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DNA Analysis of Red Grouse: An Analysis of Taxonomy and Genetic Diversity

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Summary

The taxonomic status of red grouse in Ireland has been the subject of considerable debate over the past century. Many ornithologists believe that Irish red grouse should be classified as *Lagopus lagopus scoticus*, which is the same subspecies of red grouse as that in England, Scotland, and Wales. However, there is also a considerable body that believes the red grouse in Ireland merit unique subspecific status, namely *L. lagopus hibernicus*. The aim of this study was to compare DNA sequences from red grouse in Ireland and Great Britain to see whether there was evidence of genetic distinction between the two putative subspecies, because this would provide a strong basis for classifying them as separate subspecies. However, we found no evidence of genetic differentiation between red grouse in Ireland and Great Britain, and therefore our data do not support the classification of Irish red grouse as *L. lagopus hibernicus*. Furthermore, the ecological and morphological evidence do not present a strong case in support of *hibernicus*, and therefore we do not currently recommend that Irish red grouse be awarded unique subspecific status. However, it is important to note that we cannot entirely rule out the possibility that *hibernicus* is a distinct subspecies because there are a number of evolutionary and demographic reasons which could explain why subspecies or even species share the same DNA sequences.

We also compared DNA sequences from current and museum samples of Irish red grouse, and found no reason to conclude that the current genetic similarity of grouse from Ireland and Great Britain is a result of hybridization and introgression following the repeated introduction of British red grouse into Ireland over the past century. We did, however, find evidence that levels of genetic diversity in Irish red grouse have been declining over the past century, presumably as a result of diminishing population sizes. The loss in habitat, combined with the declining numbers and genetic diversity of Irish red grouse, justify their designation as a Northern Ireland Priority Species for conservation.

Introduction

The willow ptarmigan (*Lagopus lagopus*) has a circumpolar distribution that encompasses northern Canada, Alaska, Britain, Ireland, northern Scandinavia, and Russia. The habitat of North American and Eurasian willow ptarmigan is tundra, and individuals living in these areas alternate between a dappled brown summer plumage and a white winter plumage. In contrast, *L. lagopus* within the U.K. lives in upland moors, bogs, and heaths that are dominated by heather (*Calluna vulgaris*), and lack a white winter plumage. These differences in habitat and plumage are two of the reasons why the willow ptarmigan in the U.K. are usually classified as a distinct subspecies, *L. l. scoticus*, commonly known as red grouse.

Today the subspecific status of red grouse in the U.K. is still widely accepted, although some researchers maintain that it should be classified as a separate species (*Lagopus scoticus*; Madge and McGowan 2002). More contentious is the question of whether red grouse populations from Ireland should be classified as the same subspecies as the red grouse in Britain. In Ireland, birds tend to be paler and more yellowish than in England, Wales, and other parts of Scotland with the exception of the Outer Hebrides, where they resemble the Irish red grouse (Witherby, Jourdain et al. 1941; Madge and McGowan 2002). It has been suggested that these plumage differences may reflect locally adaptive camouflage to the heather moors of Britain versus the blanket bogs of Ireland (Hutchinson 1989), and therefore some taxonomists maintain that the Irish red grouse should be classified as a separate subspecies (*Lagopus lagopus hibernicus*). However, this assertion has not been tested in any vigorous way, and it is also possible that plumage differences between Irish and Scottish red grouse result from environmental factors, particularly diet, in which case there would be less support for the classification of separate subspecies in Ireland and Britain (Cramp and Simmons 1979).

The controversial subspecific status of Irish red grouse has been further complicated by a series of deliberate introductions of Scottish red grouse into Ireland over the last two centuries, which may have resulted in interbreeding between the two forms (Vaurie 1965). Putative *hibernicus* on the Outer Hebrides may also have been influenced by introductions from the mainland (Bannerman 1963). However, there is essentially no evidence that either supports or refutes the suggestion that interbreeding between the two forms has occurred in the past, and in fact the importance of hybridization between Scottish and Irish or Hebridean forms has been downplayed by some ornithologists who point out that the Irish red grouse have maintained their distinctive pale plumage.

Resolving the taxonomy of red grouse in Ireland is particularly pressing because populations are declining there, most likely as a result of disease of both birds and foodplants and, perhaps more importantly, the loss of suitable habitat. A recent survey commissioned by EHS (Allen *et al.*, 2004) revealed a total of around two hundred pairs of red grouse in Northern Ireland. In addition to the total number being very small, the distribution of these birds is highly fragmented. As a result, connections between populations are often poor, and local population sizes are often extremely small. This means that the red grouse in this region may be at risk with respect to their long-term survival, which is why the red grouse is a Northern Ireland Priority Species for conservation. Low population sizes and fragmented habitat also suggest that it may be sensible to monitor the genetic diversity of Irish red grouse, since decline in population sizes and genetic diversity are not necessarily correlated.

Molecular systematics

One of the most straightforward methods for ascertaining the taxonomic status of a group of individuals is with DNA sequence data. Such data allow us to compare the genetic similarity of particular populations to each other, and also to other closely related taxonomic groups (e.g. sibling species or subspecies). In general terms, individuals that

are genetically similar to one another, in other words that have a high percentage of matching DNA sites, may be considered members of the same species, whereas more genetically divergent individuals may be considered as distinct species. This approach has been used for a number of years, although has recently been formalized with the DNA barcoding project, which is being undertaken by an international collaboration known as the Consortium for the Barcoding of Life. This is currently hosted by the Smithsonian's National Museum of Natural History in Washington, D.C., and is promoting the eventual acquisition of genetic barcodes for all living species. Part of this project has looked at the feasibility of using mitochondrial DNA sequences to identify species of birds.

As part of the DNA barcoding project, Hebert et al. (2004) concluded from a comparison of 260 bird species that a particular stretch of DNA sequence known as cytochrome oxidase was useful for differentiating between species because the sequences were never shared by more than one species. The ability to discriminate between species on the basis of DNA sequences has been demonstrated in a wide range of taxonomic groups, and has some important practical applications for example in wildlife forensics. However, DNA barcoding remains somewhat controversial since not everyone agrees that taxonomic decisions should be made on the basis of a single DNA sequence. We will return to this controversy later in the report.

Aims of this study

In this study we have used mitochondrial DNA sequence data to address the following questions:

1. Is there any genetic evidence to support the idea that Irish red grouse are a unique species or subspecies, or are they genetically indistinguishable from red grouse in Scotland, England, and Wales?

2. Is there any evidence that the Scottish red grouse that were introduced into Ireland multiple times over the last century have hybridized with Irish red grouse?
3. What are the taxonomic relationships between Irish and Scottish red grouse and other members of the genus and family?
4. Have there been any noticeable changes in the levels of genetic diversity within Irish red grouse over the past century?

Methods

DNA extraction

Current samples: Feathers that were collected from 36 individual birds during the 2004 survey of red grouse (Allen *et al.*, 2004) were sent to us in labeled envelopes. We extracted DNA from these feathers using a Qiagen DNAeasy kit (Qiagen Ltd., Crawley, U.K.), following the manufacturer's protocol. Extracted DNA was run out on a 0.8% agarose gel and visualized using an ethidium bromide stain.

Museum samples: We removed either a small piece of footpad or a piece of feather (including shaft) from 66 museum birds using a single-use razor blade, and the sample was placed into a sterile 0.5ml eppendorf. DNA was extracted using a Qiagen DNAeasy kit (Qiagen Ltd., Crawley, U.K.), with the following protocol:

1. Grind sample (in the case of grouse feathers use the region containing the blood spot) in liquid nitrogen and add 180 μ l manufacturer's buffer ATL
2. Add 20 μ l of supplied proteinase K (or 20 μ l of 20mg/ml concentration of standard proteinase K)
3. Add 20 μ l 1M DTT (Dithiothrietol; Sigma-Aldrich, U.K.) and vortex
4. Incubate at 55°C for 12-24 hours, vortexing every half hour for the first three hours
5. Vortex for 15 secs and add 300 μ l manufacturer's buffer AL.

6. Add 1µl of carrier RNA to every 300µl AL buffer
7. Vortex and incubate at 70°C for 10 mins
8. Add 300µl ethanol and vortex
9. Follow manufacturer's protocol from Step 5 onwards

Extracted DNA was run out on a 0.8% agarose gel and visualized using an ethidium bromide stain.

PCR and sequencing

All successful DNA extractions were then amplified using polymerase chain reaction (PCR). We targeted the 5' hypervariable domain of the grouse control region since in birds this is typically the most variable region of mitochondrial DNA and should therefore be most likely to reveal any sequence differences between closely related species or subspecies. We used primers that were designed from the chicken mitochondrial sequence (Desjardins and Morais 1990), and which have previously been shown as suitable for use on red grouse (Piertney, MacColl et al. 2000). The primer sequences were as follows:

Heavy primer: 5'-GTGAGGTGGACGATCAATAAAT-3'

Light primer: 5'-TTGTTCTCAACTACGGGAAC-3'

Each PCR reaction was set up as follows: 5 µl DNA, 1U Taq polymerase (Bioline, London, U.K.) in the manufacturer's buffer { 160mM (NH₄)₂SO₄, 670mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20}, 1.5mM MgCl₂, 0.1mM dNTPs, and 0.4µM each primer

PCR cycling conditions were as follows:

Current samples: 1 cycle of 3 minutes at 90°C followed by 30 cycles of 30 seconds at 90°C, 30 seconds at 55°C and 30 sec 72°C, followed by a final extension of 5 minutes at 72°C

Museum samples: 1 cycle of 3 minutes at 90°C followed by 35 cycles of 30 seconds at 90°C, 30 seconds at 55°C and 30 sec 72°C, followed by a final extension of 5 minutes at 72°C

Amplification reactions were verified by running out on a 0.8% agarose gel and then visualizing with an ethidium bromide stain. Successful reactions were cleaned up using QIAquick PCR purification kit (Qiagen Ltd, U.K.), following the manufacturer's protocol. Samples were then sequenced by Macrogen Inc. using a 3730xl DNA analyzer. A total of 85 sequences was obtained (Appendix 1).

Supplementary material

Additional control region sequences from GenBank, at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), were downloaded for comparison with our sequences. These included thirteen grouse species and two additional ptarmigan species (*Lagopus mutus* and *Lagopus leucurus*). These GenBank sequences were aligned with the sequences that we obtained in order to identify overall levels of similarity within and between genera.

We also compared our data from Irish red grouse to some data on Scottish red grouse that were published by Piertney *et al.* (2000). This publication was based on control region sequences of 247 grouse individuals, and provided us with a comparison of the overall levels of genetic diversity in Irish and Scottish grouse.

Sequence analysis

All sequences were manually compared to the electropherograms that resulted from automated sequencing, and verified sequences were aligned using ClustalX (Thompson, Gibson *et al.* 1997). Each unique sequence was assigned its own haplotype identity. The genetic distances between haplotypes were calculated as % sequence

divergence (i.e. the percentage of nucleotide sites that differed in a comparison of any two sequences).

Red grouse and willow ptarmigan phylogeny

Because the sequences that we identified– which came from red grouse and willow ptarmigan individuals from Ireland, Scotland, England, Wales, Norway, Sweden, and Russia – had a high overall level of similarity, we used a statistical parsimony network (Templeton, Crandall et al. 1992) to infer evolutionary relationships. This is because a traditional phylogenetic tree will be relatively uninformative, and might even be misleading, when sequences have relatively few differences between them (Freeland 2005). A network analysis, on the other hand, links haplotypes to one another through a series of evolutionary steps. The general approach is to first connect haplotypes that differ by a single mutation, followed by haplotypes that differ by two mutations, three mutations, and so on. The final product is a single network showing the interrelationships of all haplotypes in a way that requires the smallest number of mutations. We generated a network using the software TCS, version 1.13 (Clement, Posada et al. 2000).

Genetic differentiation within Tetraonidae

As mentioned earlier, in order to increase the likelihood of detecting variation within species, we targeted a highly variable region of DNA sequence. This worked to our advantage when comparing red grouse/willow ptarmigan from different areas, but proved to be more problematical when comparing grouse from different species and genera because the high variability made alignments difficult at that level of genetic differentiation. As a result, species- and genus- level phylogenetic trees inferred from this variable region are unlikely to be reliable. We therefore limited our comparison between species to calculations of the percentage sequence divergence as a basis for comparing within- versus between- species genetic differentiation.

Genetic diversity in red grouse

In order to identify any changing levels of genetic diversity within Irish red grouse species we first pooled all samples from i) Irish red grouse museum samples (1875-1924), and ii) Irish red grouse current samples (2003-2004) (Figure 1). This gave us a comparison based on 22 museum samples and 36 current samples. Using these data, we calculated haplotype and nucleotide diversity values for each time period using the software DnaSP version 4.0 (Rozas and Rozas 1999). Haplotype diversity is a measure of the numbers and frequencies of different haplotypes; the higher the value, the more diverse is the group of individuals being assessed. Nucleotide diversity is a measure of the mean divergence between sequences, in other words it measures how different sequences are, on average, from one another. The higher the nucleotide diversity, the more different the sequences are within the group of individuals that are being compared.

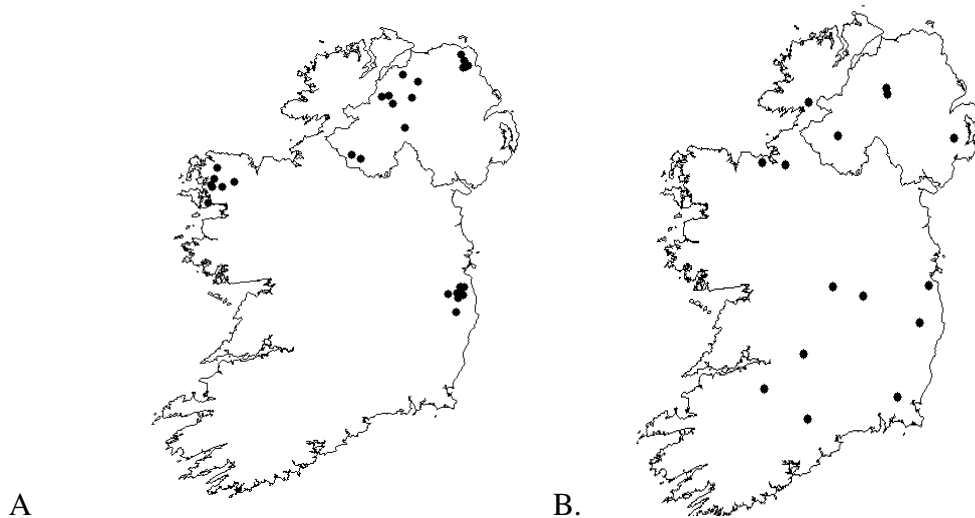


Figure 1. Origins of A) current Irish red grouse samples, and B) museum Irish red grouse samples.

Once we had calculated haplotype and nucleotide diversity of the current and museum red grouse samples, we then compared these values to estimates for current Scottish red grouse based on data from Piertney *et al.* (2000). This publication provides a nucleotide diversity value for each of ten populations. In addition, we calculated the overall haplotype diversity value for Scottish red grouse using the method of Nei (1973). As another measure of genetic diversity, we also identified the frequency of the most common haplotype in each of the three comparisons (Irish museum, Irish current, and Scottish current).

Results

We were able to extract, amplify, and sequence a total of 36 current Ireland samples; this was a 100% success rate since it represents the total number of sampled individuals that was sent to us from the survey of 2003/2004. We had a total of 66 museum samples, from which we were able to extract and amplify DNA from 55 for a success rate of 83% which is consistent with other studies. Of these, a total of 41 provided reliable sequences; the remainder were re-sequenced two or three times but still did not generate unambiguous sequence at all sites. In total, we obtained data from 22 museum samples of Irish red grouse, and 19 museum samples of red grouse and willow ptarmigan from other locations (Scotland, England, Wales, Norway, Russia, Sweden). The summary of dates and locations from which sequences were obtained are given in Appendix 1. Note that an additional eight sequences were provided by Stuart Piertney (University of Aberdeen) for a total of 85 sequences.

Phylogenetic relationships

In total, we identified twelve unique haplotypes (Table 1), each comprising 310 nucleotides. Overall similarity was high; levels of sequence divergence within all red grouse and willow ptarmigan samples ranged from 0.3-2.3%. The most common haplotype (H1) was shared by red grouse from Ireland and Great Britain (museum and

current, including both *scoticus* and putative *hibernicus*), and also by willow ptarmigan from Norway (museum). The second most common haplotype (H2) was shared by red grouse from Ireland and Great Britain (museum and current, including *scoticus* and putative *hibernicus*). The remaining haplotypes, which were all less common, were found only in one location, i.e. Ireland, the Outer Hebrides, mainland Scotland, Russia, or Sweden. The evolutionary relationships between the twelve haplotypes are shown in Figure 2.

Table 1. Regional locations from which the twelve different haplotypes have been identified. More detailed information on locations and dates is provided in Appendix 1. Note that haplotypes 1 and 2 (H1 and H2) were found in both *scoticus* and *hibernicus* individuals.

Haplotype	Sample details: location, museum versus current (number of samples)
H1	Ireland – museum (10)
	Ireland – current (31)
	Wales – museum (2)
	Scotland – museum (3)
	Scotland – current (3)
	Outer Hebrides – museum (1)
	England – museum (1)
	England – current (3)
	Norway – museum (2)
	H2
Ireland – current (2)	
Outer Hebrides – museum (4)	
Scotland – museum (1)	
England – museum (1)	
H3	Ireland – museum (2)
H4	Ireland – museum (5)
	Ireland – current (1)
H5	Outer Hebrides - museum (1)
H6	Scotland – current (1)
H7	Ireland – current (1)
H8	Scotland – current (1)
H9	Ireland – museum (2)
H10	Russia – museum (1)
H11	Russia – museum (1)
H12	Sweden – museum (1)

A comparison of our sequence data with that of other *Lagopus* species reveals clear interspecific genetic distinction. The sequence divergence between our *L. lagopus* samples (including *scoticus* and putative *hibernicus*) and *L. mutus* (rock ptarmigan) was 12.8-13.4%, whereas that between our *L. lagopus* samples (including *scoticus* and putative *hibernicus*) and *L. leucurus* (white-tailed ptarmigan) was 12.1-13.0% (*L. mutus* and *L. leucurus* data from Drovetski 2002). The levels of genetic divergence between *L. lagopus* and other species within the family Tetraonidae were even larger, and in fact could not be obtained with accuracy because alignments between these more distantly related species could not be guaranteed on the basis of the hypervariable domain of the control region that we sequenced.

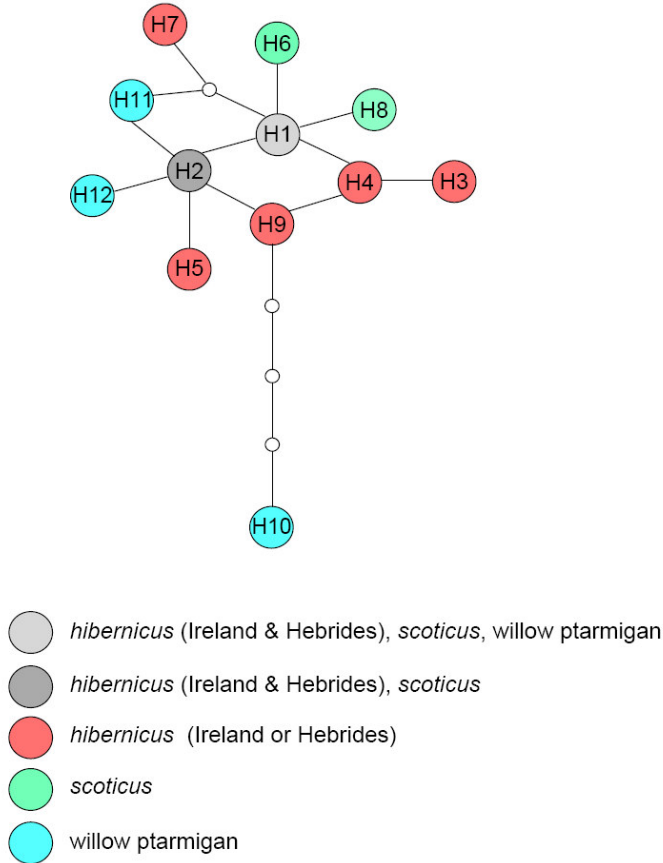


Figure 2. Maximum parsimony network showing the evolutionary relationships between the twelve haplotypes identified in this study. Each coloured circle represents a unique haplotype. The number of line segments between a haplotype (multiple segments are denoted by the small, white circles) represents the number of mutations that have occurred.

A comparison between Scotland (current populations), Ireland (museum samples), and Ireland (current samples) revealed substantial differences in genetic diversity (Figures 3, 4, and 5). Scotland consistently showed the highest level of diversity. Within Ireland, there is evidence that genetic diversity has been declining over the past 130 years. Today, overall diversity in Ireland is very low. This is particularly true when measured as haplotype diversity, and the low level of haplotype diversity in Ireland today is a result of the fact that red grouse in Ireland are dominated by a single haplotype (H1), which was found in 89% of the birds in our sample. Irish red grouse have not seen a substantial decline in nucleotide diversity which was relatively low even 130 years ago; however, in this context the lack of substantial change in genetic diversity may be less meaningful when measured as nucleotide diversity which is a reflection of the overall similarity of the different lineages, and not of the numbers of those lineages.

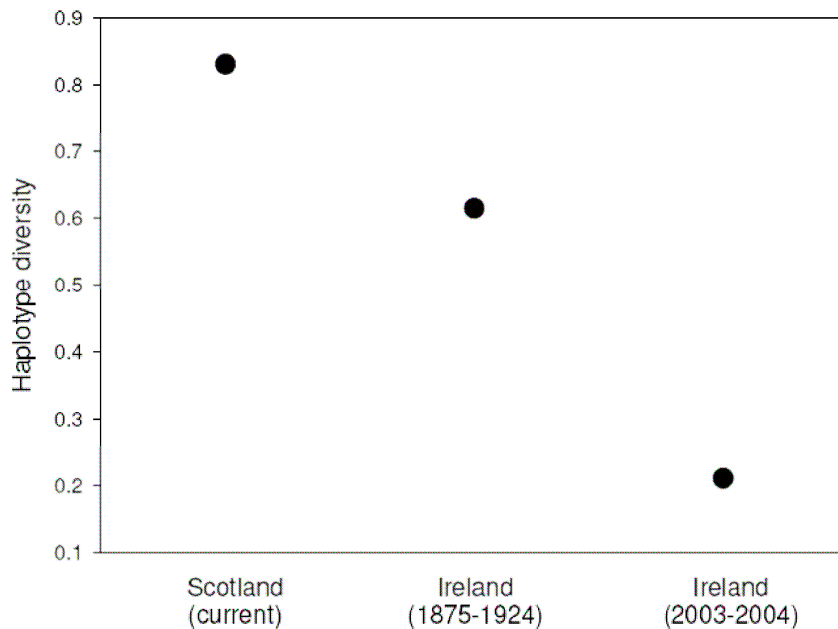


Figure 3. A comparison of haplotype diversity values in Scotland (current), Ireland (museum), and Ireland (current). The haplotype diversity in red grouse in Ireland today is considerably lower than in the other two comparisons. The haplotype diversity for Scotland was calculated using the data in Piertney *et al.* (2000).

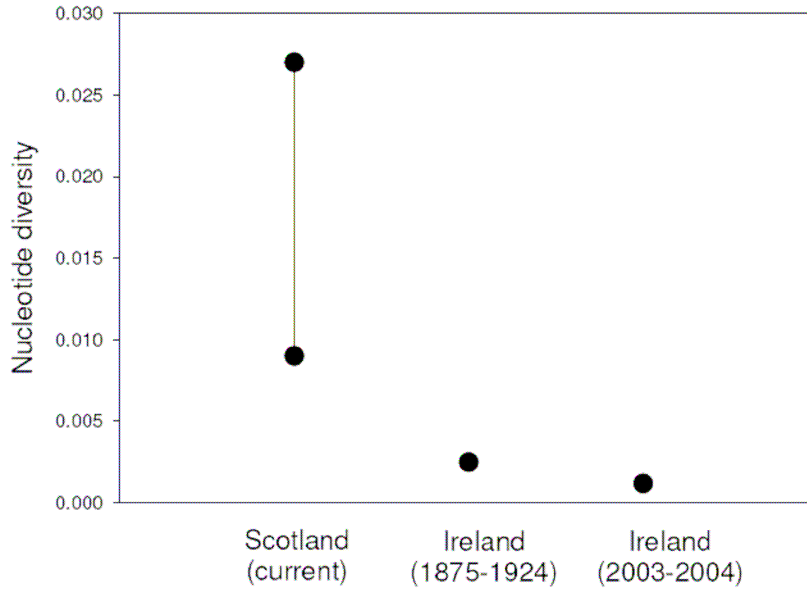


Figure 4. A comparison of nucleotide diversity values in Scotland (current), Ireland (museum), and Ireland (current). The nucleotide diversity in red grouse in Ireland has been considerably lower than in Scotland for the past 130 years. The nucleotide diversity for Scotland is presented as a range, based on the data provided by Piertney *et al.* (2000).

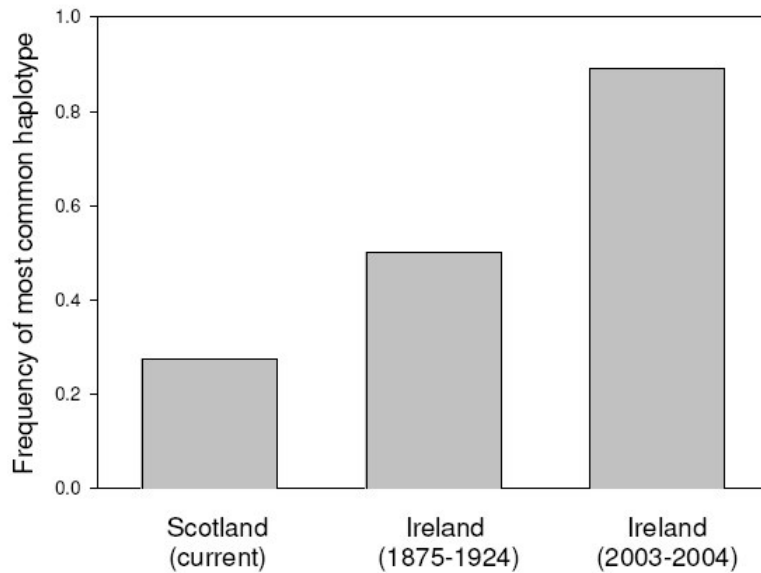


Figure 5. A comparison of the frequency of the most common haplotype found in Scotland (current), Ireland (museum), and Ireland (current). The low haplotype diversity found in Ireland today can be attributed to the fact that the most common haplotype in our samples occurred at a frequency of 89%. The haplotype frequency data for Scotland are from Piertney *et al.* (2000).

Discussion

Taxonomy and phylogeny

The results of this study present no support for the proposal that red grouse native to Ireland and the Outer Hebrides constitute a unique species or subspecies. There are two lines of evidence that have led us to this conclusion. First, some haplotypes were shared by red grouse from Ireland (putative *hibernicus*) and from Great Britain (*scoticus*). The most common haplotype (H1) was found in a total of 57 red grouse from Ireland (museum and current) and Great Britain (museum and current), and also in willow ptarmigan from Norway (museum). The second most common haplotype (H2) was found in red grouse in Ireland (current and museum), and also in red grouse from Great Britain. Although there have been exceptions to this rule (Johnson and Cicero 2004), we generally expect distinct species or subspecies to have distinct genetic lineages, which is clearly not the case in *scoticus* and putative *hibernicus*. The second reason why our data provide no support for the subspecies *hibernicus* is that *hibernicus* haplotypes are not more closely related to each other than they are to *scoticus* haplotypes (Figure 1). If putative *hibernicus* from Ireland and the Outer Hebrides were taxonomically distinct then we would expect them to form a distinct cluster on the evolutionary network.

It is also worth noting that there is a lack of ecological or behavioural data that would support the idea of taxonomic distinction between *scoticus* and *hibernicus*. The main reason why the subspecies *hibernicus* has been suggested in the past has been on the basis of a more yellow plumage in the red grouse that are native to Ireland and the Outer Hebrides compared to the darker plumage in British red grouse. However, there are numerous examples of birds whose plumage colouration along the red-orange-yellow spectrum has been caused by different levels of carotenoids in their diet (reviewed in Olson and Owens 2005), and this may therefore provide us with an environmental explanation for the differences in red grouse plumage across the United Kingdom. Relevant to this explanation is the fact that the grouse habitat on the Outer Hebrides is more similar to Ireland than to mainland Scotland, consisting largely of bogs. Flora that

are common to Ireland and the Outer Hebrides may explain the lighter plumage in birds native to these regions. However, there still remains the question of why *scoticus* that are introduced to Ireland can apparently maintain their relatively dark plumage (Hutchinson, 1989).

While we have no reason to support the designation of *L. lagopus hibernicus* as a distinct subspecies, it is important to note that at this stage we cannot unequivocally rule out this possibility. A recent review of the literature found that there was little agreement between the number of subspecies identified within a species (an average of 5.5), and the number of unique mitochondrial genetic lineages within that species (an average of around two; Zink 2004). Broadly speaking, genetic data are most convincing when they lead to a positive result, in other words a clear signal of genetic differentiation which can only have resulted from reproductive isolation. A negative result will always be harder to interpret because it could have been influenced by a number of relatively recent evolutionary or demographic phenomena. It is also possible in this case that a greater level of differentiation would be found in a non-neutral gene that may be involved in the evolution of alternative, adaptive plumage colouration, because it has been suggested that the plumages provide camouflage in their respective habitats: heather (*Calluna vulgaris*) dominated moors in Britain versus the blanket bogs of Ireland and the Outer Hebrides (Hutchinson 1989). However, we currently have no way of targeting such a gene even if it does exist; such an investigation would be expensive, time-consuming, and would have no guarantee of success. Therefore, while evidence (both genetic and ecological) in support of the idea of *hibernicus* as a unique subspecies is currently weak, we cannot yet rule out that possibility.

The range of sequence divergences within and between different species of ptarmigan (*L. lagopus*, *L. mutus*, and *L. leucurus*) present no doubt that these species are taxonomically distinct. However, within *L. lagopus* (red grouse and willow ptarmigan), the sharing of haplotype 1 with red grouse and willow ptarmigan from Norway raises an additional set of questions. Unfortunately, the Norwegian museum samples provided no specific information on where these specimens were collected. Nevertheless, we can

conclude from these data that genetic divergence between red grouse and willow ptarmigan is extremely low, as evidenced by the fact that other willow ptarmigan genetic lineages are nested with the haplotype network (Figure 2). The genetic similarity across Ireland, the United Kingdom, and western Europe demonstrate an overall very low level of genetic divergence within *Lagopus lagopus*, which is a reflection of the fact that they have all shared a recent common ancestor. There is currently some uncertainty regarding the number of subspecies that have been identified across the range of *Lagopus lagopus*, for example Johnsgard (1983) (Johnsgard 1983) lists 16 subspecies, and del Hoyo et al. (1994) (Del Hoyo, Elliott et al. 1994) suggest 19 subspecies worldwide (without recognizing *hibernicus*); a more detailed phylogeny based on *Lagopus lagopus* sampled from across its range may provide insight into this question.

Hybridization

There has been some concern that past introductions of red grouse from mainland Great Britain into Ireland and the Outer Hebrides have genetically swamped native *hibernicus*, and we addressed this question by comparing the haplotypes of current red grouse to those from museum samples. Haplotype 1, which was shared by red grouse from across Ireland and the United Kingdom, was already present in Ireland by 1881. While it is possible that introductions pre-dated 1881, we would under this scenario still be left with the need to explain why this haplotype was also found in Norway in 1897; if we were to conclude that it was in Ireland simply as a result of hybridization between Irish red grouse and introduced birds from Great Britain then we would be implying that this haplotype was unique to Great Britain, but its presence in Norway illustrates that this is clearly not the case. We therefore conclude that the genetic similarity between red grouse in Ireland and Scotland cannot be entirely attributable to hybridization and genetic introgression following past introductions.

Genetic diversity

Perhaps the most troubling result from our work has been the identification of declining levels of genetic diversity in Irish red grouse. Regardless of their taxonomic status, haplotype diversity has been dwindling over the last century or so, with a single haplotype (H1) appearing to be headed rapidly towards fixation (the current frequency is 89%). This decline was less apparent in a comparison of nucleotide diversity, but in this case haplotype diversity may be a more appropriate measure since it reflects the diversity of genetic lineages, whereas nucleotide diversity reflects the extent to which these lineages differ from one another which is less important in a closely related taxonomic complex such as that described by red grouse/willow ptarmigan.

The decline in genetic diversity that we have identified is perhaps not very surprising when we consider that red grouse habitat has been reduced markedly over the past century; currently there is only an estimated 12% of peatland habitat remaining (Tomlinson 1997). Habitat loss, along with other possibly relevant factors such as climate, predation, and disease (reviewed in Allen *et al.*, 2004) has led to a concomitant decline in the numbers of red grouse in Ireland. A recent survey concluded that there were around 200 pairs in Northern Ireland (Allen, Mellon *et al.* 2004) and, while estimates from Ireland are less precise, the total population for Ireland has been estimated at between 1,000 and 5,000 birds (Sharrock 1976; Gibbons, Reid *et al.* 1993).

Although not particularly surprising in light of ongoing reductions in the numbers of Irish red grouse, this decline in genetic diversity should not be lightly dismissed. While the loss of apparently non-adaptive genotypes may have little effect in the short-term, the loss of genetic lineages in general may nevertheless prove detrimental in the long-term. This is because environments are constantly changing, and genetic diversity is necessary if populations are to continue to evolve and adapt to changing conditions. Low levels of genetic diversity can also increase the likelihood of inbreeding which can result in inbreeding depression (the reduced ability of inbred individuals to survive and successfully breed). Therefore, the long-term survival of populations may only be

possible if levels of genetic diversity are kept sufficiently high. While there are no clear guidelines on what these levels need to be, we should be particularly concerned about populations or species in which substantial levels of genetic diversity have been lost over a relatively short period of time. In our study a comparison of museum and current samples provided a clear example in which this has occurred, in other words a substantial number of genetic lineages have been lost from Irish red grouse over the past century. If numbers of Irish red grouse continue to decline then genetic diversity will increasingly be lost; at the same time, as genetic diversity is lost, inbreeding depression may accelerate the rate at which populations are reduced in size. This could potentially accelerate the local extinction of populations, and introduces an element of uncertainty regarding the long-term effects of dwindling genetic diversity. The establishment of red grouse in Ireland as a species in need of some level of protection is therefore entirely justifiable.

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Appendix 1. Sample ID, location and date where samples were collected, sex of bird (if known), comments on taxonomy (if available), and source of sample for the 85 sequences that were used in this study.

Sample ID	Location collected ^a (Grid Ref)	Date collected	Sex	Source of sample	Comments on taxonomy ^b	Haplo-type
158-LLA	Brougher Mount (H359529)	29/03/2004	?	Survey		H1
160-LLA	Lough Fea (H739874)	08/03/2004	?	Survey		H1
161-LLA	Tappaghan (H290690)	10/04/2004	?	Survey		H1
163-LLA	Knocklayd (D113368)	08/03/2004	?	Survey		H1
167-LLA	Cushleake (D220350)	24/03/2004	?	Survey		H2
176-LLA	Sperrins (H640980)	17/04/2004	?	Survey		H1
203-LLA	Tievebulliagh (D181267)	15/02/2004	?	Survey		H1
210-LLA	Orra More (D145258)	29/03/2004	?	Survey		H1
216-LLA	Benbradagh (C731117)	28/03/2004	?	Survey		H1
217-LLA	Agangarrive (D145308)	26/03/2004	?	Survey		H1
218-LLA	Agangarrive (D156307)	26/03/2004	?	Survey		H1
222-LLA	Mullaghsallagh (H684950)	30/03/2004	?	Survey		H1
232-LLA	Tievenameenta (H210370)	11/06/2005	?	Survey		H1
233-LLA	Dunbrook Mountain (C590160)	10/06/2005	?	Survey		H4
235-LLA	Tievenameenta (H155755)	11/06/2005	?	Survey		H1
239-LLA	Belmore Mountain (H130414)	12/06/2005	?	Survey		H1
241-LLA	Muinguinan (F898303)	26/05/2004	?	Survey	<i>hibernicus</i>	H1
290-LLA	Corslieve (F840010)	25/03/2004	?	Survey	<i>hibernicus</i>	H1
291-LLA	Ballycroy National Park (F860190)	22/07/2003	?	Survey	<i>hibernicus</i>	H2
294-LLA	Owenglass (F839144)	21/03/2003	?	Survey		H7
302-LLA	Scardaun Lough (F930120)	20/03/2003	?	Survey		H1
311-LLA	Gorfinbar (H627667)	10/05/2005	?	Survey		H1
312-LLA	Koram Hill (H405952)	13/07/2005	?	Survey		H1
314-LLA	Owensoy Nature Reserve (G050160)	26/04/2005	?	Survey		H1
319-LLA	Lugalla (O140080)	04/04/2005	?	Survey		H1
333-LLA	Enniskerry (O190180)	13/04/2005	?	Survey		H1
336-LLA	Tonduff (O150130)	02/04/2005	?	Survey		H1
342-LLA	Knocknagun (O160180)	Spring 2005	?	Survey		H1
343-LLA	Prince William's Seat (O170180)	15/03/2005	?	Survey		H1

353-LLA	Powerscourt Paddock (O190100)	18/03/2005	?	Survey		H1
355-LLA	Derrybawn Mount (T120950)	17/03/2005	?	Survey		H1
357-LLA	Prince William's Seat (O170180)	Spring 2005	?	Survey		H1
362-LLA	Derrybawn (T120950)	25/04/2005	?	Survey		H1
363-LLA	Powerscourt Paddock (O190100)	Spring 2005	?	Survey		H1
369-LLA	Powerscourt Paddock (O190100)	14/03/2005	?	Survey		H1
SP-LLA1	Orkney	2003	?	Piertney	<i>scoticus</i>	H1
SP-LLA2	Mainland Scotland	2003	?	Piertney	<i>scoticus</i>	H6
SP-LLA3	Mainland Scotland	2003	?	Piertney	<i>scoticus</i>	H8
SP-LLA4	Mainland Scotland	2003	?	Piertney	<i>scoticus</i>	H1
SP-LLA5	Mainland Scotland	2003	?	Piertney	<i>scoticus</i>	H1
SP-LLA6	North Yorkshire	2003	?	Piertney	<i>scoticus</i>	H1
SP-LLA9	North Yorkshire	2003	?	Piertney	<i>scoticus</i>	H1
SP-LLA11	North Yorkshire	2003	?	Piertney	<i>scoticus</i>	H1
MD01-LLA	County Mayo	2005	?	Murray	<i>hibernicus</i>	H1
MD07-LLA	Wicklow	1915	M	UM	<i>leucistic</i>	H9
MD10-LLA	Ballina, County Mayo	1881	F	NMI	<i>hibernicus</i>	H4
MD11-LLA	Limerick	19 th century	F	NMI	<i>hibernicus</i>	H2
MD13-LLA	Laois	1881	?	NMI	<i>scoticus</i>	H1
MD16-LLA	Wicklow	1881	M	NMI		H1
MD17-LLA	Laois (Portarlington)	1881	F	NMI	<i>hibernicus</i>	H4
MD18-LLA	Wexford	1881	M	NMI	<i>hibernicus</i>	H3
MD19-LLA	Sligo	1881	F	NMI	<i>hibernicus</i>	H1
MD20-LLA	Ballina, County Mayo	1881	M	NMI		H4
MD21-LLA	Sligo	1881	F	NMI	<i>hibernicus</i>	H1
MD23-LLA	Tpperary	1881	M	NMI	<i>hibernicus</i>	H4
MD24-LLA	Killarney	1881	M	NMI	<i>hibernicus</i>	H1
MD25-LLA	Offaly	1882	M	NMI	<i>hibernicus</i>	H1

LLA							
MD27-LLA	Tipperary	1881	M	NMI			H1
MD27-LLA	N.Ulster	1923	F	NMS	<i>hibernicus</i>		H1
MD28-LLA	Norway	1897	M?	NMI			H1
MD29-LLA	Norway		M	NMI			H1
MD30-LLA	Uist	c. 1900	M	NMS	<i>hibernicus</i>		H2
MD31-LLA	Uist	1923	M	NMS	<i>hibernicus</i>		H1
MD32-LLA	Isle of Harris	1988	F	NMS	<i>hibernicus</i>		H2
MD33-LLA	Ireland	1908	M	NMS	<i>hibernicus</i>		H4
MD34-LLA	Ireland	1908	F	NMS	<i>hibernicus</i>		H1
MD35-LLA	Stornoway	1923	M	NMS	<i>hibernicus</i>		H3
MD36-LLA	Stornoway	1923	F	NMS	<i>hibernicus</i>		H2
MD37-LLA	Uist	1923	F	NMS	<i>hibernicus</i>		H2
MD38-LLA	Binscarth	1873	F	NMS	<i>scoticus</i>		H1
MD39-LLA	Perthshire	1907	M	NMS	<i>scoticus</i>		H2
MD41-LLA	Perthshire	1959	M	NMS	<i>scoticus</i>		H1
MD42-LLA	Wales	1907	M	NMS	<i>scoticus</i>		H1
MD44-LLA	Cumberland	1916	M	NMS	<i>scoticus</i>		H2
MD45-LLA	Lancashire	1908	M	NMS	<i>scoticus</i>		H1
MD46-LLA	Sweden	1907	M	NMS	<i>Lagopus lagopus</i>		H12
MD48-LLA	Pechora Valley, Russia	1875	M	NMS	<i>Lagopus lagopus</i>		H10
MD49-LLA	Archangel, Russia	1876	M	NMS	<i>Lagopus lagopus</i>		H11
MD50-LLA	Midlothian	1895	M	NMS	<i>scoticus</i>		H1
MJ03-LLA	Outer Hebrides	1924	M	Tring	<i>hibernicus</i>		H5

MJ05-LLA	Outer Hebrides	1932	M	Tring	<i>hibernicus</i>	H2
MJ12-LLA	Wales	1907	F	Tring	<i>scoticus</i>	H1
MJ17-LLA	Londonderry	1924	F	Tring	<i>hibernicus</i>	H1
MJ18-LLA	Londonderry	1924	F	Tring	<i>hibernicus</i>	H1
MJ20-LLA	Donegal	1911	M	Tring	<i>hibernicus</i>	H9

^aAll survey samples were collected in Ireland

^bMost likely subspecies; based on a combination of plumage characteristics, location where bird was sampled, and comments on museum labels

NMI = National Museum of Ireland

NMS = National Museum of Scotland

UM = Ulster Museum

NHM = British Natural History Museum