction programme at Newborough. Asterisks (*) indicate wild-caught individuals selected by default, crosses (+) indicate squirrels that died between sampling and selection

Individual	Sex	Origin	mtDNA Haplotype	Microsatellite Locus Allele Scores						Selected
				SCRV3	SCRUB	SCRV9	SCRV10	RSU4	RSU5	
R1	F	Captive	wmz 1	205/209	202/204	197/199	71/73	277/289	132/138	Y
R2	F	Captive	wmz 1	209/211	204/204	197/199	79/81	289/289	138/142	Y
R3	F	Cumbria	ah178	201/205	204/204	197/197	73/81	269/273	134/138	Y*
R4	M	Cumbria	ah178	201/203	202/204	197/201	73/81	269/269	134/140	Y*
R5	M	Captive	c17	209/211	202/204	195/199	73/81	273/273	132/138	Y
R6	M	Captive	c17	203/205	202/202	197/199	73/81	273/273	132/132	Y
R7	M	Captive	c17	209/211	202/204	197/199	81/81	273/273	132/138	Y
R9	F	Captive	ah163	205/207	202/202	197/197	73/81	265/273	136/138	Y
R10+	F	Captive	s6	209/211	202/202	195/201	73/81	269/269	136/138	N
R11	M	Captive	s6	209/211	202/204	197/201	73/73	269/269	138/138	N
R13	F	Captive	wmz2	203/205	202/202	197/197	71/73	273/273	138/138	N
R14+	M	Captive	wmz2	201/203	202/204	195/199	73/81	273/281	138/138	N

Table 6. Matrix of pairwise relatedness between the eight individuals selected for reintroduction at Newborough, indicating the pairs chosen starting with the lowest value of *r*. These *n*DNA-selected pairs also lead to the separation of mtDNA lineages. Mean allelic diversity (*A*) far exceeds levels found at Pentraeth and mean heterozygosity (H_E) exceeds levels previously found in any wild UK population

Individual	Pairwise *r*								Pairings		mtDNA
(Sex)	R1	R2	R3	R4	R5	R6	R7	R9			Haplotypes
Rl (F)	*								1st	R2&R6	wmz1, c17
R2 (F)	0.21	*							2nd	R1&R4	wmz1, ah178
R3 (F)	0.22	0.07	*						3rd	R5&R9	c17, ah163
R4 (M)	**−0.47**	−0.27	0.19	*					4th	R3&R7	ah178, c17
R5 (M)	−0.14	−0.03	−0.10	−0.55	*						
R6 (M)	0.02	**−0.49**	0.03	−0.20	0.34	*				Mean (A) = 4.50	
R7 (M)	−0.17	0.19	**0.16**	−0.38	0.70	0.39	*			Mean H_E = 0.686	
R9 (F)	−0.15	−0.42	0.03	−0.22	**−0.25**	0.27	0.02	*			

tation, PHVA predicts a 41% loss of heterozygosity and a 74% risk of extinction during the same 20-year period. Furthermore, the supplemented population retained more than twice the number of alleles ($n = 9$) than the non-supplemented population ($n = 4$).

Discussion

The emergence of conservation genetics as a recognised research field over the past decade has established the foundations for its widespread application to wildlife management today. Having previously been limited to the high-budget conservation of flagship species (e.g. Palsboll *et al.* 1999), genetic techniques are becoming increasingly available to conservation management of less charismatic taxa at a local scale. The accelerated publication of baseline phylogenies and population genetic studies for many species of flora and fauna, combined with the development of theoretical genetic management, have paved the way for molecular data to be incorporated into everyday conservation strategy. This paper reflects this broadening use of conservation genetic approaches in small-scale projects and demon-

Table 7. Results of PHVA simulations run for the Pentraeth and Newborough populations over 20 years under current management conditions. The effect of supplementation on the probability of population survival ($P(S)$), population numbers (N), expected heterozygosity (H_E), and number of alleles (A) shows the beneficial genetic effect of translocation into the Pentraeth population and the necessity to supplement the Newborough population in terms of genetic diversity and population survival

Population	Scenario	$P(S)$	N	H_E	A
Pentraeth	Not Supplemented	0.82	51	0.81	11
	Supplemented	0.97	60	0.89	21
Newboroughs	Not Supplemented	0.26	45	0.59	4
	Supplemented	0.75	52	0.75	9

strates the benefit of genetic data for making informed management decisions.

Genetic diversity on Anglesey

Prior to the recovery project at Pentraeth, the spread of grey squirrels across Anglesey resulted in the rapid loss of red squirrels over a 20-year period. The observation of an mtDNA haplotype present in the 20-year-old Anglesey specimen, but absent from the remaining population sample today, strongly indicates that red squirrel decline has reduced genetic diversity on Anglesey. The remnant population at Pentraeth is likely to have remained due to the favoured coniferous habitat type (Lurz *et al.* 1995; Skelcher 1997), rather than as a result of any genetic advantage displayed by the group. Indeed, there is no historical evidence to suggest that the population was isolated from the wider island population. As such, it can be assumed that loss of genetic diversity from Anglesey represents a reduction in potential adaptive genetic variation rather than a purge of deleterious alleles.

The results of microsatellite analysis at Pentraeth further indicate reduced genetic diversity in the population. Allelic diversity was less than half that observed in populations of red squirrel from northern England (Hale *et al.* 2001b). The mode-shifted allele frequency distribution is characteristic of a recent bottleneck in the population, in which rare alleles are rapidly lost during population decline (Luikart *et al.* 1998). Levels of heterozygosity were similarly reduced from those found in northern England and can also be shown to be concordant with a sustained bottleneck of the Pentraeth population. If mean levels of heterozygosity in northern England are taken as an stimate of pre-bottleneck heterozygosity at Pentraeth (H_o), then it is possible to calculate the reduction in heterozygosity at Pentraeth as

$$H_t/H_o = 0.224/0.541 = 0.41 \text{ or a } 59\% \text{ reduction in } H_E \quad (1a)$$

The expected loss of heterozygosity over time under a sustained bottleneck can be calculated for a population (Frankham *et al.* 2002, Equation lb). Based on a minimum size at Pentraeth of $N = 39$ adults and an N/N_e ratio of 0.1, we may assume an effective population size of $N_e = 3$. Over 5 years the expected loss of heterozygosity under such a bottleneck would then be

$$H_t/H_o = [1 - 1/(2N_e)]^t = [1 - 1/(2 \times 3.9)]^5 = 0.50 \text{ or a } 50\% \text{ reduction in } H_E \quad (1b)$$

The level of assumption behind such estimates prevents any quantitative interpretation of the equations, however, the similarity between observed loss of heterozygosity and loss expected under bottleneck conditions does support the occurrence of a recent bottleneck at Pentraeth and indicates the severity of its effect on levels of population genetic diversity.

Population decline is often accompanied by deviations from Hardy–Weinberg Equilibrium (HWE) as fragmentation effectively leads to inbreeding within the population as a whole. However on Anglesey, as only a single population persists, the observation of HWE at Pentraeth was to be expected, despite the reduction in heterozygosity.

The occurrence of three monomorphic loci at Pentraeth from known polymorphic microsatellites, combined with DNA extraction from hair follicles, raises the issue of allelic dropout as an

alternative explanation to allelic loss (Taberlet and Luikart 1999). However, the use of multiple hairs for red squirrel DNA recovery has previously been shown to produce accurate genotypes for these microsatellites (Hale *et al.* 2001a) and the loci yielded multiple alleles for individuals from Newborough, therefore allelic dropout is not considered a likely cause of low diversity.

Loss of diversity together with a known reduction in census size raises concern over the level of genetic variability remaining within this wild population. Given that its isolation as a remnant group is probably the result of a non-selective sweep through the Anglesey red squirrels, reduced genetic variation poses a potential threat to the future viability of the population. However, despite evidence of a recent genetic bottleneck event and current low levels of nuclear and mitochondrial variation, there have been no immediate signs of inbreeding depression at Pentraeth (Shuttleworth *et al.* 2002). This observation is consistent with a previous study recording the effects of a similar, if less severe reduction in red squirrel population genetic diversity in Europe (Wauters *et al.* 1994), prompting the question of whether or not to augment the Pentraeth population with individual squirrels from elsewhere. There are clear risks associated with translocation, including the spread of disease, and incompatible behavioural and habitat adaptations. Nevertheless, if the decision is delayed until deleterious heritable traits begin appearing in an inbred population, it may be a much more difficult task to redress the problem.

Demographic modelling can be a useful tool in assessing future threats to a population, including increased levels of inbreeding (Brook *et al.* 2000). The PHVA at Pentraeth demonstrated the effect of translocating females into the population, reducing the loss of heterozygosity and increasing the overall probability of survival. Use of PHVA in a relative, rather than an absolute sense, is often recommended (Beissinger and Westphal 1998), as although PHVA may not be an accurate predictor of population response, it does bring clarity to a problem by identifying the key aspects of management strategy requiring attention (Brook *et al.* 2002). As is shown here, the identification of genetic diversity as a future management issue helps reinforce the conclusions of past molecular observation. At the time of writing, no decision has been made over whether or not to introduce females to Pentraeth. Nevertheless, it would be prudent to consider which populations offer the best candidates for future translocation.

The phylogenetic relationships between mtDNA control region haplotypes in the UK and northern Europe carry little geographic information. This was evident from the unresolved nature of the reconstructed trees presented here and by Barratt *et al.* (1999) and also from the median-joining network analysis. The network analysis also revealed a relatively large number haplotypes missing from the main frame of the network. This is likely to be an artefact of translocations into the UK being from a subset of extant continental European populations rather than the widespread loss of eleven haplotypes from the species. The overall lack of phylogeographic structure can also be explained by the frequent introduction of continental red squirrels into the UK during the 19th century, following native population declines (Shorten 1954). Such haplotype admixture has led to the suggestion that all UK populations would probably be genetically suitable sources of individuals for augmentation (Barratt *et al.* 1999). However, results of the current study indicate a limited degree of phylogeographic structure among Welsh haplotypes and this in turn may influence the choice of candidates for translocation into the Pentraeth population. The observed bootstrap support for the mid-Wales and two Anglesey haplotypes, although low, would suggest the mid-Wales population as genetic source for translocation into Pentraeth. A mid-Wales individual may also be favoured in terms of proximity, habitat similarity and political will. Of the previously recorded Clocaenog haplotypes, one of the samples (wc3) could also be considered a suitable translocation candidate. Despite the lack of bootstrap support in the reconstructed MP phylogeny, wc3 appears closely related to the mid-Wales sample from both the network analysis and haplotype distance matrix, indicating a relatively recent common ancestor. However, the presence of an unrelated haplotype at Clocaenog (wc9) suggests that this population is mixed with red squirrels originating elsewhere, compromising Clocaenog as a source of 'pure Welsh stock' for translocation into Pentraeth.

Newborough reintroduction

Prior to the commencement of the project, the selection of an entirely Welsh founder population was proposed for the reintroduction project at Newborough. This would have the advantages of regional habitat adaptation, maintenance of a 'Welsh' stock on the island and consequently greater local conservation support. However, following the discovery of very low genetic diversity at Pentraeth, and the likely low level of diversity in the smaller mid-Wales population, it was concluded that use of Welsh squirrels was not a suitable option. Furthermore, the trapping, screening and translocation of wild red squirrels in the UK can only be carried out under strict licence and was not an option available to the programme. This effectively limited the choice of candidates to captive squirrels, 20 of which were available to the project. Of these, only 12 were deemed suitable on the bases of age, behaviour and health. A larger candidate pool containing a much higher percentage of wild-born squirrels would clearly have been preferable.

The results of mitochondrial and microsatellite screening from the Newborough squirrels demonstrated that the group contained relatively high genetic diversity compared to both the Pentraeth population and populations from the north of England. This is the result of selecting for maximum diversity in the founders and is an encouraging sign for the reintroduction programme. It should be noted however that with such a low founder population size ($n = 8$), heterozygosity will rapidly decrease within the new population. The results of PHVA modelling demonstrate that continual augmentation of the population will be required to slow the build-up of inbreeding in subsequent generations. The attempt to minimize inbreeding by selecting breeding pairs was dependent upon the diversity and sex of candidate squirrels. Despite the limited availability of individuals, the selected breeding pairs are each comprised of a diverse combination of mitochondrial and nuclear genotypes. It is interesting to note that selecting breeding pairs based on minimising relatedness at nuclear loci, led to the separation of mitochondrial lineages in each pairing. This result provides some support for phylogeographic structure being maintained in the species, despite the widespread admixture of populations following serial introductions of the red squirrel into the UK.

The difference in levels of genetic diversity between supplemented and non-supplemented scenarios at Newborough demonstrates the importance of considering genetic management in reintroductions, and highlights the sensitivity of populations to supplementation at this stage. However, although modelling genetic augmentation of a population allows the effects on heterozygosity to be investigated, in very small populations such as the founding group at Newborough, it is not possible to examine the effects of increased heterozygosity on the probability of survival. The relative impact on population numbers means that supplementation directly affects population survival, explaining the three-fold difference observed between scenarios at Newborough.

The future success of the population at Newborough is uncertain, but incorporating genetic management into the project at an early stage increases its chances. There is now a tremendous opportunity to examine the growth of a population in the wild based on known individual genotypes. It is proposed to employ more microsatellite markers to individually tag founders and offspring, allowing a genetic stud-book to be established over the first few generations. This will provide the basis for further genetic management, but also enable behavioural studies to examine aspects of breeding structure and reproductive success, hopefully providing more information for the conservation of the species in the UK.

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