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Population genetic structure and systematics of the Irish Hare

Quercus Project QU03-04

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by

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Executive Summary

1. An analysis of the genetics of Irish hares was undertaken to determine their evolutionary relationships to other hares (systematic status) and to describe their population structure.

2. Hares were collected from throughout Ireland and, for the evolutionary study, samples from Eurasia and data from other species were included for comparison. Genetic samples from approximately 1200 hares were collected and analysed. Broadly, two approaches were adopted. To analyse systematic status, four parts of the mitochondrial genome and one nuclear gene were investigated. To analyse population structure, 11 microsatellite markers were used.

3. Systematic analyses highlighted the distinctness of the Irish hare. Irish hares possess a comparatively high number of unique genetic forms (mitochondrial haplotypes) that are not shared by any other mountain hare (or other hare species) outside Ireland. The phylogeny of the Irish hare is characteristic of species that survived the Ice Age in a glacial refuge. Evidence of in breeding and introgression of non-native brown hare DNA into that of the Irish hare was not found.

4. The Irish hare's unique morphology, ecology and behaviour in relation to other mountain hares is the result of genetic adaptation (i.e. speciation) as the result of isolation for at least 30,000–60,000 years. The unique evolutionary history and continued genetic isolation of the Irish hare challenges its present taxonomic classification as a subspecies. The Irish hare certainly fulfils the criteria for a full species under the evolutionary and phylogenetic species concept.

5. Examining population structure, observed levels of microsatellite genetic variation are similar to what has been observed among other population of lagomorphs. No genetic differences were found between samples collected from the same location over two successive seasons, suggesting temporal genetic stability. No evidence for a genetic bottleneck was found in any of the samples examined.

6. The pattern of population structure is characteristic of a historically large population that used to occupy a large contiguous area and is now highly fragmented. The observed genetic differences are most likely to the random effect of genetic drift acting on small isolated groups.

Contents

Introduction

The Irish hare is thought to be one of Ireland's longest established mammals. It is a priority for conservation action in Northern Ireland and is the subject of an all-Ireland Species Action Plan.

The mountain hare (*Lepus timidus* Linnaeus 1758) is currently widely distributed in northern latitudes of Europe and Asia ranging from Ireland, Scotland, Russia and Japan. *L. timidus* is renowned for its high degree of variability in many aspects of its morphology, ecology, and behaviour. The recognition that some of this phenotypic plasticity has a genetic basis, has led earlier workers to question the taxonomy of the species.

Ognev (1940) recognised 16 distinct subspecies of mountain hares throughout the range of the species including the Irish and the Scottish hares. More recently, Corbet (1978) challenged this earlier classification, and proposed a taxonomic revision based only five subspecies (again including Irish and Scottish hares). The main problem with current taxonomy of mountain hares is associated with the lack of diagnostic morphological characters.

The Irish hare's status as the subspecies *Lepus timidus hibernicus* Bell 1837 is currently justified on the basis of considerable differences in a number of morphological, ecological and behavioural features in comparison to other mountain hares from outside Ireland:

- Irish hares do not usually change into the characteristic white winter coat observed in mountain hares elsewhere. In Ireland, only a few individuals turn white or patchy.
- While mountain hares are often found inhabiting high grounds, tundra and open forest habitats and feed on birch, juniper and/or willow, in Ireland hares are common in lowland areas comprised of grasslands, lowland heaths and bogs where they mostly rely on grasses for food.
- The Irish hare is also characterised by having smaller home ranges in comparison to mountain hares from elsewhere.

The postglacial origins of Irish hares, at the end of the last Ice Age are still the subject of much controversy. Two contrasting hypotheses have emerged. The first one is based on the assumption that no mammals would have survived in Ireland. Thus, the Irish hare would have colonised Ireland from southern glacial refugia following the retreat of the ice some 14,000 - 10,000 years ago, across putative land bridges connecting Ireland to Britain and the main European continent.

Carbon dating for cave fossils indicates that hares were present in Ireland as far back as 14,000 and 30,000 years ago. The most recent ice age started ~78,000 BP and reached its maximum extent ~20,000 BP with a warmer period between 65,000 and 35,000 BP. During the maximum extent of this glaciation, contrary to most of NW Europe that was covered by ice, an ice free, cold tundra-like landscape covered the southern part of Ireland from Kerry to Waterford. Furthermore, around 18,000 years ago, the sea level was ~140 m lower than the present. Thus, the land configuration would have been very different in relation to the present day. The area now covered by the Celtic Sea and the English Channel would have consisted of relatively ice free dry land. The second hypothesis suggests that the Irish hare, as a cold tolerant species, could have survived in such an environment.

If the Irish hare survived the last glaciation in an putative Irish/Celtic Sea refugium, current populations could be the only remaining descendents of an older genetic lineage that was probably common in Europe prior to the last ice age. If this is the case, the current subspecies designation of the Irish hare is questionable since it is mostly based on a recent colonisation history.

From a genetic perspective, a number of predictions can be made to test the two hypotheses.

- If the present Irish hare population is descended from ancestors that colonised Ireland since the end of the last Ice Age some 10,000 – 14,000 years ago, this should translate into relatively shallow levels of genetic divergence between the Irish population and mountain hares from outside Ireland (in particular those from Scotland). Thus, taking into consideration random effects (e.g. lineage sorting and genetic drift), the levels of genetic divergence should be relatively low (i.e. compatible with a period of isolation dating back the end of the last ice age).
- Colonisation would have taken place through putative land (or ice) bridges connecting Ireland to Britain and the main European continent. All evidence suggests that such land bridges were short lived. Thus, as the ice retreated, the raising sea level quickly filled the Celtic Sea and the Irish Sea cutting all access to Ireland. The prediction for this pattern of quick colonisation is a reduced level of genetic diversity brought about by a relatively low number of individuals in the founding population.
- However, if the present day Irish hares are the descendents of an ancestral population that survived the last ice age in a putative glacial refugium in southern Ireland/Celtic Sea, this should be evident from increased levels of genetic diversity, that are characteristic of glacial

refugia. Thus, following the retreat of the ice, populations of species that have survived in glacial refugia are often characterised by sudden increases in population size, which in turn is associated with an increased level of genetic diversity. Furthermore, a higher level of genetic divergence should be observed in relation to other mountain hares outside Ireland.

All these predictions can be tested with modern molecular markers.

Here we provide a non-technical report on a comprehensive survey of Irish hare genetics, based on mitochondrial and DNA markers. Our aims were:

1. To elucidate the genetic and taxonomic relationships of the Irish hare in relation to mountain hares from outside Ireland
2. To assess current levels and patterns of geographic distribution of genetic diversity within and between Irish hare populations
3. To assess the possible genetic impact of the limited introduction of brown hare (*L. europaeus*) to Ireland on the Irish hare.

Methods

Sample collection

The sampling design for the project required the extensive co-operation of numerous parties including the Environment and Heritage Service (EHS), the National Parks and Wildlife Service (NPWS), Irish Coursing Club (ICC) and the Countryside Alliance (CA). In particular, the ICC has been the main cornerstone for the delivery of the population genetic component of the study by providing invaluable information, field assistance, and access to samples.

Over two sampling seasons (2003/2004 and 2004/2005), 918 hare specimens have been collected for genetic work using non-invasive methods with the assistance of ICC personnel. M. Hughes coordinated and led all sampling carried out in this study.

Ruth Hamill (UCD) kindly provided hare follicles from 183 Irish hare specimens from four locations in the Republic of Ireland collected during her previous investigation of the genetics of Irish hares. DNA for an additional 48 specimens were available from previous study carried out by QUB personnel on behalf of the EHS. Additional samples (road kills) were also obtained with the kind assistance of EHS staff and other interested members of the public.

To address the evolutionary component of the project, additional mountain hare samples were obtained from a number of locations outside Ireland including: the Isle of Man (N=1), Scotland (N=13), Switzerland (N=4), Norway (N=6), Sweden (N=8), Finland (N=7), and Russia (N=20). Biopsy tissue samples from the two sister species of mountain hares, the Arctic hare (*L. arcticus*), and the Alaskan hare (*L. othus*), and from the close related species the snowshoe hare (*L. americanus*), were also obtained for comparison (N=3 in each case).

In total, over 1200 hare samples were collected for this study.

Genetic analysis

New microsatellite markers were developed for the study of population genetics. A total of 11 microsatellite markers are now available for Irish hare population genetic work.

For the phylogenetic/systematic analysis, three mitochondrial DNA (mtDNA) genes, the mtDNA control region, and one nuclear gene were amplified by Polymerase Chain Reaction (PCR), sequenced and analysed.

To allow for comparison with relevant data available in the literature, a representative number of Irish hare specimens were screened using available mtDNA PCR primers for the D-loop region (179 base pairs) and the cytochrome b gene (563 base pairs). Novel mtDNA PCR primers were developed to amplify the full complement of the ND-4 and ND-5 mtDNA genes (2510 bp).

PCR primers available in the literature were used to amplify a region consisting of 405 base pairs of the nuclear transferrin (tf) gene. The rationale for the screening of the tf nuclear gene, in addition to one mtDNA control region and three mtDNA genes was to account for the possibility of interspecific introgression as reported in the literature, where mtDNA genome were found to cross the barrier of species causing difficulties in recovering phylogenetic signals. Under this scenario, the nuclear gene can be used to identify the species involved. In addition, the congruence of phylogenetic information from different mtDNA and nuclear genes allows for higher confidence in inferring species phylogeny/systematic status.

The three mtDNA genes, the mtDNA control region, and the nuclear transferrin gene were analysed separately using appropriate analytical approaches for sequencing data. In each case, in addition to the novel data generated in this study, a number of relevant sequences from other mountain hare and from other related species of the genus *Lepus* were downloaded from the GeneBank database to allow for a more comprehensive and rigorous analysis.

For the D-loop region, a total of 583 sequences (26 generated in this study) representing 14 species of the genus *Lepus* were analysed. For cytochrome b 216 sequences (30 generated in this study) representing six *Lepus* species were analysed. For the ND-4/5 genes, 100 sequences (99 generated in this study) representing three *Lepus* species were analysed. For the tf gene, a total of 55 sequences (30 generated in this study) representing six Lepus species in addition to one specimen of rabbit

(*Oryctolagus cuniculus*) and one specimen of eastern cottontail (*Sylvilagus floridanus*) were analysed.

Results

Evolutionary relationships of Irish hares

The main common feature of the independent analyses of mtDNA regions/ genes is the distinctness of the Irish hare specimens (Figs. 1, 2, and 3). Although, numbers are not always comparable, in all instances, Irish hares possess a comparatively high number of unique mtDNA haplotypes which are not shared by any other mountain hare (or other hare species) outside Ireland.

Another relevant feature of the mtDNA data is the 'star shape' phylogeny of Irish hare mtDNA haplotypes. This is characterised by one or two central node haplotypes to which most other haplotypes are connected by only a few mutations. This pattern is obvious from the analysis of the three mtDNA genes and the mtDNA control region. This 'star shape' topology is characteristic of glacial refugia. It basically typifies population expansion from glacial refugia following the retreat of the ice.

While comparing the topologies (i.e. arrangement) of the genetic trees generated by the three mtDNA genes and the mtDNA control region, a very interesting observation was noted. It appears that the cytochrome b gene, which has been widely used in the literature for phylogenetic studies of lagomorphs, is of limited usefulness to infer genetic relationships among closely related species of the genus *Lepus*. Thus while all species are clearly differentiated on the basis of the D-loop region (for which similar sampling coverage is available, Fig. 1), the pattern is not clear for the cytochrome b topology (Fig. 2).

Individuals from different species share the same mtDNA haplotypes (see 'combo' haplotype in Fig. 2). This confirms early suggestion in the literature that cytochrome b gene is not suitable for phylogenetic studies of recently divergent species of hares. This observation also challenges the hypothesis for the occurrence of excessive genetic introgression between hare species, as it has been reported in the literature from the screening of this particular gene.

Another interesting feature of the cytochrome b topology is related to link of the 'Sco2' mtDNA haplotype with the remaining of the predominantly Irish haplotypes (Fig. 2). A close examination of the origin of the particular "Scottish" specimen reveals that it came from the Isle of Mull, a location where Irish hares are known to have been introduced in the past.

The time since population expansion for the Irish hare based on the D-loop region and ND4/5 genes was estimated to be 57,716 years (95%

confidence intervals 26,916 - 84,203) and 35,446 years (95% C.I. 25,636 - 46,988) respectively assuming an average generation time of four years. In both instances, the estimates are not compatible with the hypothesis of recent colonisation. For comparison, the time since population expansion for the brown hare based on comparable D-loop data was estimated to be 21,697 years (95% C.I. 16,723 - 27,565).

The elapsed time since separation from a common ancestral population, which was estimated from genetic divergence values calculated for D-loop data for samples with N >20, is also incompatible with hypothesis of colonisation within the past 10,000-14,000 years (Table 1). The degree of divergence between the Irish hare and other *Lepus* species follows expected phylogenetic pattern. Thus, the two sister species (*L. arcticus* and *L. othus*) are the closest species to the mountain hares.

Pairwise comparison	*da*	*T*
Irish hare v.s. mountain hare (*L. timidus* - Russia)	0.04129	362,193
Irish hare v.s. Artic hare (*L. arcticus* - Canada and Greenland)	0.06576	576,842
Irish hare v.s. Alaskian hare (*L. othus* - Alaska)	0.07637	669,912
Irish hare v.s. brown hare (*L. europeaus* - Europe)	0.09254	811,754
Irish hare v.s.Yunnan hare (*L. comus* - southern China)	0.09760	856,140
Irish hare v.s. Japenese hare (*L. brachyurus* - Japan)	0.11902	1,044,035
Irish hare v.s. Yarkand hare (*L. yarkandensis* - Chinese Turkestan)	0.12291	1,078,158
Irish hare v.s. snowshoe hare (*L.americanus* - US)	0.16294	1,429,298

Table 1. Estimates of nucleotide differences (*da*) and time of divergence (*T*) between samples. T is calculated assuming a constant rate of nucleotide substitution from the formula da = 2λT, where $\lambda = 7x10^{-9}$ per site per year.

Close examination of the distribution of mountain hare mtDNA haplotypes in Europe indicate lack of geographic structuring possibly as the result of genetic mixing between interglacial periods. Large genetic differences among mtDNA haplotypes (i.e. large number of mutations) are suggestive of large historical population sizes.

Limited sampling outside Ireland limits the amount of conclusions that can be drawn about relationships to other mountain hares. Nonetheless, despite limited sampling, mountain hares from Scotland also appear to be significantly distinct from mountain hares found in main continental Europe.

The tree topologies also indicate the existence of taxonomic problems of other Lepus species. For instance, *L. capensis* from China and from the Southern Europe are clearly genetically distinct (see Fig. 1).

Analysis of the transferrin gene adds valuable additional evidence for the uniqueness of the Irish hare genetic lineage. One of the haplotypes that occurs at a very high frequency in Ireland is ancestral to other transferrin haplotypes found not only in other mountain hares outside Ireland, but also in other *Lepus* species (Fig. 4). Thus 'hap2' is ancestral to haplotypes in *L. europaeus*, *L. granatensis*, *L. castroviejoi*, *L. corsicanus* as well as of other derived haplotypes found in other mountain hare.

Close examination of the data suggests other interesting trends. For instance, the transferrin 'hap2' is also found in Norway. An examination of the ND-4/5 tree topology shows two Norwegian haplotypes that are more related to Irish haplotypes than with haplotypes of other European mountain hares. However, the genetic differences between these two Norwegian haplotypes with the rest of the Irish haplotypes is considerable (seven mutations, see Fig. 3) implying a long time since separation from a common ancestor. One possibility is that the representatives of the 'ancestral population' would have also survived in other refugia in Europe. They are now being replaced and/or mixed with lineages from different refugia.

There is no evidence of genetic introgression of brown hare DNA into the Irish hare. Earlier evidence for introgression might have been based on the use of inappropriate marker (i.e. cytochrome b). This would be easy to test by screening putative "introgressed individuals" with for the D-loop region and/or ND4/5 gene.

Outside Ireland, brown hares have been shown to be able to displace mountain hares. Thus, the potential problem of introductions comes from habitat displacement and not necessarily from genetic introgression. Even so, despite repeated reports of brown hare introductions in Ireland, this species has not expanded to the same extent as elsewhere in Europe. This appears to provide additional support for the genetic distinctness of Irish hares. It would be important, however, to examine brown hare specimens for the possibility of unidirectional genetic introgression from mountain hare into brown hare as backcrosses (i.e. mating involving putative cross-species hybrids individuals) into Irish hares cannot be ruled out.

All genetic data seems to suggest that the Irish hare is more genetically divergent than previously thought. Observed levels of divergence and haplotypic diversity can not be explained by recent colonisation. Instead, the most parsimonious explanation is that the Irish hare survived the last glacial maximum in refugia possibly in the southern parts of Ireland and/or other areas further south now covered by water.

The Irish hare's unique morphology, ecology and behaviour in relation to other mountain hares is thus the result of genetic adaptation (i.e. speciation) as the result of continuous isolation for at least for the past 30,000 – 60,000 years (or more considering times of divergence between Irish hares and other mountain hares, see Table 1) . The unique evolutionary history and continued genetic isolation of the Irish hare challenges its present taxonomic classification as a subspecies.

The Irish hare certainly fulfils the species criteria under the evolutionary and phylogenetic species concept. Notwithstanding differences in sampling efforts, approximately 37% of the haplotypic diversity observed in mountain hare throughout its range is restricted to Ireland.

Population structure

A total of 931 Irish hare specimens were screened for seven microsatellite loci. A subset consisting of 542 individuals were screened for an additional four makers (11 in total) to test whether the additional number of markers would increase resolution of analysis.

Observed levels of microsatellite genetic variation are similar to what has been observed among other population of rabbits and hares. No genetic differences were found between samples collected from the same location over two successive seasons suggesting temporal genetic stability.

No evidence for genetic bottleneck(s) was found in any of the samples examined.

A small, but statistically significant overall level of population structuring was observed among samples screened. However, there was no major correlation between genetic and geographic distance among populations. Thus, samples from geographically close areas were found to be significantly different from one another, while other samples, from geographically distant sites were found to be genetically similar. Additional preliminary analysis suggested the existence of some regional groupings that has to be investigated further.

The present pattern of population structure is characteristic of a historical large population that used to occupy a large area and is now highly fragmented. The observed genetic differences are most likely to the random effect of genetic drift acting on small isolated groups. This finding is in agreement to what has been observed in the close related species the snowshoe hare where only minor genetic differences were observed among samples from widely separated areas. The differences were largely attributed to contemporary habitat changes.

The high individual discriminatory power provided by the microsatellite makers were found to be useful in tracking the movements and the whereabouts of particular individuals examined during this investigation. Thus among our data set, we identified seven instances of individuals that were used in coursing events either in the same year or over multiple years. This data provides important information about the fate of hares that are coursed and subsequently released back into the wild. Thus data from this investigation suggests that hares that have participated in coursing events are not only surviving, but they are also not moving from the areas were they are released.

The sedentary behaviour of individuals is probably associated with their release into suitable habitat with availability of shelter and food. This behaviour is also probably contributing for the patterns of population structuring observed.

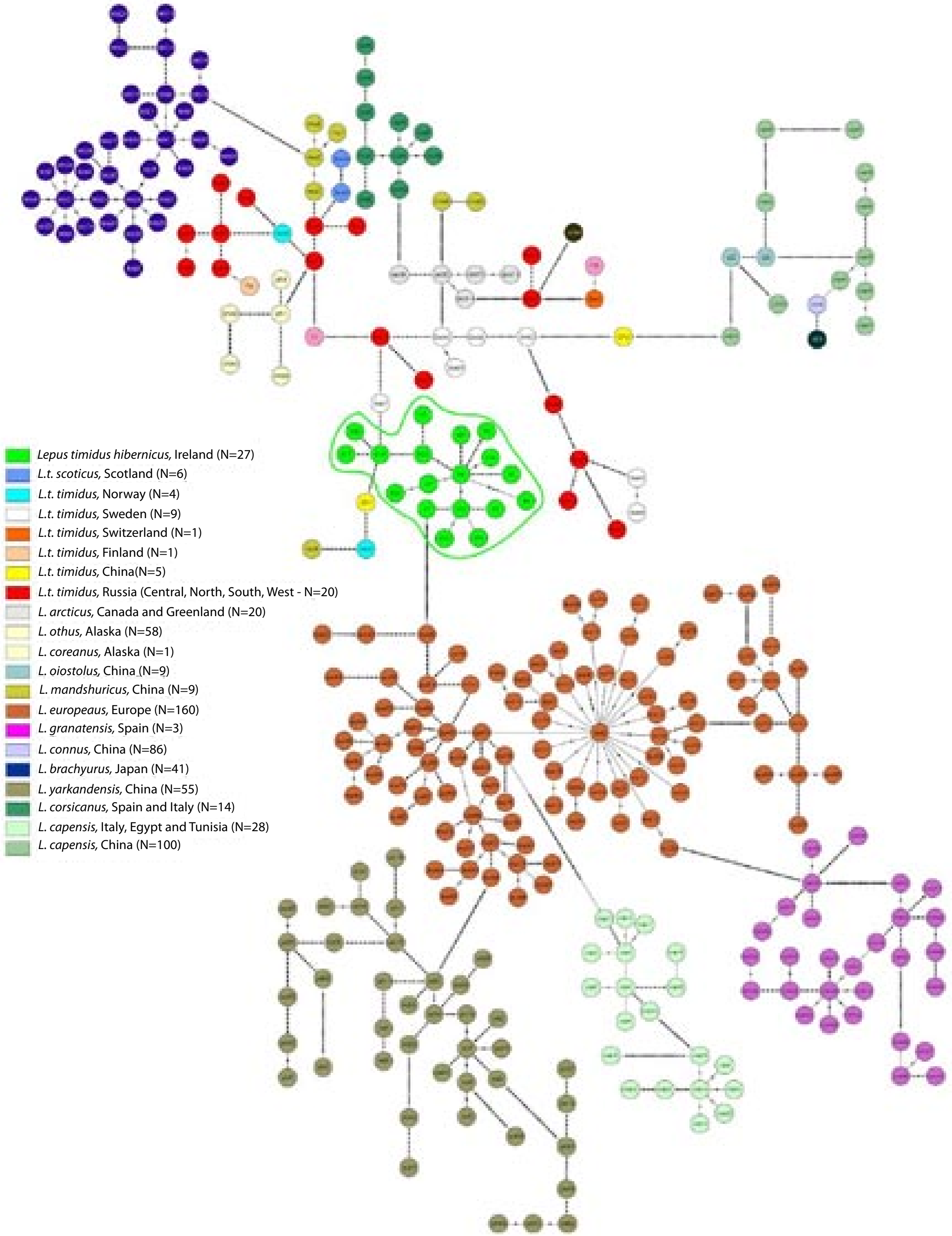

Fig 1. Minimum spanning tree for 337 mtDNA haplotypes observed in the D-loop region among 584 hare specimens from 13 species of the genus *Lepus*. Unique *L. t.hibernicus haplotypes* are highlighted by a circle. Black filled circles in between connection lines represent inferred haplotypes not actually observed in the data. There is one mutation separating each haplotype (observed or inferred). Thus, for instance, the Irish haplotypes Ir10 and Ir13 differ by two mutations.

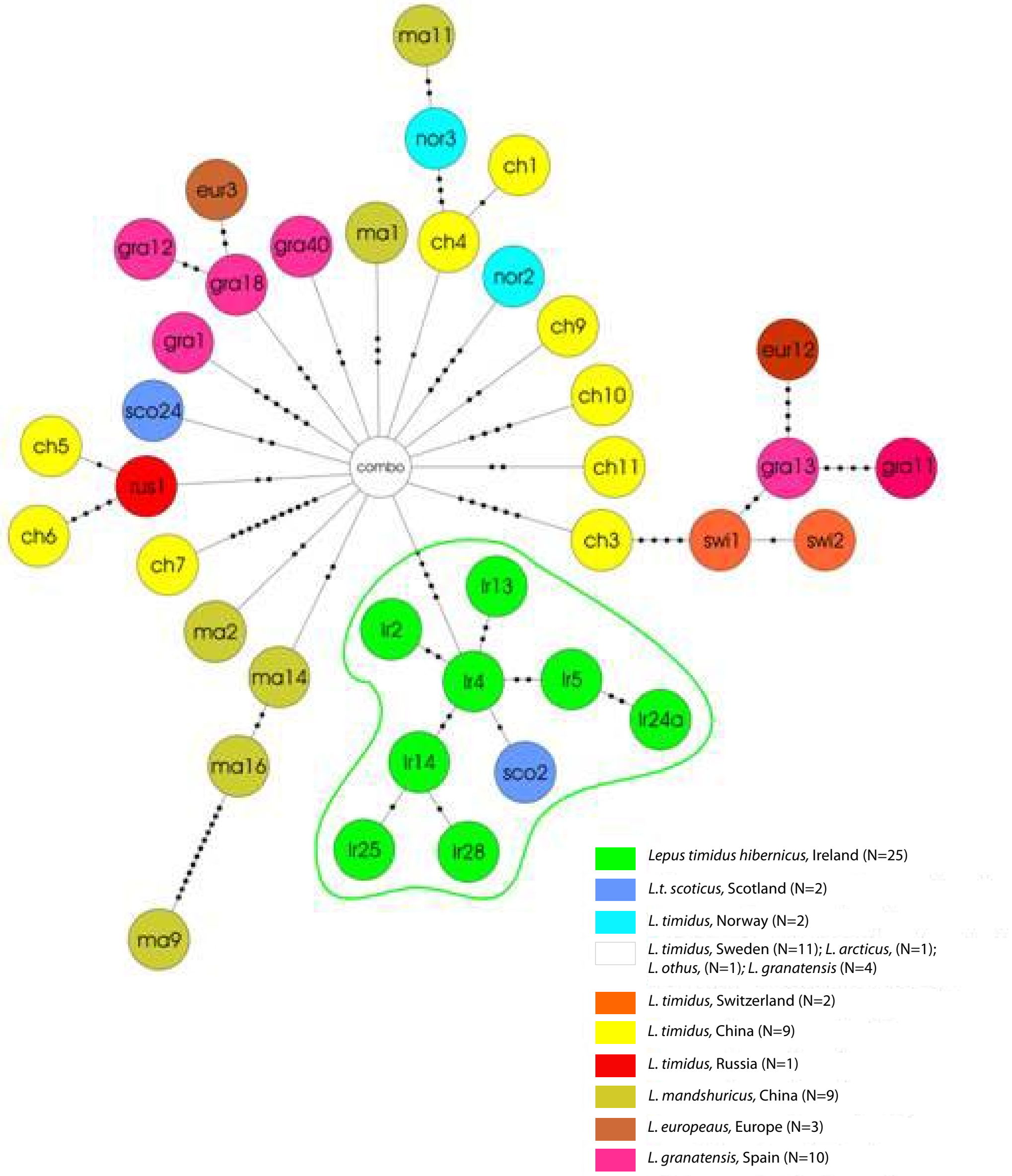

Fig 2. Minimum spanning tree for 39 mtDNA haplotypes observed in the cytochrome b region among 73 hare specimens from six species of the genus *Lepus*. Unique *L. t.hibernicus* haplotypes are highlighted by a circle. Black filled circles in between connection lines represent inferred haplotypes not actually observed in the data. There is one mutation separating each haplotype (observed or inferred). Thus, for instance, the Irish haplotypes Ir14 and Ir25 differ by two mutations.

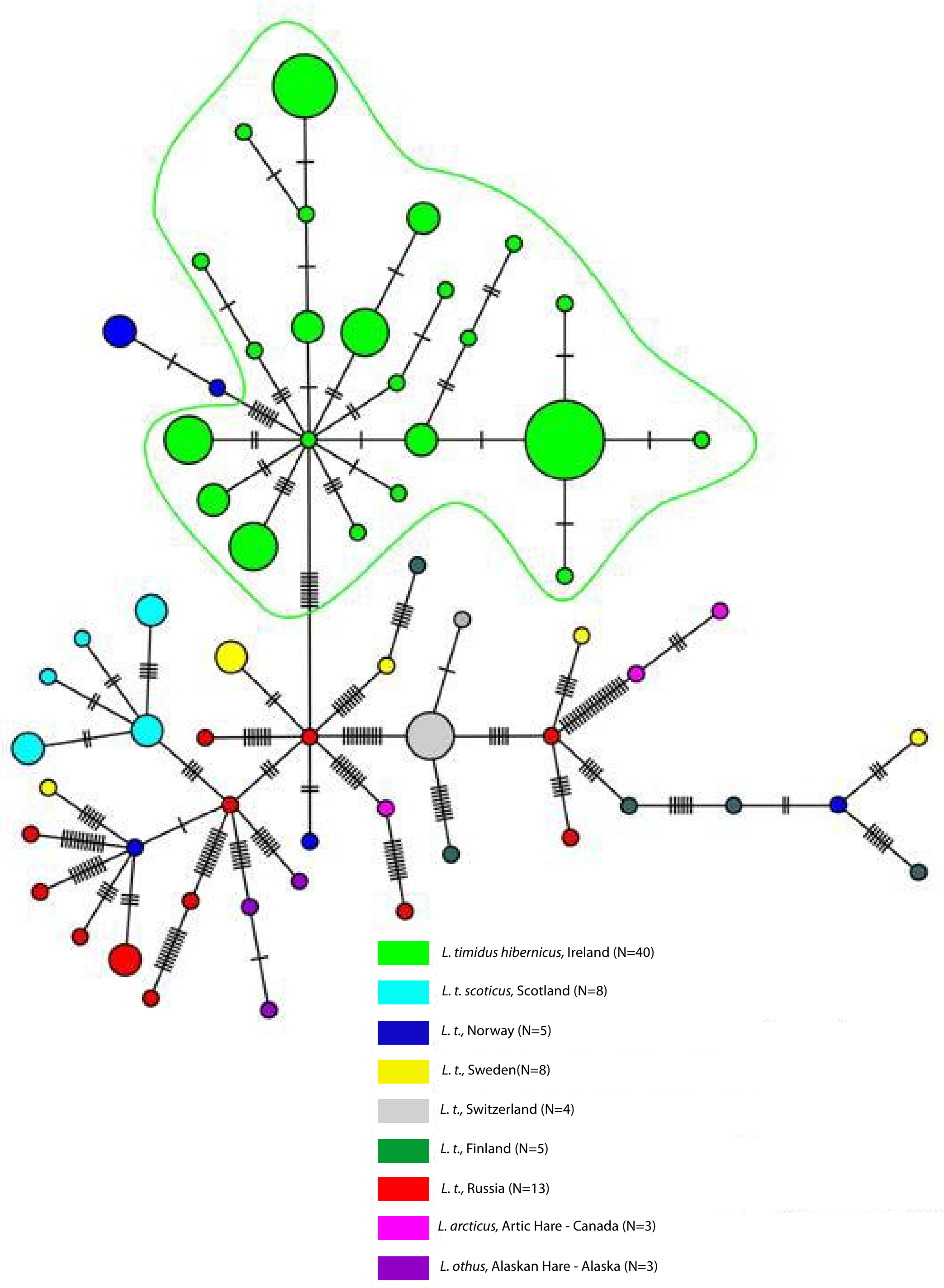

Fig 3. Minimum spanning tree for 64 myDNA haplotypes observed in the ND4/ND5 (2.5Kb) region among 89 mountain hares from throughout the range of the species in northern Europe. Three Artic and three Alaskan hares are included for comparison. Unique *L. t.hibernicus* haplotypes are highlighted by a green circle.

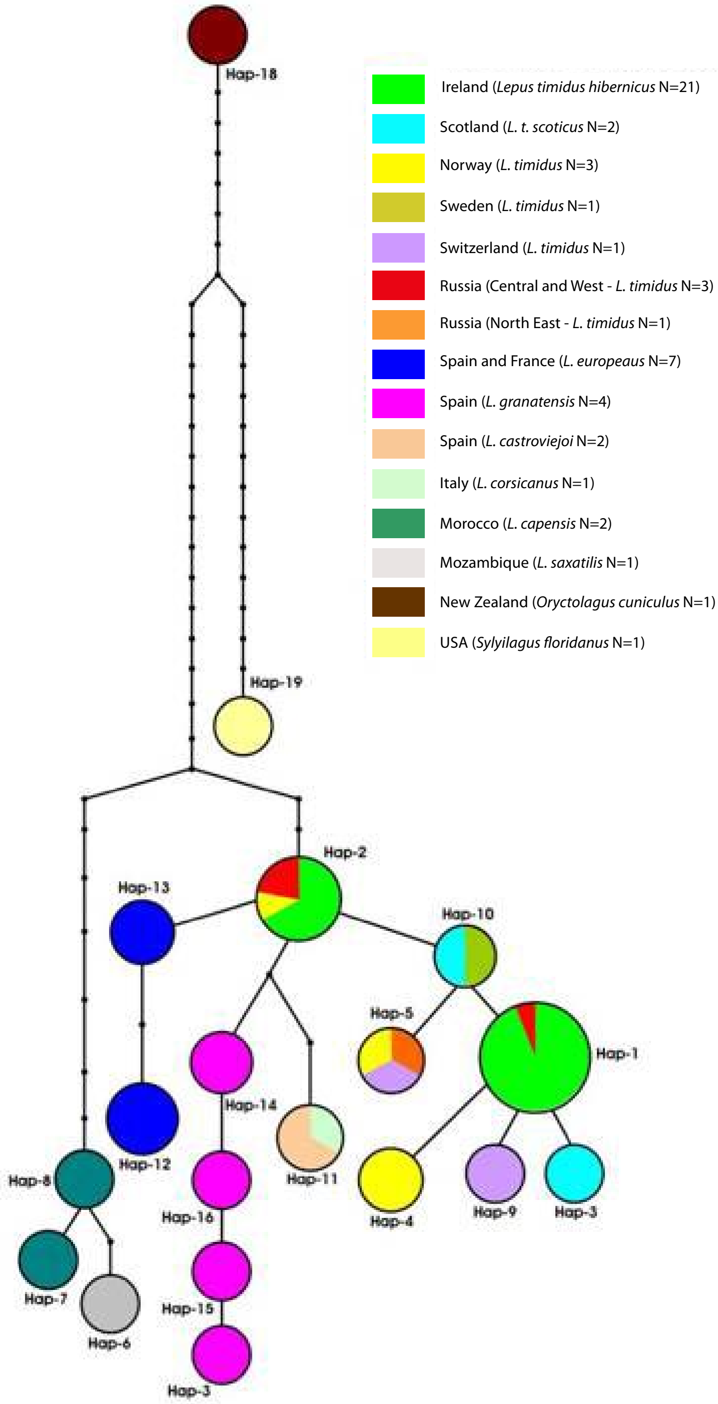

Fig 4. Minimum spanning tree for 51 nuclear haplotypes observed in the transferrin region sequenced among 51 hare specimens from six species of the genus Lepus. O. cuniculus and S. floridanus specimens are also included for comparison. Relative frequencies of particular haplotypes among all samples examined (size of the pie charts) and within each species/subspecies (pie slices with distinct colours) are also illustrated in the Small black filled circles in between connection lines represent inferred haplotypes not actually observed in the data. There is one mutation separating each haplotype (observed or inferred). Thus, for instance, the tranferrin haplotypes 2 and 1 (Hap-2 and Hap-1) differ by two mutations.

Discussion

Genetic approaches have been taken to understanding the evolutionary origins of Irish hares and their contemporary population structure.

These analyses have revealed the unique nature of the Irish hare. Irish hares form an ancient and diverse genetic lineage, many characters of which are not found outside of Ireland. It is likely that Irish hares survived the last glaciation in a refuge in, or close to, Ireland. This genetic evidence corroborates ecological, morphological and behavioural evidence of the uniqueness of the Irish hare.

Although it is currently considered an endemic sub-species *L. timidus hibernicus*, we believe there is substantial evidence for the full species status of the Irish hare.

We found no evidence of the introgression of brown hare DNA into that of Irish hares, though it remains possible that brown hares in Ireland may contain DNA from Irish hares.

Finer scale examination of Irish hare microsatellites, provided no evidence of a genetic bottleneck in Irish hare populations and only limited evidence of population structure. However, there was evidence of the effects of fragmentation of populations from a previously large, contiguous population. This could be the result of habitat loss, in combination with the sedentary nature of the species.

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