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Phylogeographic structure of brown trout *Salmo trutta* in Britain and Ireland: glacial refugia, postglacial colonization and origins of sympatric populations

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The phylogeographical structure of brown trout *Salmo trutta* in Britain and Ireland was studied using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of four mitochondrial DNA segments (16S/ND1, ND5/6, COXIII/ND5 and ND5/12S). Analysis of 3636 individuals from 83 sites–morphotypes revealed a total of 25 haplotypes. These haplotypes were nested in seven two-step clades. Although there was a clear geographical patterning to the occurrence of derived clades, admixture among ancestral clades was extensive throughout the studied area. A relevant feature of the data was that some populations contained mixtures of highly divergent clades. This type II phylogeographic pattern is uncommon in nature. Clade intermixing is likely to have taken place during earlier interglacials as well as since the Last Glacial Maximum. The anadromous life history of many *S. trutta* populations has probably also contributed to clade mixing. Based on the data presented here and published data, postglacial colonization of Britain and Ireland most likely involved *S. trutta* from at least five potential glacial refuges. Probable locations for such refugia were: south of England–western France, east of the Baltic Sea, western Ireland, Celtic Sea and North Sea. Ferox *S. trutta*, as defined by their longevity, late maturation and piscivory, exhibited a strong association with a particular clade indicating that they share a common ancestor. Current evidence indicates that the Lough Melvin gillaroo *S. trutta* and sonaghen *S. trutta* sympatric types diverged prior to colonization of Lough Melvin and, although limited gene flow has occurred since secondary contact, they have remained largely reproductively isolated due to inlet and outlet river spawning segregation. Gillaroo *S. trutta* may reflect descendents of a previously more widespread lineage that has declined due to habitat alterations particularly affecting outlet rivers. The mosaic-like distribution of mtDNA lineages means that conservation prioritization in Britain and Ireland should be based on the biological characteristics of local populations rather than solely on evolutionary lineages.

Key words: brown trout; mitochondrial DNA; PCR–RFLP; phylogeography; sympatric populations.

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INTRODUCTION

The brown trout *Salmo trutta* L. displays extensive genetic, ecological, morphological and life-history variation (Ferguson, 1989), which has resulted in a long-standing debate on the evolutionary origins and taxonomic implications of this variability (Günther, 1866; Behnke, 1972; Kottelat & Freyhof, 2007). The Atlantic group (*sensu* Bernatchez, 2001) of *S. trutta* is currently distributed from Morocco northwards to Iceland, together with the Baltic and White Seas. Based on the degree of mitochondrial DNA (mtDNA) divergence, Bernatchez (2001) suggested that this group split from the more eastern *S. trutta* groups during the early to mid-Pleistocene, some 700 000 years before present (B.P.). In NW Europe, this period since separation of the Atlantic group encompasses major climatic oscillations that resulted in several glacial and interglacial periods (Webb & Bartlein, 1992; Clark *et al*., 2004). Many studies of fish and other organisms have revealed the dramatic effects of these Pleistocene glaciations on evolution and current distribution of species (Hewitt, 2004). The most recent glacial period started *c*. 30 000 B.P. and reached a maximum (Last Glacial Maximum, LGM) some 23 000 to 18 000 B.P. (Bowen *et al*., 2002; Clark *et al*., 2004; Knight *et al*., 2004).

During the LGM ice covered much of Britain and Ireland, although some peripheral regions such as the south and west of Ireland, and south of England remained free from glaciation (Clark *et al*., 2004). In addition, as a result of the lowered sea level, the land mass was considerably extended in comparison with today (Clark *et al*., 2004), providing possible refugia in areas that are currently marine. *Salmo trutta* is potentially anadromous and this would have allowed both rapid range retreat into refugia during glacial periods and subsequent expansion during interglacial periods. Similarly, the sea would not have presented a barrier to postglacial colonization of current freshwater catchments. Thus, most, if not all, native *S. trutta* populations in Britain and Ireland, irrespective of current life history, colonized as anadromous *S. trutta* (Ferguson, 2006). Anadromy has allowed continued gene flow between populations in some adjacent catchments. These interlinked factors suggest that the phylogeographic structure of *S. trutta* in NW Europe is likely to be more complex than that described for many other organisms. Overall, there have been repeated opportunities for genetic divergence in allopatric refuges, followed by interbreeding, or varying degrees of reproductive isolation, on secondary contact after postglacial colonization. This prediction is supported by studies of *S. trutta* that have revealed a complex phylogeographic structure within NW Europe. Although the consensus is that postglacial colonization involved multiple lineages originating from separate refuges, hypotheses differ in terms of the number, origin and dispersal dynamics of these lineages (Ferguson & Fleming, 1983; Hamilton *et al*., 1989; Hynes *et al*., 1996; García-Marín *et al*., 1999; Weiss *et al*., 2000; Bernatchez, 2001; Cortey *et al*., 2009). More extensive sampling and increased phylogeographic resolution are therefore necessary to clarify the evolutionary history of *S. trutta* in this region, especially given that, to date, only limited studies have been undertaken in Britain and Ireland.

In several waters throughout the range of the species, two or more populations of *S. trutta* have been found to co-occur (Allendorf *et al*., 1976; Ferguson & Mason, 1981; Crozier & Ferguson, 1986; Sušnik *et al*., 2005; Duguid *et al*., 2006). Typically these sympatric populations show significant interpopulation differences in allele

frequencies and, since only limited gene flow is necessary to result in homogeneity of neutral alleles (Morjan & Rieseberg, 2004), a high degree of reproductive isolation must be present. One of the best studied examples is the sympatric gillaroo *S. trutta*, sonaghen *S. trutta* and ferox *S. trutta* populations in Lough Melvin, NW Ireland (reviewed by Ferguson, 2004). Based on their genetic, morphological and ecological differentiation (Ferguson & Mason, 1981; Ferguson, 1986; Cawdery & Ferguson, 1988; Ferguson & Taggart, 1991; Prodöhl, 1993; McVeigh *et al*., 1995), and to highlight their conservation importance, Ferguson (2004) considered these as distinct biological species. He proposed reinstatement of the 19th century scientific names of gillaroo *Salmo stomachicus* Günther, sonaghen *Salmo nigripinnis* Günther and ferox *Salmo ferox* Jardine. This classification is also used by Kottelat & Freyhof (2007). A fuller understanding of the evolutionary origins and homologies to other *S. trutta* populations, however, is necessary for validation of this classification and implementation of conservation plans. In particular, it is essential to elucidate whether these sympatric populations in Lough Melvin are the result of colonization by two or more genetically distinct lineages, or of colonization by a single lineage followed by splitting within the lake under the influence of selective forces. Ferox *S. trutta* (long-lived, late maturing and piscivorous) also occur in many other lakes (Greer, 1995) and, based on nuclear and mitochondrial markers, have been shown to be genetically differentiated from sympatric *S. trutta* in Lochs Laggan and Awe (Scotland) (Duguid *et al*., 2006).

In this study, brown trout were sampled extensively throughout Britain and Ireland, including sympatric populations where feasible, and assayed for mtDNA variation using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). As homologies to *S. trutta* in other waters are not known, the Melvin *S. trutta* types and ferox *S. trutta* from various waters are treated as populations from the standpoint of this study. The geographical distribution of mtDNA haplotypes was then used to: (1) infer the glacial refuge areas and patterns of postglacial colonization of Britain and Ireland and in doing so permit broader inferences on the evolutionary history of *S. trutta* in NW Europe; and (2) investigate the origins of available sympatric populations.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Salmo trutta specimens ($N = 3636$) were obtained from 83 sites–sympatric morphotypes (Table I and Fig. 1) throughout Britain and Ireland, with samples being obtained as far as possible from waters with no known history of supplemental stocking with non-native farm-reared *S. trutta*. In order to control for possible stocking in some locations, however, samples were obtained from two fish-farm strains that have been the predominant strains used for such stocking in Britain and Ireland (Ferguson, 2007). In addition, 90 specimens from four regions in Iceland and 18 specimens from Poland were used for comparison with the results of Hynes *et al*. (1996), and together with the samples from Britain and Ireland are collectively referred to here as NW Europe. One or two individuals from each of Spain (Atlantic drainage), France (Mediterranean drainage), Corsica, Turkey, Albania (Ohrid) and Slovenia (marbled trout, *Salmo marmoratus* Cuvier) were used as phylogenetic outgroups. Genomic DNA was isolated from frozen or ethanol-preserved trout tissue as described by Taggart *et al*. (1992).

TABLE I. Location, sample size, mtDNA haplotype frequencies, number of haplotypes (Nhap) and haplotype diversity (h ± S.E.) of the trout samples from NW Europe. Numbers preceding sample names correspond to Fig. 1. Geographical regions are italicized and regional values are reported, with haplotypes, with frequencies <0·01 being rounded to 0·01 (spread across the facing page)

		Haplotypes											
Sample	*N*	13·7	3·7	4·7	10·7	14·3	15·7	3·8	16·8	22·8	9·3	6·5	17·1	1·2
North-west Ireland														
1. Derg	100		0·38			0·01						0·34		
2. Lackagh	16		0·63			0·19								
3. Eske	32		0·16									0·13		
4. Behy	46													
5. Erne	50													
6. Melvin ferox	49													
7. Melvin gillaroo	50		0·08											
8. Melvin sonaghen	42													0·14
Total	385		0·15			0·01						0·10		0·02
West Ireland														
9. Conn	33					0·27								
10. Carrowsallagh	63		0·41									0·06		
11. Burrishoole	307		0·66								0·13			
12. Crumlin	145		0·19								0·32	0·01		
13. Corrib	41		0·02			0·02								
14. Mask	73		0·15											
15. Corrib–Mask ferox	43		0·07			0·02								
16. Ennel	31			0·10										
17. Ree	33													
18. Croneen	33													
Total	802		0·34	0·01		0·01					0·11	0·01		
South-west Ireland														
19. Laune	54		0·13									0·06		
20. Caragh	34		0·24								0·06	0·06		
21. Coomasaharn	14													
22. Currane	54		0·39			0·06					0·20			
23. Inchiquin	12		0·58			0·17								
Total	168		0·26			0·03					0·08	0·03		
South-east Ireland														
24. Coumshingaun	36													
25. Glendalough	15					1·00								
Total	51					0·29								
North-east Ireland														
26. Glynn	102											0·01		
27. Glenariff	42	0·05		0·02								0·81		
28. Neagh	30		0·33											
Total	174	0·01	0·06	0·01								0·20		
North-west England														
29. Eden	29			0·93										
30. Kent	30		0·73			0·17								
31. Lune	30		0·57	0·23										
Total	89		0·44	0·38		0·06								
West Scotland														
32. Doon	14		0·86											
33. Eck	22											0·36		
34. Awe	31		0·10									0·26		
35. Awe ferox	10		0·10									0·10		
36. Avich	40											0·05		
37. Awe isolated	105					0·10					0·01	0·49		
38. Mull	10		0·20			0·10								
39. Laggan	103		0·02	0·13										
40. Laggan ferox	8		0·13											
41. Pattack	85		1·00											

TABLE I. Continued

Haplotypes														
1·3	8·3	11·3	12·3	21·3	1·1	1·4	1·5	2·6	5·9	7·6	19·6	20·6	Nhap	h ± s.e.
0·27													4	0·674 ± 0·013
										0·19			3	0·575 ± 0·112
0·03										0·69			4	0·502 ± 0·093
0·98										0·02			2	0·043 ± 0·041
										1·00			1	–
										1·00			1	–
0·26					0·08	0·20	0·06	0·02		0·30			7	0·802 ± 0·027
0·10					0·14			0·29		0·33			5	0·776 ± 0·031
0·23					0·03	0·03	0·01	0·03		0·40			10	0·753 ± 0·014
0·48										0·24			3	0·652 ± 0·044
0·41										0·11			4	0·653 ± 0·032
0·12										0·09			4	0·529 ± 0·029
0·05		0·37								0·06			6	0·722 ± 0·018
0·22								0·02		0·71			5	0·461 ± 0·079
0·12										0·73			3	0·441 ± 0·062
0·07										0·84			4	0·296 ± 0·087
0·35			0·03							0·52			4	0·617 ± 0·056
0·64										0·36			2	0·477 ± 0·050
0·27			0·06							0·67			3	0·492 ± 0·075
0·18		0·07	0·01					0·01		0·28			10	0·761 ± 0·007
0·80										0·02			4	0·352 ± 0·077
0·65													4	0·535 ± 0·081
1·00													1	–
0·35													4	0·693 ± 0·028
0·25													3	0·621 ± 0·118
0·60										0·01			6	0·569 ± 0·033
0·83							0·17						2	0·286 ± 0·084
													1	–
0·59							0·12						3	0·565 ± 0·051
0·92	0·07												3	0·147 ± 0·046
0·05										0·07			5	0·343 ± 0·092
0·67													2	0·460 ± 0·061
0·67	0·04									0·02			7	0·513 ± 0·039
0·07													2	0·133 ± 0·081
0·10													3	0·439 ± 0·097
0·20													3	0·605 ± 0·067
0·12													4	0·651 ± 0·027
0·14													2	0·264 ± 0·136
0·45										0·18			3	0·658 ± 0·052
0·13										0·52			4	0·662 ± 0·064
0·10										0·70			4	0·533 ± 0·180
0·90				0·05									3	0·190 ± 0·081
0·34				0·06						0·01			6	0·640 ± 0·030
0·50										0·20			4	0·733 ± 0·120
0·19										0·66			4	0·515 ± 0·047
										0·88			2	0·250 ± 0·180
													1	–

TABLE I. Continued (spread across the facing page)

Sample	*N*	Haplotypes 13·7	3·7	4·7	10·7	14·3	15·7	3·8	16·8	22·8	9·3	6·5	17·1	1·2
42. Ewe	65		0·23			0·02						0·25		
43. Langavat	55		0·22	0·18		0·24						0·07		
44. Laxford	68		0·51			0·12				0·06		0·01		
45. Crocach	52											1·00		
Total	668		0·25	0·03		0·05				0·01	0·01	0·21		
Orkney & Shetland														
46. Boardhouse	27		0·48									0·41		
47. Harray	86		0·81			0·03					0·01	0·10		
48. Shetland	44		0·45			0·05		0·02				0·02		
Total	157		0·66			0·03		0·01			0·01	0·13		
East Scotland														
49. Shin	114											0·25		
50. Spey	21		0·90								0·05			
51. Lee	32		0·06									0·22		
52. Rannoch	84		0·27									0·13		
53. Tay	59		0·37			0·03						0·41		
54. Forth	62		0·06			0·02								
55. Tweed	51		0·10			0·06					0·18			
Total	423		0·18			0·01					0·02	0·17		
East England														
56. Coquet	40		0·05			0·05					0·15	0·03		
57. Tyne	18		0·44			0·06								
58. Wear	44		0·18								0·05			
59. Ouse	14										0·21			
60. Witham	16						0·25							
Total	132		0·14			0·02	0·03				0·08	0·01		
South-east England														
61. Thames	18			0·06								0·06		
62. Medway	23		0·04			0·17						0·09		
63. Stour	30			0·37										
64. Rother	30			0·30										
65. Dour	11			0·64										
66. Brede	8		0·13	0·50								0·38		
67. Ibour	12		0·17									0·08		
Total	132		0·03	0·24		0·03						0·05		
South-west England														
68. Lymington	22			0·23		0·05								
69. Frome	18		0·06											
70. Teign	38		0·55						0·13			0·11		
71. Yealm	12		0·50									0·08		
72. Tamar	20		0·55									0·35		
73. Fowey	66		0·53					0·02	0·08			0·29		
74. Valency	40		0·83									0·13		
Total	216		0·50	0·02		0·01		0·01	0·05			0·17		
Wales														
75. Severn	15		0·53									0·13		
76. Tywi	30		0·30									0·13		
77. Taf	27		0·26											
78. Teifi	30		0·20									0·13		
79. Conwy	29		0·24									0·14	0·03	
80. Clwyd	15		0·13			0·20						0·07		
81. Dee	24		0·46	0·08		0·04						0·08		
Total	170		0·29	0·01		0·02						0·10	0·01	
82. Iceland	90													
83. Poland	18		0·06	0·11	0·11							0·17		
84. Howietoun farm	46		0·07											
85. Rosscrae farm	23			0·30							0·09	0·04		

TABLE I. Continued

Haplotypes														
1·3	8·3	11·3	12·3	21·3	1·1	1·4	1·5	2·6	5·9	7·6	19·6	20·6	Nhap	h ± S.E.
0·08										0·42	0·02		6	0·718 ± 0·030
0·18										0·11			6	0·828 ± 0·017
0·01										0·13	0·15		7	0·688 ± 0·049
													1	–
0·19				0·01						0·22	0·02		10	0·802 ± 0·005
0·11													3	0·613 ± 0·050
										0·03			5	0·328 ± 0·062
0·23										0·23			6	0·703 ± 0·044
0·08										0·08			14	0·540 ± 0·043
0·36			0·02							0·38			4	0·674 ± 0·014
0·05													3	0·186 ± 0·110
0·03								0·66				0·03	5	0·532 ± 0·087
0·04										0·56			4	0·601 ± 0·040
0·08										0·10			5	0·688 ± 0·035
0·89								0·02		0·02			5	0·212 ± 0·068
0·29								0·08		0·29			6	0·792 ± 0·028
0·29			0·01					0·06		0·26		0·01	32	0·787 ± 0·008
0·23								0·28		0·23			7	0·815 ± 0·027
0·11									0·06	0·33			5	0·712 ± 0·074
0·34								0·02		0·41			5	0·697 ± 0·036
										0·79			2	0·363 ± 0·130
0·50										0·25			3	0·667 ± 0·075
0·26								0·09	0·01	0·36			22	0·772 ± 0·021
0·06									0·06	0·78			5	0·405 ± 0·143
0·57										0·13			5	0·652 ± 0·094
0·60										0·03			3	0·522 ± 0·056
										0·70			2	0·434 ± 0·070
										0·36			2	0·509 ± 0·101
													3	0·679 ± 0·122
										0·75			3	0·439 ± 0·158
0·24									0·01	0·39			23	0·728 ± 0·020
0·73													3	0·437 ± 0·105
0·94													2	0·111 ± 0·096
0·21													4	0·639 ± 0·066
0·33										0·08			4	0·682 ± 0·102
0·10													3	0·595 ± 0·073
0·08										0·02			6	0·634 ± 0·045
0·05													3	0·309 ± 0·087
0·25										0·01			25	0·665 ± 0·023
0·27										0·07			4	0·626 ± 0·110
0·53										0·03			4	0·628 ± 0·063
0·74													2	0·399 ± 0·084
0·67													3	0·515 ± 0·087
0·59													4	0·599 ± 0·077
0·53										0·07			5	0·695 ± 0·109
0·21							0·04			0·08			7	0·754 ± 0·074
0·53							0·01			0·03			29	0·625 ± 0·028
1·00													1	–
0·06										0·50			6	0·732 ± 0·096
								0·07		0·87			3	0·241 ± 0·080
0·09								0·04	0·26	0·17			7	0·826 ± 0·046

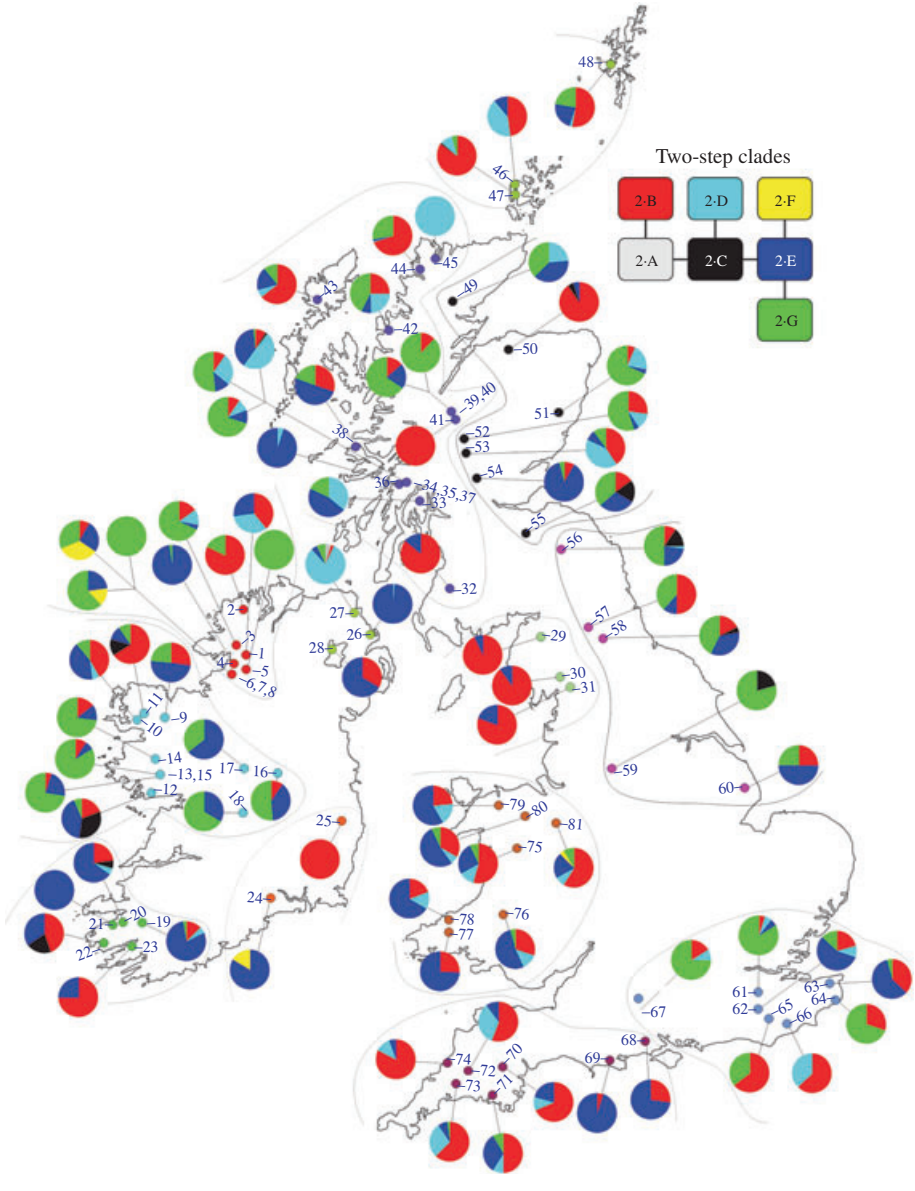

FIG. 1. Map of Britain and Ireland showing locations of wild population sample sites and the frequencies of two-step clades at each site. Regions, as defined in Table I, are indicated with feint lines.

MITOCHONDRIAL DNA RFLP ANALYSIS

PCR primers, which were developed on the basis of homologous sequences of rainbow trout *Oncorhynchus mykiss* (Walbaum) (Zardoya *et al*., 1995), were used initially to amplify brown trout mtDNA. These primers were: (1) QUB*Omy*mt16S-F 5′-TGCCCCCATGGAAGCGA TTAT-3′ and QUB*Omy*mtND1-R 5′-AGTGGGAGCTGGCAAAGGTGA-3′, which amplify a *c*. 2·35 kilobase (kb) fragment containing the 16S rRNA and ND1 genes (referred to subsequently as 16S/ND1 and corresponding to a region between positions 1757 and 4109 base pairs

(bp) of the complete *S. trutta* mitochondrion genome (Duc *et al*., 2007), which was submitted to Genbank, accession number NC_010007, after the QUB primers had been designed); (2) QUB*Omy*mtND5-F 5′-CCACACCCGAGAACACCTACTTAT-3′ and QUB*Omy*mtND6-R 5′-GAGGTCGACTAGTGCGTCATTAGC-3′ which amplify a 2·75 kb fragment containing the ND5 and ND6 genes (ND5/6, corresponding to a region between positions 11 649 and 14 445 bp of the complete *S. trutta* mitochondrion genome). Based on the study by Hynes *et al*. (1996), and a preliminary survey involving *S. trutta* from 12 geographically widespread waters in NW Europe, 10 restriction enzymes were chosen for RFLP screening of these regions (16S/ND1: *Ava*II, *Hae*III, *Alu*I and *Hpa*II; ND5/6: *Ava*II, *Xba*I, *Hin*fI, *Hae*III, *Dde*I and *Taq*I).

PCR–RFLP screening of the 16S/ND1 and ND5/6 regions identified unambiguously only four of the total mtDNA haplotypes described by Hynes *et al*. (1996). Haplotypes II, V, VI, VII and VIII, which previously had been found exclusively in Melvin sonaghen *S. trutta* and gillaroo *S. trutta*, however, were lumped into a single PCR–RFLP haplotype (haplotype 1). As the resolution of these haplotypes was essential for the elucidation of the phylogeography of gillaroo and sonaghen *S. trutta*, other mtDNA regions were screened for the presence of their defining restriction sites (R. Hynes & P. Prodöhl, unpubl. data). These were then assayed in two additional fragments named according to their flanking genes. The *Ava*II and *Mbo*I restriction sites distinguishing total mtDNA haplotypes II, VII and VIII were RFLP typed in the COXIII/ND5 fragment (4·4 kb, corresponding to a region between positions 8833 and 13 247 bp of the complete *S. trutta* mitochondrion genome), which was amplified using the primers QUB*Str*mtCOXIII-F (5′-CAAGCCCCTGACCCCTAACTGG-3′) and QUB*Str*mtND5-R (5′-CCCGGAGGCTATAGACGGCAGTGAA-3′). The *Hin*fI restriction sites distinguishing total mtDNA haplotypes V and VI were RFLP typed in the ND5/12S fragment(4·1 kb, corresponding to a region between positions 13 435 and 15 556 bp of the complete *S. trutta* mitochondrion genome) amplified using the primers QUB*Str*mtND5-F (5′-AGCCGCCCTCCTAGTTA CCATC-3′) and QUB*Str*mt12S-R (5′-CGCGCCTCAGAGCCAGTTTC-3′). RFLP analyses of these fragments were performed on all individuals identified initially as haplotype 1 as well as representatives (one to six individuals from as geographically widespread sites as possible) of all other 16S/ND1 and ND5/6 composite haplotypes. RFLP analysis of the four fragments was also performed on representative specimens of all haplotypes identified by analysis of total mtDNA (Hynes *et al*., 1996). Specimens of these same haplotypes were also analysed by Bernatchez (2001), and thus some homologies can be established.

PCRs for RFLP analysis consisted of *c*. 100 ng template DNA, 10 pmol of each primer, 200 μM dNTP mix, 2 mM (16S/ND1) or 2·5 mM (ND5/6, COXIII/ND5, ND5/12S) $MgCl_2$, 1× *Taq* polymerase buffer and 2 U of *Taq* polymerase (Invitrogen; www.invitrogen.com). Amplifications were performed in 50 μl volumes with a 5 min denaturation at 94° C, 30 cycles of 94° C for 1 min, 60° C (16S/ND1), 63° C (ND5/6, COXIII/ND5) or 65° C (ND5/12S) for 1 min and 72° C for 2·5 min, followed by a final extension at 72° C for 10 min. Restriction digests were carried out in 12 μl volumes using 8 μl of PCR product, 3 U of restriction enzymes and the appropriate buffer and conditions as recommended by the manufacturer (NEB or Promega; www.promega.com). Restriction fragments were separated by electrophoresis in Tris–Borate–EDTA buffered agarose gels containing ethidium bromide. Resultant mtDNA fragment profiles were visualized using UV light. The fragment sizes of each restriction morph were calculated by comparing migration distances relative to 1 kb and 100 bp DNA Ladders (Invitrogen).

COMPOSITE HAPLOTYPE NOMENCLATURE

Each RFLP pattern for the 13 endonuclease–region combinations was assigned a letter thus generating a 13 letter composite mtDNA individual haplotype (Table II). Since only limited screening of the COXIII/ND5 and ND5/12S regions has been undertaken so far in comparison with the more comprehensive screening of the 16S/ND1 and ND5/6 regions in this and published studies, the numerical composites for the two sets of regions are given separately with a stop between them (Table II). Thus, QUB 1.1 refers to a mtDNA haplotype from this laboratory comprising composite haplotype 1 for the 16S/ND1 and ND5/6 regions and composite haplotype 1 for the COXIII/ND5 and ND5/12S regions. This nomenclature

TABLE II. PCR–RFLP composite haplotypes together with homologies to those reported by Hynes *et al*. (1996) and Bernatchez (2001). See text for explanation of the QUB composite haplotype nomenclature. The Duguid (2002) & McKeown (2005) column refers to numbering of composite 16S/ND1, ND5/6 haplotypes used by Duguid (2002) and McKeown (2005) (spread across the facing page)

	Composite genotype												
	16S/ND1			ND5/6							COXIII/ND5		ND5/12S
mtDNA haplotype	*Ava*II	*Hae*III	*Alu*I	*Hpa*II	*Ava*II	*Xba*I	*Hin*fI	*Hae*III	*Dde*I	*Taq*I	*Ava*II	*Mbo*I	*Hin*fI
QUB 1.1	A	A	A	A	B	A	A	A	A	A	B	A	A
QUB 1.2	A	A	A	A	B	A	A	A	A	A	C	A	E
QUB 1.3	A	A	A	A	B	A	A	A	A	A	C	A	A
QUB 1.4	A	A	A	A	B	A	A	A	A	A	B	B	A
QUB 1.5	A	A	A	A	B	A	A	A	A	A	B	C	A
QUB 2.6	A	A	A	A	A	B	B	A	A	A	A	A	A
QUB 3.7	A	B	A	A	B	A	C	A	A	B	C	A	C
QUB 4.7	A	C	B	A	B	A	C	A	A	B	C	A	C
QUB 5.9	A	A	A	A	A	B	D	A	A	A	A	A	B
QUB 6.5	B	C	A	B	C	A	A	B	B	A	B	C	A
QUB 7.6	A	A	A	A	A	B	A	A	A	A	A	A	A
QUB 8.3	A	A	A	B	B	A	A	A	A	A	C	A	A
QUB 9.3	B	C	A	A	B	A	A	A	A	A	C	A	A
QUB 10.7	A	C	B	A	C	A	C	A	A	B	C	A	C
QUB 11.3	A	A	A	A	B	A	A	A	C	A	C	A	A
QUB 13.7	B	C	A	B	B	A	C	A	A	B	C	A	C
QUB 14.3	A	C	A	A	B	A	C	A	A	B	C	A	C
QUB 3.8	A	B	A	A	B	A	C	A	A	B	C	A	D
QUB 9.10	B	C	A	A	B	A	A	A	A	A	D	D	A
QUB 12.3	A	A	A	A	B	A	A	A	D	A	C	A	A
QUB 15.7	A	A	B	A	B	A	C	A	A	B	C	A	C
QUB 16.8	A	D	A	A	B	A	C	A	A	B	C	A	D
QUB 17.1	B	E	A	B	C	A	A	B	B	A	B	A	A
QUB 18.11	B	F	A	B	B	A	C	A	A	E	C	A	I
QUB 19.6	A	C	A	A	A	B	A	A	A	A	A	A	A
QUB 20.6	A	A	A	A	A	B	B	A	A	F	A	A	A
QUB 21.3	D	G	A	A	B	A	A	A	A	A	C	A	A
QUB 22.8	A	B	A	A	B	A	C	B	A	B	C	A	D
QUB 50.50	B	C	A	B	A	B	E	A	A	C	E	E	F
QUB 51.51	B	C	A	C	D	A	F	A	A	C	B	A	G
QUB 51.52	B	C	A	C	D	A	F	A	A	C	B	C	G
QUB 52.53	C	B	A	C	D	A	F	A	A	C	B	A	H
QUB 53.54	B	C	A	B	A	B	G	A	A	D	F	A	G

system easily allows additional information to be added, both in the form of new haplotypes in these regions, as well as potentially for other mtDNA regions.

DATA ANALYSES

Based on the mtDNA restriction fragment profiles, a presence or absence matrix of restriction sites in composite haplotypes was produced (using the GENERATE option in REAP, McElroy *et al*., 1992). A minimum spanning network was constructed (Arlequin 3.1, Excoffier *et al*., 2005) with ambiguities (loops) being resolved using the criteria proposed by Pfenninger & Posada (2002). The network was converted into a hierarchical nested clade design following the rules of Templeton *et al*. (1987) and Templeton & Sing (1993). In this nesting procedure, hypothetical intermediate haplotypes were treated the same as extant haplotypes, resulting in some clade redundancy at consecutive levels when multiple intermediates are inferred. For the samples from Britain and Ireland only, the association of these clades with geography

TABLE II. Continued

Hynes *et al*. (1996) total mtDNA haplotypes	Duguid (2002) & McKeown (2005)	Bernatchez (2001) haplotypes	Region of origin
II	2	ATs4r2	NW Atlantic
V	2	ATs1r6	NW Atlantic
VI	2	ATs1r2	NW Atlantic
VII	2	ATs1r2	NW Atlantic
VIII	2	ATs1r2	NW Atlantic
III and IV	3	ATs1r2(III), ATs1r10 (IV)	NW Atlantic
IX and X	4	ATs1r1	NW Atlantic
X	5	ATs1r1	NW Atlantic
XI	6	ATs1r11	NW Atlantic
XII	7	ATs1r3	NW Atlantic
I and XIII	8	ATs1r2	NW Atlantic
XIV	9	ATs1r2	NW Atlantic
XX	10	ATs8r14	NW Atlantic
X	14	ATs1r1	NW Atlantic
VI	15	ATs1r2	NW Atlantic
X	16	ATs1r1	NW Atlantic
–	19	–	NW Atlantic
–	4.I	–	NW Atlantic
–	–	–	NW Spain
–	15.I	–	NW Atlantic
–	160	–	NW Atlantic
–	162	–	NW Atlantic
–	167	–	NW Atlantic
–	–	–	NW Spain
–	18	–	NW Atlantic
–	23	–	NW Atlantic
–	99	–	NW Atlantic
–	100	–	NW Atlantic
–	–	–	Doubs
–	–	–	Corsica
–	–	–	Ohrid
–	–	–	*marmoratus*
–	–	–	Turkey

was examined with nested-clade phylogeographic analysis (NCPA) (Templeton *et al*., 1995; Templeton, 2004) (GEODIS 2.4, Posada *et al*., 2000). Interpopulation geographical distances were measured as minimum sea distances between catchment estuaries.

Levels of mtDNA variation were estimated as the number of haplotypes and haplotype diversity (Arlequin 3.1). Given that mainly restriction enzymes already known to show RFLP in *S. trutta* were used in this investigation, nucleotide diversity and related statistics were not calculated due to this ascertainment bias. Samples were grouped into geographical regions as defined in Table I, and the extent of geographical structuring of genetic variation assessed by a hierarchical analysis of molecular variance (AMOVA in Arlequin 3.1, with 1000 permutations). Haplotype analyses were conducted both with and without interhaplotype distances. The Spearman rank correlation test was used to test for a relationship between the number of haplotypes found and the sample size.

To examine genetic relationships between samples, a neighbour-joining (NJ) (Saitou & Nei, 1987) phylogram was produced from a distance matrix based on net nucleotide substitutions

between all pairs of populations (D_A), calculated using Arlequin 3.1. The NJ phylogram was constructed using the NEIGHBOR programme in PHYLIP 3.6 (Felsenstein, 1995).

RESULTS

HAPLOTYPE PHYLOGENY

Screening of the four mtDNA segments revealed 33 composite haplotypes, of which 28 were found in the Atlantic samples and 25 in Britain and Ireland (Table II). The minimum spanning network (Fig. 2) involved 19 mutations between the two most divergent Atlantic mtDNA haplotypes. Differences between *c*. 60% of adjacent haplotypes reflected a single mutation. From two to seven mutations were required to explain the differences between the remaining haplotypes, and a minimum of nine mutations was found between Atlantic and non-Atlantic haplotypes. Non-Atlantic haplotypes linked to the network at haplotype 13·7.

In two cases alternative network links were possible. Thus haplotype 6·5 could have arisen from either 9·3 or 1·5. Taking the outgroup rooting of the network and the results by Bernatchez (2001) into account, 9·3 is ancestor to both 6·5 and 1·5. Since to derive 6·5 directly from 9·3 requires seven mutations but *via* 1·5 requires 13 mutations, this latter pathway is considered unlikely. This is further supported by the population data (Table I), which show that 1·5 was only found in two populations and is thus likely to be of recent origin, while 9·3 is geographically widespread, a feature commonly associated with ancestral types. Similar arguments support the derivation of 7·6 from 1·3 directly rather than *via* 1·1.

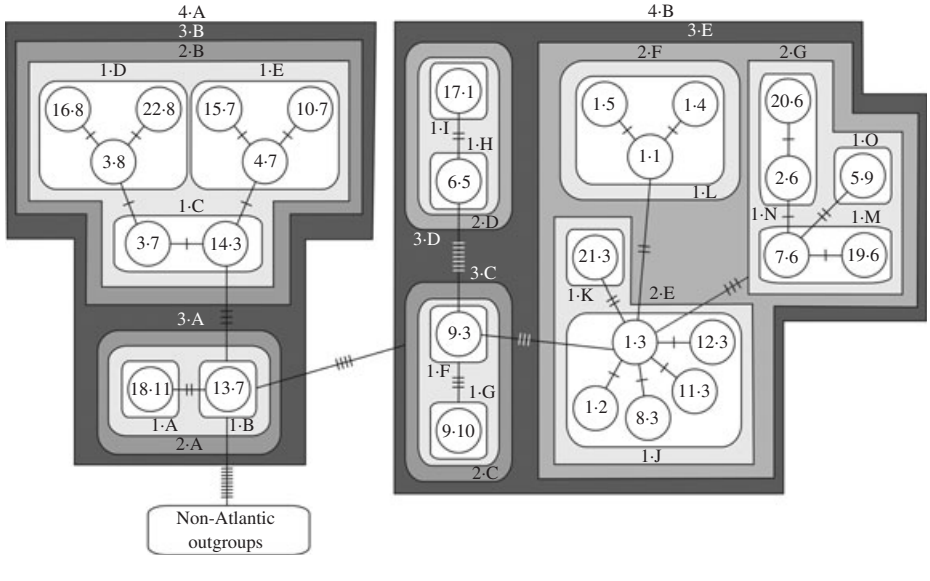

FIG. 2. Nested clades superimposed on the minimum spanning network of haplotypes found in *Salmo trutta* specimens from the Atlantic region. The network is rooted using non-Atlantic outgroups. Haplotypes are show as circles and higher-level clades as rectangles. Each cross bar represents one mutation.

The nested clade arrangement of the minimum spanning network (Fig. 2) grouped the haplotypes (0 step clades) found in the Atlantic *S. trutta* samples into two four-step clades. Clade 4.A was further divided into two three-step clades and Clade 4.B was split into three three-step clades. Four of the seven two-step clades were identical in haplotype composition with three-step clades as a result of multiple hypothetical intermediate haplotypes with only 3.E being resolved into three two-step clades (2.E, 2.F and 2.G).

GEOGRAPHICAL DISTRIBUTION OF HAPLOTYPES AND CLADES

One to seven haplotypes (mean 3·7) were found in individual samples from NW Europe, with four and seven geographically widespread samples showing the maximum and minimum number of haplotypes, respectively (Table I). There was no significant correlation ($P < 0{\cdot}05$) between the number of haplotypes found and sample size when sample size was >15. The highest diversity in a catchment was in the Lough Melvin system with eight haplotypes overall, seven in gillaroo *S. trutta*, five in sonaghen *S. trutta* and one in ferox *S. trutta*. The overall level of haplotype diversity in NW Europe was 0·793 (±0·003) and the mean haplotype diversity per sample was 0·49 (range: 0–0·83). Among the *a priori* defined geographical regions, the lowest levels of haplotype diversity were observed in SW Ireland, SE Ireland, NE Ireland and Orkney & Shetland (Table I). Three haplotypes (1·3, 3·7 and 7·6) predominated, being present, respectively, in 30, 27 and 22% of all individuals, and 82, 66 and 64% of samples. With the exception of haplotype 6·5 (11% of individuals and 50% of the samples), the remaining haplotypes were each found in <3% of all individuals and 17% of samples. Twelve haplotypes were restricted to a single catchment, with three of these (1·1, 1·4 and 1·2) being present only in Melvin sonaghen *S. trutta* and gillaroo *S. trutta*.

AMOVA revealed that while the majority of the molecular variance (*c*. 60%) was contained within populations, significant heterogeneity was detected among samples within regions (*c*. 36%) and among regions (*c*. 3·5%) (Table III). Overall, there are few clear geographical groupings in the NJ DA phylogram (Fig. 3) with, in most

TABLE III. AMOVA with and without interhaplotype differences among wild trout population samples from Britain and Ireland. The variance components, the percentage of the total variance explained by the groupings, the fixation indices and their significance are indicated

	Pair-wise differences				Haplotype frequencies			
	Variance	% total	Φ	*P*	Variance	% total	Φ	*P*
Among regions (Φ_{CT}, F_{ST})	0·093	3·52	0·035	<0·001	0·015	3·23	0·032	<0·001
Among populations within regions (Φ_{SC}, F_{IS})	0·961	36·23	0·376	<0·001	0·137	35·39	0·366	<0·001
Within populations (Φ_{ST}, F_{IT})	1·599	60·25	0·398	<0·001	0·252	61·39	0·386	<0·001

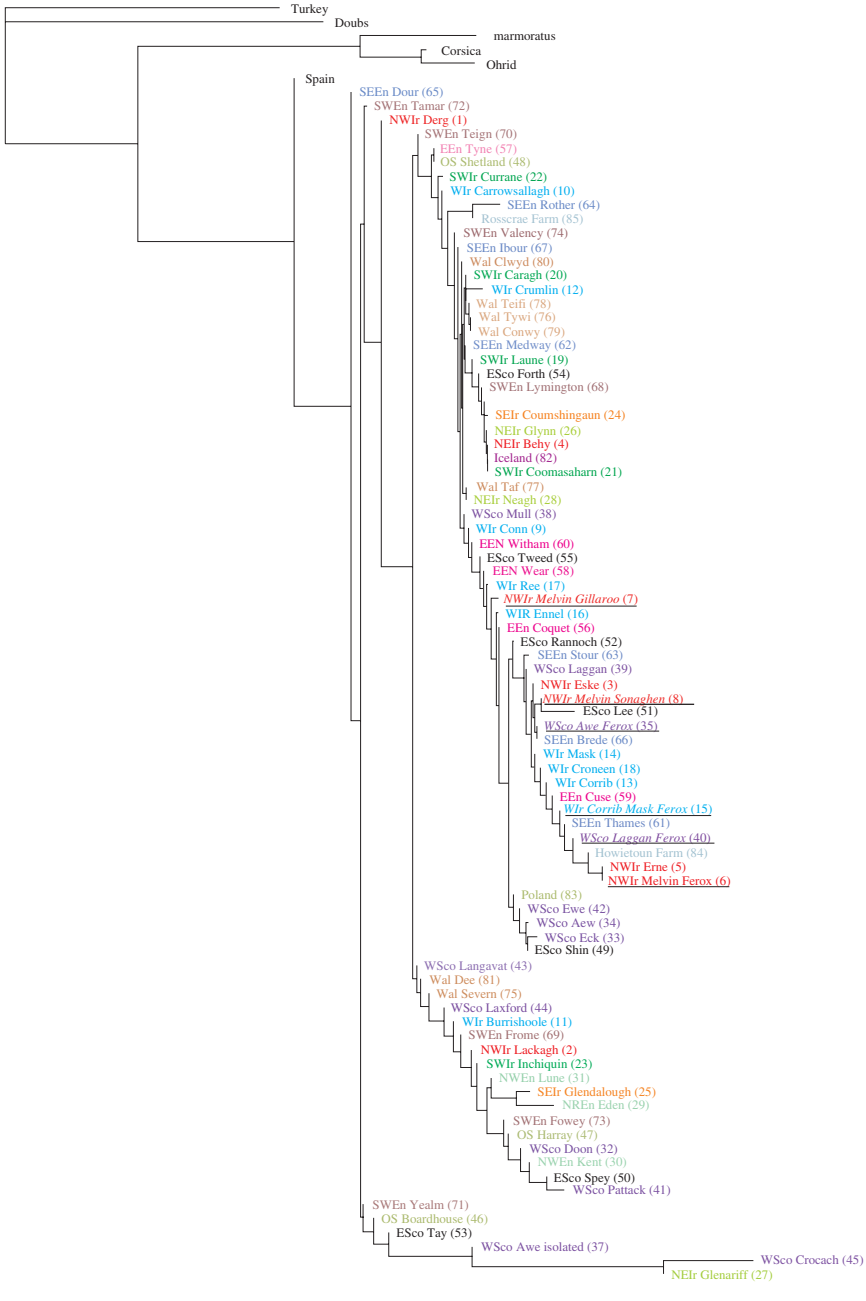

FIG. 3. Neighbour-joining phylogram based on pair-wise net nucleotide substitutions (D_A). Lough Melvin gillaroo *S. trutta*, sonaghen *S. trutta* and ferox *S. trutta* (Melvin and elsewhere) are highlighted (italics and underline). Region codes: NWIr, north-west Ireland; WIr, West Ireland; SWIr, south-west Ireland; SEIr, south-east Ireland; NEIr, north-east Ireland; NWEn, north-west England; WSco, West Scotland; OS, Orkney & Shetland; ESco, East Scotland; EEn, East England; SEEn, south-east England; SWEn, south-west England; Wal, Wales (see Table I for additional details).

cases, samples from within the same catchment (*e.g.* Melvin), or from adjacent catchments, not clustering together.

Individual samples possessed from one (five samples) to five two-step clades (four samples) (Fig. 1 and Table IV). Intermixing of clades within populations was extensive, and the average sample contained descendents of three of the two-step clades. Admixture was most apparent among clades 2-B from 4-A, and 2-E and 2-G from 4-B, and was mainly attributable to their respective high frequency component haplotypes 3·7, 1·3 and 7·6. There, however, was a significant heterogeneity of two-step clades among samples and regions (Fig. 1 and Table V), and all pair-wise heterogeneity tests of two-step clade frequencies between regions were significant with the exception of SW Ireland with SE Ireland, and adjacent Wales with both of these regions.

A summary of the NCPA results is given in Table VI. The null hypothesis of no association between haplotype variation and geography was rejected for most clades where the NCPA analysis could be carried out, with the exception of one-step clades in 2.D and 2.E. The inference at the higher clade levels (three-step and four-step) was of continuous range expansion. At the lower levels, there are several cases of restricted gene flow with isolation by distance. In the case of clades within 2-G, the inference is of past fragmentation followed by range expansion.

DISCUSSION

RFLP ANALYSIS AND RESOLUTION

In order to screen all previously identified informative haplotypes, especially those characteristic of gillaroo and sonaghen, PCR–RFLP analysis of four non-contiguous DNA segments was required. This highlights the importance of not relying on a limited section of the mtDNA genome to infer phylogeographic structure, as genealogical information in different segments of the mtDNA genome is additive rather than duplicated (Cummings *et al*., 1995; Churikov *et al*., 2001). Given that the PCR–RFLP analysis here extended over a total sequence in excess of 10 000 base pairs, population screening of these variants by direct sequencing was not practically feasible, although this is currently changing (Hudson, 2008). Given that RFLPs identified by total mtDNA analysis in Melvin *S. trutta* (Hynes *et al*., 1996) were preferentially screened in the COXIII/ND5 and ND5/12S segments, this may have resulted in an ascertainment bias. This, however, should not affect the determination of relationships of the Melvin sympatric populations to other populations in Britain and Ireland, one of the main foci of this study.

The practical necessity of using RFLP analysis limited the data analyses that could subsequently be carried out. The results of study, however, considerably extend the phylogenetic resolution within the Atlantic group compared with previous studies. In the most extensive trout mtDNA study published to date, Bernatchez (2001) combined sequencing data from a 313 bp segment of the D-loop, with PCR–RFLP analysis of the ND 5/6 region and a second region comprising the cytochrome oxidase B gene and the D-loop. Despite examining a much wider geographical area, Bernatchez (2001) identified only 16 composite sequencing–RFLP haplotypes compared with the 28 Atlantic haplotypes reported here. Bernatchez (2001) recognized

TABLE IV. Geographical distribution of the two, three and four-step clades. Numbers preceding sample names corresponds to Fig. 1. Geographical regions, as defined in Table I, are italicized and regional values are reported

		Clades									
										4.A	4.B
		3.A	3.B	3.C	3.D				3.E		
Sample	*N*	2.A	2.B	2.C	2.D	2.E	2.F	2.G			
North-west Ireland											
1. Derg	100		0·39		0·34	0·27			0·27	0·39	0·61
2. Lackagh	16		0·81					0·19	0·19	0·81	0·19
3. Eske	32		0·16		0·13	0·03		0·69	0·72	0·16	0·84
4. Behy	46					0·98		0·02	1·00		1·00
5. Erne	50							1·00	1·00		1·00
6. Melvin ferox	49							1·00	1·00		1·00
7. Melvin gillaroo	50		0·08			0·26	0·34	0·32	0·92	0·08	0·92
8. Melvin sonaghen	42					0·24	0·14	0·62	1·00		1·00
Total	385		0·16		0·10	0·25	0·06	0·43	0·74	0·16	0·84
West Ireland											
9. Conn	33		0·27			0·49		0·24	0·73	0·27	0·73
10. Carrowsallagh	63		0·41		0·06	0·41		0·11	0·52	0·41	0·59
11. Burrishoole	307		0·66	0·13		0·12		0·09	0·21	0·66	0·34
12. Crumlin	145		0·19	0·32	0·01	0·42		0·06	0·48	0·19	0·81
13. Corrib	41		0·05			0·22		0·73	0·95	0·05	0·95
14. Mask	73		0·15			0·12		0·73	0·85	0·15	0·85
15. Corrib/Mask ferox	43		0·09			0·07		0·84	0·91	0·09	0·91
16. Ennel	31		0·10			0·39		0·52	0·90	0·10	0·90
17. Ree	33					0·64		0·36	1·00		1·00
18. Croneen	33					0·33		0·67	1·00		1·00
Total	802		0·36	0·11	0·01	0·25		0·28	0·53	0·36	0·65
South-west Ireland											
19. Laune	54		0·13		0·06	0·80		0·02	0·82	0·13	0·87
20. Caragh	34		0·24	0·06	0·06	0·65			0·65	0·24	0·77
21. Coomasaharn	14					1·00			1·00		1·00
22. Currane	54		0·44	0·20		0·35			0·35	0·44	0·56
23. Inchiquin	12		0·75			0·25			0·25	0·75	0·25
Total	168		0·29	0·08	0·03	0·60		0·01	0·61	0·29	0·71
South-east Ireland											
24. Coumshingaun	36					0·83	0·17		1·00		1·00
25. Glendalough	15		1·00							1·00	
Total	51		0·29			0·59	0·12		0·71	0·29	0·71
North-east Ireland											
26. Glynn	102				0·01	0·99			0·99		1·00
27. Glenariff	42	0·05	0·02		0·81	0·05		0·07	0·12	0·07	0·93
28. Neagh	30		0·33			0·67			0·67	0·33	0·67
Total	174	0·01	0·06		0·20	0·71		0·02	0·72	0·08	0·93
North-west England											
29. Eden	29		0·93			0·07			0·07	0·93	0·07
30. Kent	30		0·90			0·10			0·10	0·90	0·10
31. Lune	30		0·80			0·20			0·20	0·80	0·20
Total	89		0·88			0·12			0·12	0·88	0·12

TABLE IV. Continued

		Clades									
										4.A	4.B
		3.A	3.B	3.C	3.D				3.E		
Sample	*N*	2.A	2.B	2.C	2.D	2.E	2.F	2.G			
West Scotland											
32. Doon	14		0·86			0·14			0·14	0·86	0·14
33. Eck	22				0·36	0·46		0·18	0·64		1·00
34. Awe	31		0·10		0·26	0·13		0·52	0·65	0·10	0·90
35. Awe ferox	10		0·10		0·10	0·10		0·70	0·80	0·10	0·90
36. Avich	40				0·05	0·95			0·95		1·00
37. Awe isolated	105		0·10	0·01	0·49	0·40		0·01	0·41	0·10	0·91
38. Mull	10		0·30			0·50		0·20	0·70	0·30	0·70
39. Laggan	103		0·15			0·19		0·66	0·85	0·15	0·85
40. Laggan ferox	8		0·13					0·88	0·88	0·13	0·88
41. Pattack	85		1·00							1·00	
42. Ewe	65		0·25		0·25	0·08		0·43	0·51	0·25	0·75
43. Langavat	55		0·64		0·07	0·18		0·11	0·29	0·64	0·36
44. Laxford	68		0·69		0·02	0·02		0·28	0·29	0·69	0·31
45. Crocach	52				1·00						1·00
Total	668		0·34	0·00	0·21	0·21		0·24	0·44	0·34	0·66
Orkney & Shetland											
46. Boardhouse	27		0·48		0·41	0·11			0·11	0·48	0·52
47. Harray	86		0·85	0·01	0·11			0·04	0·04	0·85	0·15
48. Shetland	44		0·52		0·02	0·23		0·23	0·46	0·52	0·48
Total	157		0·69	0·01	0·13	0·08		0·08	0·17	0·69	0·31
East Scotland											
49. Shin	114				0·25	0·38		0·38	0·75		1·00
50. Spey	21		0·91	0·05		0·05			0·05	0·91	0·10
51. Lee	32		0·06		0·22	0·03		0·69	0·72	0·06	0·94
52. Rannoch	84		0·27		0·13	0·04		0·56	0·60	0·27	0·73
53. Tay	59		0·41		0·41	0·09		0·10	0·19	0·41	0·59
54. Forth	62		0·08			0·89		0·03	0·92	0·08	0·92
55. Tweed	51		0·16	0·18		0·29		0·37	0·67	0·16	0·84
Total	423		0·19	0·02	0·17	0·29		0·33	0·62	0·19	0·81
East England											
56. Coquet	40		0·10	0·15	0·03	0·23		0·50	0·73	0·10	0·90
57. Tyne	18		0·50			0·11		0·39	0·50	0·50	0·50
58. Wear	44		0·18	0·05		0·34		0·43	0·77	0·18	0·82
59. Ouse	14			0·21				0·79	0·79		1·00
60. Witham	16		0·25			0·50		0·25	0·75	0·25	0·75
Total	132		0·19	0·08	0·01	0·26		0·46	0·72	0·19	0·81
South-east England											
61. Thames	18		0·06		0·06	0·06		0·83	0·89	0·06	0·94
62. Medway	23		0·22		0·09	0·57		0·13	0·70	0·22	0·78
63. Stour	30		0·37			0·60		0·03	0·63	0·37	0·63
64. Rother	30		0·30					0·70	0·70	0·30	0·70
65. Dour	11		0·64					0·36	0·36	0·64	0·36
66. Brede	8		0·63		0·38					0·63	0·38
67. Ibour	12		0·17		0·08			0·75	0·75	0·17	0·83
Total	132		0·30		0·05	0·24		0·40	0·64	0·30	0·70

TABLE IV. Continued

		Clades									
										4.A	4.B
		3.A	3.B	3.C	3.D				3.E		
Sample	*N*	2.A	2.B	2.C	2.D	2.E	2.F	2.G			
South-west England											
68. Lymington	22		0·27			0·73			0·73	0·27	0·73
69. Frome	18		0·06			0·94			0·94	0·06	0·94
70. Teign	38		0·68		0·11	0·21			0·21	0·68	0·32
71. Yealm	12		0·50		0·08	0·33		0·08	0·42	0·50	0·50
72. Tamar	20		0·55		0·35	0·10			0·10	0·55	0·45
73. Fowey	66		0·62		0·29	0·08		0·02	0·09	0·62	0·38
74. Valency	40		0·83		0·13	0·05			0·05	0·83	0·18
Total	216		0·57		0·17	0·25		0·01	0·26	0·57	0·43
Wales											
75. Severn	15		0·53		0·13	0·27		0·07	0·33	0·53	0·47
76. Tywi	30		0·30		0·13	0·53		0·03	0·57	0·30	0·70
77. Taf	27		0·26			0·74			0·74	0·26	0·74
78. Teifi	30		0·20		0·13	0·67			0·67	0·20	0·80
79. Conwy	29		0·24		0·17	0·59			0·59	0·24	0·76
80. Clwyd	15		0·33		0·07	0·53		0·07	0·60	0·33	0·67
81. Dee	24		0·58		0·08	0·21	0·04	0·08	0·33	0·58	0·42
Total	170		0·33		0·11	0·53	0·01	0·03	0·57	0·33	0·67
82. Iceland	90					1·00			1·00		1·00
83. Poland	18		0·28		0·17	0·06		0·50	0·56	0·28	0·72
84. Howietoun farm	46		0·07					0·94	0·94	0·07	0·94
85. Rosscrae farm	23		0·30	0·09	0·04	0·09		0·48	0·57	0·30	0·70

TABLE V. AMOVA among *Salmo trutta* population samples from Britain and Ireland based on two-step clade frequencies. The variance components, the percentage of the total variance explained by the groupings, the fixation indices, and their significance are indicated

	Two-step clade frequencies			
	Variance	% total	Φ	*P*
Among regions (Φ_{CT}, F_{ST})	0·018	4·67	0·048	0·026
Among populations within regions (Φ_{SC}, F_{IS})	0·132	35·02	0·367	<0·001
Within populations (Φ_{ST}, F_{IT})	0·229	60·31	0·396	<0·001

two major clades in the Atlantic haplotypes, AT3-1 and AT3-2, which, on the basis of haplotype homologies (Table II) are equivalent to clades 4.B and 4.A in this study. Only five haplotypes could be unambiguously related to the Bernatchez haplotypes, with several of the latter being further resolved into multiple haplotypes in this study. For example, the Bernatchez ATs1r2 haplotype was split into six haplotypes resulting in the Bernatchez one-step clade AT1-1 being resolved into three two-step clades (2.E, 2.F and 2.G) and six one-step clades.

TABLE VI. Summary results of NCPA testing association between haplotype–clade distribution and geographical distances. Only haplotypes–clades with geographical and genetic variation are tested

Haplotypes or clades being tested	χ^2; P^1 value	Chain of inference	Inference
Haplotypes in clade 1.C	550; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion
Haplotypes in clade 1.D	14; $P < 0{\cdot}001$	Inconclusive outcome	Inconclusive outcome
Haplotypes in clade 1.E	160; $P < 0{\cdot}0001$	1N; 2Y; 3Y; 5N; 6N; 7Y	Restricted gene flow–dispersal but with some long-distance dispersal
Haplotypes in clade 1.J	1861; $P < 0{\cdot}0001$	1N; 2Y; 3N; 4N	Restricted gene flow with isolation by distance
Haplotypes in clade 1.L	31; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion
Haplotypes in clade 1.M	369; $P < 0{\cdot}0001$	1N; 2Y; 3N; 4N	Restricted gene flow with isolation by distance
One-step clades in clade 2.B	1097; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion
One-step clades in clade 2.G	617; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12Y; 13Y	Past fragmentation followed by range expansion
Two-step clades in clade 3.E	1697; $P < 0{\cdot}0001$	1N; 2Y; 3N; 4N	Restricted gene flow with isolation by distance
Three-step clades in clade 4.A	777; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion
Three-step clades in clade 4.B	1697; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion
Four-step clades in total cladogram	1465; $P < 0{\cdot}0001$	1N; 2N; 11Y; 12N	Contiguous range expansion

N, no; Y, yes.

CLADE DIVERGENCE AND INTERMIXING

The glacial history of NW Europe indicates that a number of potential refuges for *S. trutta* could have existed during glacial advances. Thus, the genetic discontinuities in the network probably represent haplotype sorting during allopatric isolation in glacial refugia. While it is theoretically possible that divergent mtDNA clades could arise without historical isolation by lineage sorting in a species with high N_e and gene flow, there is no empirical support for such an event (Avise, 2000). Given that *S. trutta* would have been displaced from all but peripheral areas of NW Europe during the LGM, most current populations must be the result of postglacial colonists. These have likely spread from multiple refugia since most clades depart significantly from the random geographical distribution expected if all were derived from a single refugium. Many populations, however, possess multiple clades, including in some cases highly divergent ancestral ones. This situation is different to that found in many other Eurasian freshwater fishes, where current clade distribution is largely allopatric with intermixing confined to limited contact zones (Koskinen *et al*., 2000; Gum *et al*., 2005; Østbye *et al*., 2005; Van Houdt *et al*., 2005). Avise (2000) refers to this pattern of co-distribution of distinct clades as a category II phylogeographic pattern and notes that such a pattern is uncommon. Given the small distances involved in Britain and Ireland relative to the migration capabilities of anadromous *S. trutta* and the lack of barriers to marine movement around the coasts, it is perhaps not surprising that much more clade intermixing is found in this region compared with continental areas. In addition, clade intermixing is likely to have taken place both prior to and since the LGM. That is, earlier glaciations, which resulted in clade divergence, were followed by interglacials that allowed range expansion and clade mixing.

The NCPA inference of contiguous range expansion as the dominant factor determining the geographical distribution of many clades, especially higher-level ones (three-step and four-step), and haplotypes is not unexpected, given that expansion must have occurred into most of Britain and Ireland in the postglacial period. The inference of restricted gene flow with isolation by distance for some lower level clades and haplotypes could be attributed to stable population structuring following colonization, with tip haplotypes being recent mutations that have only spread locally as a result of contemporary accurate natal homing in trout. Biological inferences from NCPA need to be used with caution, as recent simulations suggest that erroneous inferences can occur (Beaumont & Panchel, 2008; Petit, 2008; Nielsen & Beaumont, 2009). Templeton (2008, 2009*a*, *b*), however, argues convincingly that the approach is reliable having been validated both by actual data and by simulated data. The inferences presented here are compatible with the expected postglacial scenario for *S. trutta* in Britain and Ireland.

On the basis of its more southern geographical distribution of haplotypes, Bernatchez (2001) proposed that clade AT3-2 (4.A here) was the ancestral one from which AT3-1 (4.B here) evolved, which is in accordance with the polarity of the network here. Within clade 4.A, the occurrence of the ancestral clade 2.A(= 3.A) in the samples from NE Ireland and Spain supports this hypothesis as does the occurrence of haplotype clade 2.C(= 3.C) in Britain, Ireland and Spain. This wide distribution indicates that these clades evolved during an early glacial period and subsequently intermixed, as previously proposed by Bernatchez (2001) for equivalent clades.

A further factor that potentially needs to be taken into account in relation to clade and haplotype admixture is artificial transfer as a result of supplemental stocking by farm-reared *S. trutta*. While populations with no known history of such stocking were targeted in this study, records of such activities from the late 19th and early 20th centuries are often incomplete. Although stocking has been undertaken in Britain and Ireland for over 100 years, the genetic effect appears to have been very limited relative to the extent of stocking (Ferguson, 2007). Many farm strains of *S. trutta* in Britain and Ireland are based on the Howietoun and Solway strains, which were established in the late 19th century and were based on broodstock from Loch Leven, SE Scotland (Ferguson, 2007). The Rosscrea strain, which is partly based on Howietoun broodstock, and Rosscrea derivatives, is the only strain that has been used for stocking in Ireland for over 50 years. Of the two most frequent haplotypes in Rosscrea, 5·9 is not found in any Irish wild sample and 4·7 is only found in two samples at low frequency. Thus, it would appear that stocking has had little genetic effect on the populations examined in Ireland, at least in terms of female transmitted mtDNA. The occurrence of haplotype 2·6 at moderate frequency in sonaghen, however, is unusual with respect to other Irish wild *S. trutta* populations, being found otherwise only in Lough Corrib at a very low frequency. This haplotype is found in both farm strains examined and is moderately common in catchments in East Scotland and NE England where Loch Leven is situated. The occurrence of this haplotype in Corrib is likely to be the result of artificial stocking with *S. trutta* from the Rosscrea farm, which is known to have taken place in the past (M. O'Grady, pers. comm.). This then begs the question if the occurrence of this haplotype in sonaghen is also the result of artificial stocking. It should be noted that stocking could not have been responsible for the introduction of sonaghen *S. trutta* to Lough Melvin since sonaghen, gillaroo and ferox *S. trutta* were described as occurring there in the early 19th century (Newland, 1851), well before artificial culture started. While recent information from local anglers (A. Ferguson, pers. comm.) would indicate that limited stocking of Melvin with Rosscrea *S. trutta* has occurred in the past, it seems unlikely that this was of sufficient magnitude to produce a frequency of 0·29. If stocking, however, was responsible, since farm-reared *S. trutta* are of inlet river spawning origin, they would be more likely to interbreed with sonaghen rather than gillaroo. The low frequency in gillaroo could reflect very limited interbreeding between farm *S. trutta* and gillaroo or between sonaghen and gillaroo.

CLADE DISTRIBUTION AND POSSIBLE REFUGIA

Genetic drift in small isolated populations is the most likely explanation for the fixation–high frequencies of certain haplotypes in Coomasaharn, Coomshingaun, Glendalough and Pattack, all of which are currently inaccessible to upstream migrating *S. trutta*. While these waters may have been accessible to colonization in the immediate postglacial period, geological changes (*e.g.* isostatic uplift, erosion creating water falls) could subsequently have resulted in the *S. trutta* populations becoming physically isolated. The Iceland sample, which comprised sub-samples from north, south, east and west drainages, was also fixed for a single haplotype (1·3), and this probably reflects a founder effect in the original colonizing lineage during the early postglacial phase of high dispersal, together with the country's geographical isolation preventing more recent gene flow. Cortey *et al*. (2009), however,

report four control region haplotypes from a different locality in western Iceland, all haplotypes belonging to same major clade 3-1 (equivalent to 4.B in the present study).

Genetic drift, however, cannot be responsible for all population differences, as there are clear regional differences in the distribution of two-step clades and haplotypes reflecting shared ancestry. Given the likely admixture of clades prior to the LGM, subsequent discussion distinguishes between clades, which represent discrete hierarchical groups of haplotypes in the network, and lineages, which represent groups of individuals with common ancestry that colonized after the LGM from a specific glacial refuge. The pattern of the glaciation during the LGM suggests that there were a number of potential refugia in NW Europe. The following suggestions of potential refuges should not be regarded as definitive but rather as hypotheses that can be tested, as further data become available on the distribution of haplotypes and nuclear alleles that evolved during or since the LGM.

Southern refuge

The onset of glaciation would have led to a southern retreat in the *S. trutta* range. Thus, the principal refuge is likely to have been south of the main glaciated area, that is, to the south of England and western France. This area has been suggested as the main glacial refuge for *S. trutta* in NW Europe (Hamilton *et al*., 1989) as well as for many other fish species (Nesbø *et al*., 1999; Kotlik & Berrebi, 2001; Hänfling *et al*., 2002). A more southern Iberian refuge is unlikely due to the absence in NW Europe of some characteristic haplotypes and nuclear alleles found in Iberia (Weiss *et al*., 2000; Cortey *et al*., 2009). Given that this southern refuge probably contained a mixture of extant clades, it is not possible to determine the subsequent areas that were colonized from it. Thus, the ancestral haplotypes 3·7 and 1·3 were found in most samples examined. These haplotypes, however, show the highest combined frequency (>0·75) in the SW England, SW Ireland and Irish Sea samples, areas that would be the first to be colonized by a northern movement from the refuge.

North-eastern refuge

Clade 2.D(= 3.D), the most divergent of all the clades, occurs at low frequency in most regions in Britain. With the exception of Glenariff (NE Ireland), also the only sample showing clade 2.A, it is rare in Ireland. Bernatchez (2001) found that the equivalent clade (AT1-2) had a northeasterly distribution including the Baltic and White Seas, and suggested its origin in a north-eastern refuge. The giant freshwater lake covering most of the West Siberian Plain around the time of the LGM, which later became connected to the Baltic Sea, has been proposed as a refuge for Atlantic salmon *Salmo salar* L. (Koljonen *et al*., 1999; Säisä *et al*., 2005) and whitefish *Coregonus lavaretus* (L.) (Østbye *et al.,* 2005). An origin in a refuge remote from the main area studied here could explain the relatively large number of missing haplotypes linking it to 2.C, and it is likely that some of these would be found in more detailed sampling of the Baltic and White Sea areas.

Western Ireland refuge

The ancestral clade 2.E, as haplotype 1·3, is widely distributed being found in 84% of samples and is thus likely to have been present in several refuges as noted above.

The occurrence of its descendant haplotypes primarily in the west of Ireland, however, would suggest that these evolved from 1·3 in a refuge in that region, in keeping with Bernatchez's (2001) proposal of a west-central origin for his equivalent clade AT1-1. The presence of relict populations of the Arctic cisco *Coregonus autumnalis* (Pallas) only in Irish waters that were formerly linked to the River Shannon system (Ferguson *et al*., 1978; Ferguson, 2004), and absence of this species in Britain and elsewhere in NW Europe, strongly supports the occurrence of a salmonid refuge in the current Shannon Estuary region (WSW Ireland).

North Sea refuge

Clade 2.G is primarily confined to the west coasts of Ireland and Scotland and east coast of Scotland and England. This latter group of samples is characterized by the occurrence of two derived haplotypes (clade 1.N) indicating a refuge in the current North Sea area, which is thought to have been largely ice-free during the LGM (Carr, 2004). This area has also been proposed as a glacial refuge for *S. salar* (Verspoor *et al*., 1999), bullhead *Cottus gobio* L. (Hänfling *et al*., 2002; Volckaert *et al*., 2002) and grayling *Thymallus thymallus* (L.) (Gum *et al*., 2005).

SYMPATRIC POPULATIONS

The Melvin ferox *S. trutta* is fixed for haplotype 7·6 (2.G), and this haplotype has high frequency in other ferox *S. trutta* samples from Scotland and Ireland. Thus, ferox *S. trutta* from these different lakes, as well as being substantially reproductively isolated from sympatric *S. trutta* (Duguid *et al*., 2006), share a common mtDNA ancestry. Haplotype 7·6, however, is also found at a substantial frequency in many populations not known to have ferox *S. trutta* characteristics.

Gillaroo and sonaghen *S. trutta* are the only populations, apart from the Dee (Wales), to show all three clades, 2.E, 2.F and 2.G. They, however, differ in the occurrence and frequencies of many haplotypes. Haplotype sharing at significantly different frequencies could result from: (1) recent common ancestry with no subsequent gene flow but insufficient time having elapsed for haplotype sharing to be eliminated; (2) a low level of gene flow during the immediate secondary contact period in Melvin, but with this gene flow being subsequently eliminated by reinforcement of the isolating mechanisms; and (3) recurrent gene flow at such a low level that differences in haplotype frequency can be maintained. The unique occurrence of haplotypes 1·2 in sonaghen and 1·4 in gillaroo is not surprising, given that nine other haplotypes are private to individual samples and probably represent recent mutations that have not yet spread to other populations at sufficiently high frequencies to be detected. Given the possible origin of 2·6 as a result of stocking (see above), haplotypes 1·2, 1·4 and 2·6 can be dismissed as phylogeographically uninformative in respect of gillaroo and sonaghen.

The occurrence of haplotype 1·1 only in gillaroo and sonaghen could be taken as signifying that they shared a common ancestor more recently than either did with any other population. Alternatively, this sharing could be the result of limited introgression, as noted above. The occurrence of 1·5 only in gillaroo, Coumshingaun (SE Ireland) and Dee (Wales), however, suggests that they shared a common ancestor more recently than any of them did with sonaghen. It is interesting to note that individuals of the Coumshingaun and gillaroo samples cluster together on the basis

of microsatellites (McKeown, 2005). Thus, the only hypothesis compatible with all the mtDNA data, and all other available data, is that gillaroo and sonaghen are the descendants of separate lineages that independently colonized Lough Melvin postglacially, and that there has been limited gene flow since secondary contact. Given that four haplotypes and several allozyme and minisatellite nuclear alleles (Ferguson & Taggart, 1991; Prodöhl, 1993) are not shared, however, the extent of this gene flow must have been very limited.

Sonaghen *S. trutta* from Melvin appears to be equivalent to *S. trutta* from many other catchments with the genetic, morphological and ecological features (reviewed by Ferguson, 2004) that differentiate it from these other *S. trutta* having occurred within Melvin following colonization. In particular, gillaroo and sonaghen differ in morphological traits associated with exploitation of different food sources (Ferguson, 1986; Cawdery & Ferguson, 1988) as expected from divergent selection in sympatry. Thus, sonaghen is likely to be derived from the western Ireland refuge discussed above.

Celtic Sea refuge

Overall, gillaroo is characterized by clade 2.F, being the only population to possess all three haplotypes of this clade. Thus, the gillaroo lineage probably evolved in a glacial refuge in the Celtic Sea (where the river from Coumshingaun drains), an area known to have been ice-free during the LGM (Clark *et al*., 2004). Postglacially, this lineage expanded northwards up to the west coast of Ireland and into the Irish Sea. If this was the case, it would be expected that haplotypes characteristic of gillaroo would have been found in other catchments in these areas. On the basis of morphology, gillaroo have formerly been described as occurring in other Irish lakes including Loughs Neagh, Corrib, Mask, Derg and Conn (Günther, 1866; Day, 1887) but are now absent or rare in these lakes. The rare *LDH-A1*QO* allele, which is present in Lough Melvin gillaroo *S. trutta* at a frequency of 0·2, but which is absent from sonaghen *S. trutta*, has also been found in Mask *S. trutta* (Ferguson & Taggart, 1991). Old photographs of gillaroo from Lough Conn show them to be very similar in colouration to Melvin gillaroo *S. trutta*, and local anglers report that, while previously common, trout of this type have been very rarely caught in recent years (A. Ferguson, pers. comm.). Reproductive isolation of gillaroo *S. trutta* from sonaghen *S. trutta* within Melvin is maintained due to the descending of gillaroo *S. trutta* from the outlet river to spawn (Ferguson & Taggart, 1991), an unusual life-history characteristic in Britain and Ireland, where spawning takes place after an upstream migration into tributaries (Frost & Brown, 1967; O'Grady *et al*., 2008), as is the case for sonaghen. In a comprehensive snorkel survey of Coumshingaun at spawning time, *S. trutta* were found spawning only in the outlet river (F. Igoe, pers. comm.). Outlet spawning thus may be a characteristic of the gillaroo lineage, perhaps having arisen as an adaptation to periglacial conditions. Outlet spawning behaviour has been shown to have high heritability in *S. trutta* (Jonsson *et al.,* 1994). As outlet spawning *S. trutta* juveniles have to ascend to the lake, rather than descend, this behaviour would result in reproductive isolation from normal inlet spawners, given that the unique adaptations required would result in hybrids having low fitness. Since outlet rivers, compared with inlets, have generally been more subject to alteration as a result of drainage and hydroelectric and other barriers, it is perhaps not surprising that outlet spawners would suffer. For example, the Lough Conn outlet river was

drained in the 1960s, which coincides with the reported rapid decline of gillaroo in that catchment (A. Ferguson, pers. obs.).

CONSERVATION CONSIDERATIONS

This study further demonstrates that the Lough Melvin situation is genetically unique with respect to the catchments examined and merits the highest conservation status. In particular, gillaroo appears to be the most singular type and the one most threatened due to its spawning only in the outlet river. The biological characteristics of ferox *S. trutta* also make it highly vulnerable to overexploitation (Duguid *et al*., 2006). It should be acknowledged that the Melvin catchment has been sampled in more detail than any other catchment in Britain and Ireland to date, and similar complexity may yet to be found elsewhere. Prioritizing other populations for conservation is less clear-cut than with the Lough Melvin system. It has been proposed that reciprocally monophyletic groups of lineages identified from mtDNA and other genetic markers could be regarded as evolutionarily significant units (ESU) for conservation purposes (Moritz, 1994). The genetic mosaic structure seen here in *S. trutta*, however, makes this difficult to apply. Nevertheless, it is clear that some populations are genetically distinct and have existed in genetic isolation since colonization and under distinct ecological conditions. Thus, it is likely that substantial local adaptation has occurred in this time, given that such adaptation can occur in a few generations (Koskinen *et al*., 2002). As Crandall *et al*. (2000) argue, ecological data and genetic variation of adaptive significance are more relevant for conservation than earlier ESU concepts based on historical legacy. In conclusion, as far as *S. trutta* in Britain and Ireland is concerned, conservation measures should be based on local populations rather than solely on evolutionary lineages or defined taxa, with biological and other local characteristics being used to determine conservation priority, as appropriate.

Given the number of glacial refuges that potentially existed in NW Europe, it is likely that a similar complexity of postglacial colonization will be found when detailed phylogeographic studies are undertaken for other fish species that can live in periglacial conditions such as Arctic charr *Salvelinus alpinus* (L.) and *S. salar*, as well as for a range of other aquatic organisms. Relative to the rest of western Europe, the geographical position of Britain and Ireland makes this area a central contact zone for colonizers emanating from multiple refugia, as proposed for Atlantic salmon by Consuegra *et al*. (2002). This position and unique pattern of glaciation of these islands, however, has resulted in salmonids having been influenced by several glaciations and not just the LGM. Thus, the phylogeny and genetic structure are potentially more complex than described in other geographical regions.

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