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Multiple origins for Scots pine (*Pinus sylvestris* L.) in Scotland: evidence from mitochondrial DNA variation

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Data from the pollen record and from two forms of nuclear genetic markers suggest that present-day populations of Scots pine (*Pinus sylvestris* L.) in Scotland were derived from more than one refugium after glaciation. In order to clarify this issue, genetic variation for maternally inherited mitochondrial DNA (mtDNA) was studied in 466 trees sampled from 20 natural populations in Scotland. A homologous probe for the *cox1* mitochondrial gene of *P. sylvestris* was constructed and used to detect mtDNA RFLP variation. Two common (**a** and **b**) and one rare RFLP variant (**c**) were distinguished. Evidence from segregation patterns of variants within a polymorphic population was consistent with maternal inheritance of the RFLP variation. A survey of Scottish populations indicates that mitotype **a** is present at all sites, but that mitotype **b** is confined to three western populations. Genetic differentiation for mtDNA, which migrates solely by seed is much greater ($F_{ST(m)} = 0.370$) than for nuclear markers ($F_{ST(b)} = 0.028$) which are dispersed by both pollen and seed. The geographical distribution of mitotype **b** in western Scotland, and its absence from populations in northern France and Germany, suggest that *P. sylvestris* has been derived not only from continental Europe via England, but also by migration from a western refugium, probably in Ireland or western France.

Keywords: mitochondrial DNA, *Pinus sylvestris*, population genetics, postglacial history.

Introduction

Much of our understanding of the postglacial invasion of Britain by tree species is derived from the pollen record (Birks, 1989). One of the most intriguing questions posed by this work concerns the origins of Scots pine, *Pinus sylvestris* L., whose natural populations in Britain are confined to highland Scotland. Scottish populations represent an outlier of this most widely distributed of all conifers (Vidakovic, 1991). This outlier is situated at the extreme north-west of the species range, separated by at least 500 km from the main distributions in Continental Europe and Scandinavia. Detailed consideration of pollen data suggests multiple origins for Scottish populations of *P. sylvestris* after glaciation (Birks, 1989). The most recent hypothesis proposes invasion both from France northwards via England, and from some second source along a more westerly route possibly via the north of Ireland into western Scotland (Bennett, 1995).

Interpretation of patterns of tree spread from pollen analysis is notoriously problematic, especially in a species such as *P. sylvestris* where copious amounts of wind-borne pollen may be deposited many tens of kilometres from the pollen source (Huntley & Birks, 1983). In these circumstances additional information may be gained by studying the distribution of genetic variation in present-day populations. Elements of genetic structure established at the time of invasion may remain and provide evidence for or against current interpretations of postglacial history (Ferris *et al*., 1993).

Two existing studies of genetic variation in remnant populations of *P. sylvestris* in Scotland provide tentative support for a multiple origin after glaciation. Analysis of genetically determined resin monoterpene composition highlights a group of native populations in Wester Ross possessing a

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significantly lower proportion of 3-carene in their cortical resin than those elsewhere in Scotland (Forrest, 1980). Data on isozyme variation indicate that the population showing greatest genetic differentiation from its fellows is located at Shieldaig within the Wester Ross group (Kinloch *et al*., 1986). The inference is that these populations in Wester Ross had a separate origin, and possessed frequencies of genetic markers distinct from those in other native populations at the time of colonization. They retain some of this distinctiveness for genetic markers some 50 generations later.

One of the problems with inferring history from present-day distributions of genetic markers is that the initial genetic structure established at colonization breaks down over time as a consequence of interpopulation gene flow. In plants this occurs most rapidly for nuclear genes, such as those coding for monoterpene and isozyme variation, where gene flow is mediated by both seed and pollen (Petit *et al*., 1993a). Pollen flow is especially extensive among populations of pine with substantial gene transfer occurring over tens of kilometres (Millar, 1983; Nagasaka & Szmidt, 1985). In contrast, maternally inherited organelle genes can only travel between populations via seed. Interpopulation gene flow for maternally inherited markers is likely to be substantially lower than for nuclear markers. Initial genetic structure will consequently be retained longer for maternally inherited organelle than for biparentally inherited nuclear genetic markers, making them especially suitable for the study of historical processes (Petit *et al*., 1993a).

Maternally inherited organelle markers may also have advantages over nuclear markers for detecting historical patterns of colonization because the extent of genetic differentiation between populations invading from different refugia is likely to have been greater for organelle than for nuclear markers. This is because effective population size for organelle genes is half that for nuclear genes in an outcrossing species (Birky *et al*., 1989). In bottlenecked refugial populations opportunities for differentiation of polymorphic organelle markers through drift would have been much greater than for nuclear markers. A greater degree of genetic structuring would therefore have resulted at maternally inherited markers than at nuclear markers when the colonizing populations met. Thus on both counts a study of maternally inherited organelle markers is likely to be more efficient for providing information on the colonization history of *P. sylvestris* than a study of nuclear markers. The objective of this research was therefore to determine the genetic structure of maternally inherited markers in native populations of *P. sylvestris* in Scotland in order to provide further insights into the colonization history of the species.

In conifers such as *P. sylvestris* only the mitochondrial genome is maternally inherited (Neale & Sederoff, 1989; Wagner *et al*., 1991). In contrast to angiosperms, the chloroplast genome is paternally inherited. Rates of sequence evolution in mitochondrial genomes of plants are very low and analysis of DNA sequence in known mitochondrial genes is unlikely to reveal intraspecific variation (Wolfe *et al*., 1987). However, rates of structural rearrangement are relatively high in the mitochondrial genome of plants (Palmer, 1992). Such variation is best detected by traditional RFLP analysis involving Southern blotting and species-specific mitochondrial DNA probes.

A mitochondrial DNA (mtDNA) RFLP marker was therefore developed to detect intraspecific variation within *P. sylvestris.* The maternal inheritance of this marker was tested. Variation and differentiation for the mtDNA marker was measured within a sample of 20 native pinewood populations covering the current distribution of the species in Scotland. The results are interpreted in terms of the colonization history of the species after glaciation and compared with existing data from nuclear markers.

Materials and methods

Development of mtDNA marker

For efficient RFLP analysis of the mtDNA in plants (which represents a small fraction of the total DNA) a homologous probe is required (Sutton *et al*., 1991). Sequences of the plant mitochondrial *cox1* gene were extracted from the EMBL DNA database and aligned. Conserved regions were identified and two primers were designed to amplify a 710 bp region of the *cox1* gene (Isaac *et al*., 1985; Glaubitz & Carlson, 1992). The sequences of the two primers were:

5′-ATTATCACTTCCGGTACTGG

3′-AGCATCTGGATAATCTGG.

PCR amplification of the *cox1* fragment was carried out using 50 ng template DNA from *P. sylvestris*, 25 μM of each primer, 200 μM of each dNTP and 1 U Taq polymerase (Promega) in a final volume of 50 μL. A Perkin Elmer Cetus 480 thermocycler was used with the following conditions: 94°C for 3 min followed by 40 cycles of 60 s at 94°C, 60 s at 55°C and 90 s at 72°C then a final extension period of 5 min at 72°C.

The PCR product was purified after separation on an agarose gel using the Geneclean II Kit (Bio 101, California) following the manufacturer's instructions. The purified PCR product ($\approx$700 bases) was then cloned into a pBluescript vector and partially sequenced (400 bases) using the Pharmacia T7 sequencing kit following the manufacturer's instructions. The partial sequence showed 88 per cent homology to the *cox1* sequence of *Zea mays*, confirming its identity (Isaac *et al*., 1985). The excised *cox1* sequence was used as a probe in subsequent RFLP analysis.

RFLP analysis

Total DNA was extracted from $\approx$1 g of dormant needle and bud tissue of *P. sylvestris* using the CTAB protocol of Murray & Thompson (1980). Five micrograms of each sample was digested to completion with a restriction endonuclease. Digested fragments were separated on 1 per cent agarose gels in TAE buffer for 16 h at 0.5 V/cm. DNA was transferred to Hybond-N membrane (Amersham) and covalently bound to the membrane by exposure to ultraviolet radiation at $0.4 \times 10^3 J/m^2$.

The *cox1* DNA fragment to be used as a probe was radioactively labelled with α^{32}P-dCTP (Amersham) by the method of Feinberg & Vogelstein (1983). The labelled mixture was then passed through a Millipore filter and an Elutip-d column (Schleicher and Schull) following the manufacturer's instructions, to purify the labelled probe.

Hybridizations with the excised *cox1* probe were conducted overnight at 65°C in a mini 10 hybridization oven (Hybaid) in a solution containing 2 $\times$ Denhart's solution, 4 $\times$ SSC, 1 per cent SDS, 10 per cent Dextran sulphate and 20 mM Tris, pH 7.6. (Sambrook *et al*., 1989). The hybridized blot was then washed twice with 4 $\times$ SSC, 1 per cent SDS for 30 min at 65°C, twice with 2 $\times$ SSC, 0.5 per cent SDS for 30 min at 65°C, and finally 2 $\times$ SSC for 30 min at room temperature. Hybridized blots were then exposed to X-ray film in the presence of Du Pont intensifying screens for 24–72 h.

Initial screening for variation

For initial screening 10 DNA samples were used, five from Scottish populations of *P. sylvestris* (Shieldaig, Glen Loy, Doire Darach, Glen Strathfarrar, Ryvoan, Table 1) and five from elsewhere in the worldwide distribution of the species (Russia, Germany, Sweden, Turkey and China). Five micrograms of each DNA sample were digested to completion with each of the restriction enzymes *Eco*RI, *Cfo*I, *Bam*HI, *Eco*RV, *Hin*dIII, *Sma*I, *Kpn*I, *Xba*I, *Taq*I and *Pst*I and analysed for RFLP variation as outlined above. Fragment sizes were calculated by comparison with a 1 kb ladder.

Inheritance of RFLP variants

In the absence of controlled crosses, segregation of RFLP variation was studied in open pollinated seed families from the Shieldaig population which showed polymorphism in mature trees for two restriction fragment patterns designated **a** and **b** that were detected by the *cox1*/*Cfo*I and *cox1*/*Bam*HI enzyme/probe combinations. Twenty paired samples of maternal bud tissue and open-pollinated seed were collected from the Shieldaig population. DNA extracted from bud tissue of each maternal tree was screened for the *cox1*/*Cfo*I restriction fragment polymorphism. One maternal parent of each restriction fragment type was chosen. Embryos were dissected from the seeds of these two parents and DNA was extracted from the embryos by a modification of the CTAB method. The DNA extracted from each embryo was sufficient to conduct RFLP analysis using two restriction enzymes. Five embryos from the maternal parent of restriction type **a**, and 16 embryos from the maternal parent of restriction type **b** were scored for their RFLP pattern using the *cox1*/*Cfo*I and *cox1*/*Bam*HI enzyme/probe combinations.

Screening of Scottish populations

Samples of needles and dormant buds were collected from at least 30 mature trees within each of 20 natural populations of *P. sylvestris* in Scotland (Table 1, Fig. 1). Samples cover the full geographical range of Scottish populations and include all the 'biochemical regions' designated by the Forestry Commission on the basis of monoterpene variation (Forestry Commission, 1989). DNA was extracted from 4 to 40 individuals per population, and scored for RFLP variation using the *cox1*/*Cfo*I and *cox1*/*Bam*HI probe/enzyme combinations that revealed polymorphism in the screening trial. The mean number of individuals scored was 24 per population.

Statistical analysis of variation

The significance of differences in frequency of mtDNA RFLP types (mitotypes) among populations

was determined with Fisher's exact test using the GENEPOP program (Raymond & Rousset, 1995). Partitioning of mtDNA variation within and among populations was quantified using Weir & Cockerham's (1984) estimator of the inbreeding coefficient F_{ST} employing the FSTAT program modified for analysis of haploid data (Goudet, 1995).

Results

Preliminary screening

RFLP variation was detected for the *cox1*/*Cfo*I and *cox1*/*Bam*HI probe/enzyme combinations. In both digests the Shieldaig sample contained an extra fragment not present in other populations. The putative mtDNA variant lacking the extra bands was designated as mitotype **a**, and that possessing the bands as mitotype **b** (Table 2).

Inheritance study

All five of the open-pollinated progeny of the mitotype **a** maternal tree at Shieldaig were also of mitotype **a**. All 16 progeny assayed from the mitotype **b** maternal parent at Shieldaig were of mitotype **b**.

Survey of Scottish populations

Three mtDNA RFLP patterns were detected within Scotland corresponding to mitotypes **a** and **b** designated above, plus a single individual with a novel mitotype **c** in the Glen Falloch population (Table 2). As before this variant **c** shows altered banding patterns for both restriction digests and may represent a recent mtDNA structural rearrangement. Table 1 shows the numbers of each mitotype scored in each population. Mitotype **b** is confined to three populations in the west where it occurs at frequencies of 0.16 (Shieldaig), 0.59 (Glen Loy) and 0.08 (Doire Darach) (Fig. 1). All other populations (with the exception of Glen Falloch with its single third variant) were monomorphic for mitotype **a**.

The frequency of mitotypes is highly significantly different among populations ($P<0.001$, Fisher's exact test). Weir & Cockerham's (1984) estimate of the F_{ST} value for the mitochondrial markers is 0.370 indicating strong genetic structuring for mtDNA variants among populations.

Table 1 Scottish populations of *Pinus sylvestris* analysed for mtDNA variation, and numbers of each RFLP mitotype scored

Population and number	Location	Elevation (m)	Number of each mitotype		
			a	**b**	Total
1. Glen Einig	57°57′N, 4°46′W	100	13	0	13
2. Rhidorroch	57°54′N, 4°58′W	100	26	0	26
3. Strath Vaich	57°45′N, 4°47′W	300	13	0	13
4. Loch Maree	57°40′N, 5°25′W	20	4	0	4
5. Shieldaig	57°31′N, 5°37′W	100	38	7	45
6. Achnashellach	57°28′N, 5°17′W	150	25	0	25
7. Glen Strathfarrar	57°24′N, 4°43′W	150	30	0	30
8. Glen Affric	57°15′N, 5°04′W	250	26	0	26
9. Loch Hourn	57°10′N, 5°28′W	50	17	0	17
10. Glen Barisdale	57°04′N, 5°30′W	150	30	0	30
11. Glen Garry	57°04′N, 4°55′W	150	13	0	13
12. Glen Loy	56°55′N, 5°08′W	150	13	19	32
13. Conaglen	56°48′N, 5°21′W	200	16	0	16
14. Rannoch	56°41′N, 4°19′W	250	20	0	20
15. Doire Darach	56°32′N, 4°47′W	200	37	3	40
16. Glen Orchy	56°27′N, 4°53′W	150	24	0	24
17. Glen Falloch	56°22′N, 4°39′W	200	29	0	29*
18. Abernethy	57°14′N, 3°39′W	300	10	0	10
19. Ryvoan	57°10′N, 3°42′W	400	29	0	29
20. Glentanar	57°01′N, 2°53′W	250	24	0	24

*One additional individual of mitotype **c** found (see Table 2).

Discussion

RFLP analysis using cloned mtDNA genes as probes has been applied a number of times to detect and survey mtDNA variation in plant populations (Belhassen *et al*., 1993; Dong & Wagner, 1993; Samitou-Laprade *et al*., 1993; Strauss *et al*., 1993; Ronfort *et al*., 1995). Interpretation of such RFLP data relies on the assumption that the fragments detected are located in the mitochondrial genome and do not represent sequences that have been transferred to the nucleus (Strauss *et al*., 1993). Evidence for a mitochondrial location must ultimately come from proof that the variation is maternally inherited.

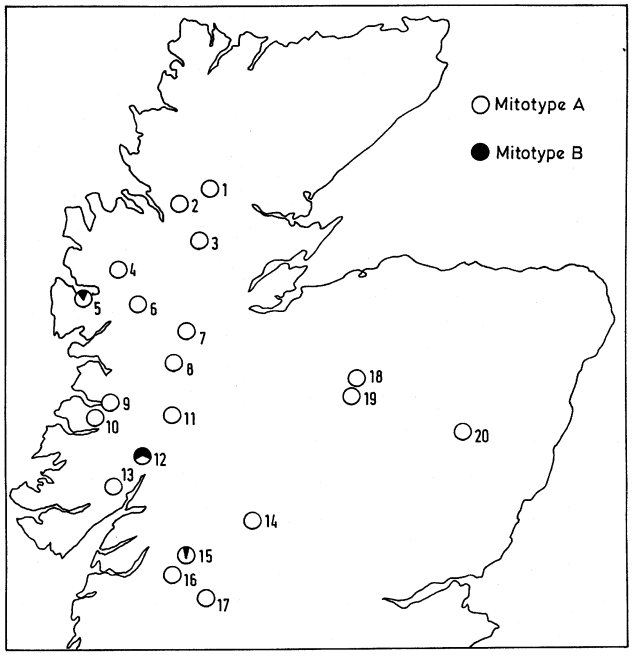

Fig. 1 Distribution of mitotypes within 20 natural populations of *Pinus sylvestris* in Scotland (Steven & Carlisle, 1959). For details of populations see Table 1. Populations are: 1, Glen Einig; 2, Rhidorroch; 3, Strath Vaich; 4, Loch Maree; 5, Shieldaig; 6, Achnashellach; 7, Glen Strathfarrar; 8, Glen Affric; 9, Loch Hourn; 10, Glen Barisdale; 11, Glen Garry; 12, Glen Loy; 13, Conaglen; 14, Rannoch; 15, Doire Darach; 16, Glen Orchy; 17, Glen Falloch; 18, Abernethy; 19, Ryvoan; 20, Glentanar.

Table 2 Mitochondrial DNA variants of *Pinus sylvestris* revealed by RFLP analysis, and their mitotype designations

Mitotype designation	Size of fragments (kb) hybridizing to *cox1* probe after digestion with:	
	*Cfo*I	*Bam*H1
a	1.6, 1.3	9.0, 7.0
b	1.6, 1.3, 0.9	9.0, 7.0, 4.5
c	2.6, 0.9, 0.7	9.0, 7.0, 2.6

Although controlled crosses were not available in this study, the pattern of segregation of genetic variants in open-pollinated progeny within the polymorphic Shieldaig population is entirely consistent with maternal inheritance. If the restriction phenotype with the extra bands (type **b**) resulted from a nuclear mutation, the only explanation for the lack of segregation among the 16 outcrossed progeny from the maternal parent of this low frequency variant would be that it is homozygous for the allele concerned. In an outcrossing species (such as *P. sylvestris*) where the frequency of a dominant phenotype with extra restriction fragments is 0.16 (as at Shieldaig), the frequency of homozygous maternal individuals is expected to be 7×10^{-3}, and the probability of choosing a homozygous maternal individual from within a sample of the variant phenotype **b** is less than 0.05. The hypothesis of nuclear inheritance is therefore an unlikely one, and maternal inheritance of the RFLP variation is a more parsimonious explanation for the results. Formal proof of maternal inheritance of *cox1* variants detected by the same form of RFLP analysis has been given for interspecific crosses involving *P. sylvestris* and related conifers (Neale & Sederoff, 1989; Sutton *et al*., 1991; Wagner *et al*., 1991; DeVerno *et al*., 1993; Wang *et al*., 1996). It is therefore reasonable to assume that the variation analysed here is maternally inherited mtDNA variation.

There is good evidence from restriction fragment analysis of PCR amplification products that the *cox1* gene is present in multiple copies in the mitochondrial genome of *P. sylvestris* (Wang *et al*., 1996). Changes in the RFLP profile of *cox1* on Southern blots are likely to be caused by rearrangements in the mitochondrial genome, relocating the *cox*1 gene copies within different flanking sequences leading to the appearance of bands of novel sizes on restriction digestion. Such rearrangements may simultaneously change the RFLP pattern for a number of restriction enzymes, as found here for the enzymes *Cfo*I and *Bam*HI. One consequence of this complex form of mutation, which occurs commonly in plant mtDNA, is that it is not possible to analyse the evolutionary relationships of different mtDNA variants. Thus although the plant mitochondrial genome is useful for assessing differences in the frequency of mater-

nally inherited variants within populations, it cannot be used in the same way as the animal mitochondrial or plant chloroplast genomes to reconstruct the history of populations using a genealogical approach (Palmer, 1992).

Two features are clearly shown by the survey of mtDNA variation in Scottish populations of *P. sylvestris*. The first is that the mtDNA variation is highly geographically structured, with large differences in mitotype frequency occurring among populations (Table 1, Fig. 1). The value of the population differentiation statistic $F_{ST(m)} = 0.37$ is high. The second feature concerns the geographical distribution of variants. Mitotype **b** is not scattered over the complete Scottish distribution, but is confined to three populations in the west (Fig. 1). One of these is Shieldaig which has been shown in previous studies to be distinct for both monoterpene and isozyme nuclear genetic markers (Forrest, 1980; Kinloch *et al*., 1986). This geographical pattern of variation is unlikely to be a sampling artifact because more than 200 samples from 12 populations to the east were scored and did not reveal a single mitotype **b** variant.

A high degree of population differentiation for maternally inherited mtDNA variants is expected even after drift/migration equilibrium has been reached, because migration among populations can only occur by seed flow (Petit *et al*., 1993a). The value of mtDNA differentiation ($F_{ST(m)} = 0.37$) is some 13 times higher than the equivalent measure of genetic differentiation for nuclear isozyme markers ($F_{ST(b)} = 0.028$) within these same Scottish populations of *P. sylvestris* (Kinloch *et al*., 1986). If populations are assumed to be at drift/migration equilibrium for these two sets of markers an estimate of the ratio of pollen to seed flow among populations can be derived from a comparison of F_{ST} values (Ennos, 1994). Using these methods, interpopulation pollen flow is calculated to be some 18.4 times greater than seed flow. These estimates are likely to be overestimates because it appears that a certain amount of structuring resulting from population history remains. Similar results have been found for estimates of pollen to seed flow in other species of pines (Dong & Wagner, 1993; Strauss *et al*., 1993; Ennos, 1994).

The presence of the mitotype **b** variant in widely dispersed populations of *P. sylvestris* in western Scotland is intriguing and may be interpreted in terms of the history of colonization after glaciation. A survey of *cox1* mtDNA variation in some 85 individuals from northern Europe (northern France, Germany, Poland, southern Sweden, Russia) reveals only mitotype **a** (Sinclair & Ennos, in prep.). Because these populations trace back to the source populations which colonized Scotland from the south, we can conclude that this cannot have been the only route by which *P. sylvestris* reached Scotland (Birks, 1989). Mitotype **b** must have been derived from some other source.

The western location of this variant suggests that it may have been associated with spread of *P. sylvestris* into Scotland from the west, and that its further spread into the east was prevented by prior colonization by another source lacking this variant. Unfortunately, putative progenitor populations for this western colonization can no longer be sampled because of the extinction of Irish populations of *P. sylvestris* between 1000 and 2000 years ago (Birks, 1989). Nevertheless, the hypothesis of a separate western colonization route ties in well with the existing data from the monoterpene and isozyme nuclear markers, which indicate the genetic distinctness of north-western populations, particularly Shieldaig (Kinloch *et al*., 1986). It is notable that the Shieldaig population is also distinct in terms of its possession of the mitotype **b** variant.

The presence of mitotype **b** in populations further south than Shieldaig suggests a more widespread colonization from the south-west than indicated by the nuclear marker data alone. The contrast between results for mitochondrial and nuclear markers may reflect a difference in the extent to which geographical structure of maternally and biparentally inherited markers has been broken down by interpopulation gene flow since colonization. More limited gene flow by seed alone may lead to longer retention of historical structure for the mitochondrial marker than for the nuclear markers, leaving more complete genetic traces of colonization routes.

This study demonstrates the utility of studies on maternally inherited mtDNA markers for elucidating the postglacial history of tree species. The relatively high rate of mutation through rearrangement in the mitochondrial genome means that intraspecific variation is sufficient for recent genetic divergence events, occurring over the period of a single glaciation, to be studied. This contrasts with the situation for chloroplast DNA where mutation rates are generally much lower and long periods of genetic isolation, amounting to many cycles of glaciation and deglaciation, may be needed for cpDNA divergence to accumulate between refugial populations (Ferris *et al*., 1993; Petit *et al*., 1993b). However, mtDNA analysis in plants cannot give the powerful phylogenetic perspective on population history

provided by analysis of cpDNA (Demesure *et al*., 1996; van Dijk & Bakx-Schotman, 1997). Nevertheless when used in conjunction with a range of other markers it is a useful tool for reconstructing recent population history.

In the present case the combination of information from nuclear and mtDNA markers strongly supports the hypothesis of two origins for *P. sylvestris* in Scotland after glaciation. However, the source of the refugial population to the west remains a question for speculation. Consideration of the glacial history of Scotland makes it highly unlikely that the western population was an endemic one, as has been suggested (Kinloch *et al*., 1986). Given the extent of the ice sheet covering Britain in the last glaciation a source in south-west Ireland or possibly western France seems far more plausible (Ballantyne & Harris, 1994; Bennett, 1995).

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