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An East–West distribution of divergent mitochondrial haplotypes in British populations of the land snail, *Cepaea nemoralis* (Pulmonata)

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Some continental European populations of the land snail *Cepaea nemoralis* have mitochondrial haplotypes that differ by up to 20% at the 16S rRNA locus. I mapped the distribution of different lineages in populations from 36 different sites in Britain and Ireland. In 93% of individuals, one of two mitochondrial lineages was found, *A* or *N*, which differ from each other by about 6% using a 16S rRNA fragment (approximately 300 base pairs). The distribution of these two types is very striking—one is confined to Wales, West and central England, and Scotland, while the other is found mainly in East and central England. The two types meet in a transition zone. The most likely explanation for the distribution is that it reflects two routes of colonization after the last ice age. *Cepaea* dispersal is leptokurtic, and only limited gene flow occurs between established populations, so that the original pattern could have been retained since the post-glacial colonization. However, many environmental gradients are orientated East–West, so alternative selective explanations are possible. A distinct mitochondrial lineage, as well as fossil evidence, suggests that Ireland was colonized separately from Britain. The implications of these distributions for the origins of the puzzling geographical patterns of shell types known as 'area effects' is discussed.

ADDITIONAL KEY WORDS:—area effects – biogeography – leptokurtic dispersal – mollusc – 16S rRNA.

CONTENTS

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INTRODUCTION

Land snails, particularly those in the genus *Cepaea*, have become model organisms for studying the mechanics of evolution and its bases in ecology (Cain & Sheppard, 1950; Cain & Currey, 1963; Johnson, 1976; Ochman, Jones & Selander, 1983). The striking genetic polymorphisms in the colour and banding of the shell, and the ease with which the snails can be collected and marked, has made them particularly valuable for the study of ecological genetics (Clarke, 1978). They also have advantages for investigating the effects of founding events on patterns of gene frequencies. Despite an average Wrightian neighbourhood of only 15–20 metres, they easily colonize vacant habitats because the distribution of their dispersal is strongly leptokurtic. Low levels of gene flow between established neighbouring populations can allow genetic traces of colonization to remain for very many generations.

The use of molecular methods to investigate the effects of colonization in snails has been delayed because mucopolysaccharides in the mucus apparently inhibit DNA polymerases, and also because many 'universal' primers (including those for mitochondrial DNA) do not work. Recently it has been found that excess mucopolysaccharides can be removed using methods of DNA extraction designed for plants. This development, in combination with the cloning and sequencing of three pulmonate mitochondrial genomes, has meant that effective primers are now available (Terrett, Miles & Thomas, 1994; Hatzoglou, Rodakis & Lecanidou, 1995; Yamazaki *et al.*, 1997).

Snail mitochondrial DNA is very diverse within and between species (Thomaz *et al.*, 1996; Douris *et al.*, 1998; Chiba, 1999). Some populations of *Cepaea nemoralis* in Europe have mitochondrial types which may differ from each other by up to 20% at the 16S rRNA locus (using a 300 base pair fragment; Thomaz *et al.*, 1996). Various hypotheses have been proposed to account for this diversity, including unusually fast evolution, the retention of deep lineages in subdivided populations, or the evolution of separate maternal and paternal lineages, as found in some of the bivalve molluscs (doubly uniparental inheritance; Skibinski, Gallagher & Beynon, 1994). Whilst the exact explanation for the retention of divergent lineages remains unknown, in the land snail *Mandarina* mitochondrial DNA may evolve very rapidly (Chiba, 1999), and a variant of doubly uniparental inheritance can be discounted in *Cepaea* because their mitochondria appear to be inherited maternally (Davison, 2000).

Limited sampling suggested that only a subset of the European variation is present in British *C. nemoralis* (Thomaz *et al.*, 1996). The two British haplotypes, A and N, differed from each other by approximately 6% using a 16S rRNA fragment. Populations of *C. nemoralis* from Crail (Scotland) and Nottingham (England) were found to be fixed for the N haplotype, whereas a site to the west of Nottingham (Deepdale, in Derbyshire) was fixed for the A haplotype.

As the pattern of differences between populations suggests only low levels of gene flow, I set out to investigate in more detail the distribution of mitochondrial haplotypes in Britain and Ireland, and to enquire into the causes of the present patterns.

TABLE 1. Sample sites of snails used in this study

Sample site (code)	Country	Grid reference	Sample size	Collector/reference
Aghada (A)	Ireland	W854468	12	P. Mordan
Anglesey-1 (A1)	Wales	SH357683	30	A. W. Davison
Anglesey-2 (A2)	Wales	SH360678	28	A. W. Davison
Bamburgh (B)	England	NU17-34-	12	A. Davison
Berrow (Be)	England	ST30-50-	10	A. Davison
Bodfari (Bo)	Wales	SJ10-70-	8	M. Hamshere
Brackley (Br)	England	SP55-29-	10	A. Davison
Brancaster (Bc)	England	TF77-44-	10	C. Wade
Broadhaven (Bd)	Wales	SR978938	9	A. Davison
Collingbourne (C)	England	SU25-54-	9	A. Davison
Craiglockart (Cr)	Scotland	NT228701	10	A. Davison
Crail (Cl)	Scotland	NO61-07-	21	Thomaz *et al.*, 1996
Deepdale (D)	England	SK10-72-	18	Thomaz *et al.*, 1996
Dovedale-1 (D1)	England	SK174514	25	A. Davison
Dovedale-2 (D2)	England	SK132527	13	A. Davison
Foxholes (F)	England	TA01-73-	10	A. Davison
Goredale (G)	England	SD911632	10	A. Davison
Great Orme (GO)	Wales	SH756841	10	D. Shuker
Hepscott (H)	England	NZ221840	25	A. Davison
Kettering (K)	England	SP86-78-	10	C. Wade
Kilmartin (Km)	Scotland	NM848015	23	A. Davison
Kirk Ireton (KI)	England	SK267501	8	E. Bailes
Lyme Regis (L)	England	SY33-91-	7	A. Davison
Letcombe Regis (LR)	England	SU395849	10	A. Davison
Ludborough (Ld)	England	TF298947	10	A. Davison
Mathon (M)	England	SO756453	9	J. Birks
Marlborough-4 (MD-4)	England	SU132743	43	Thomaz *et al.*, 1996
Marlborough-8 (MD8)	England	SU115705	51	Thomaz *et al.*, 1996
Mullaghmore (Mu)	Ireland	Not known	15	P. Mordan
Northwich (N)	England	SJ364371	8	C. Finnis
Norwich (Nw)	England	TG19-07-	9	F. Santucci
Nottingham (Nm)	England	SK6-42-	22	Thomaz *et al.*, 1996
Portland Bill (P)	England	SY680684	10	A. Davison
Rhosseli (R)	Wales	SS40-87-	10	A. Davison
Skegness (S)	England	TF51-60-	10	A. Davison
Stow-on-Wold (St)	England	SP17-22-	10	A. Davison
Tasan Beach (T)	England	SW869328	3	E. Bailes
Trowell (Tr)	England	SK48-39-	10	D. Parkin
Tring (Tg)	England	SP933123	10	D. Shuker
Wellsbourne (W)	England	SP276539	10	A. Davison
Whittington (Wh)	England	SO875527	10	A. Davison

MATERIAL AND METHODS

Sample collection

C. nemoralis is a widespread species in Britain and Western Europe, and thus easy to collect in large numbers. For this study, 36 population samples were collected from widely scattered sites around Britain and Ireland (mainly in England and Wales; Table 1). In each sample snails were taken from as small an area as possible, usually less than 100 m^2.

DNA extraction

A sliver of foot tissue was cut from the snails using a sterile scalpel and DNA was extracted using Nucleon Phytopure kits (Nucleon Biosciences).

PCR and SSCP analysis

Primers for PCR were based upon an alignment of *C. nemoralis* and *C. hortensis* mitochondrial 16S rRNA sequences (Thomaz *et al.*, 1996). These primers, 16S-1a (5′-GACGAGAAGACCCTAGAAGC-3′) and 16S-2a (5′-CCTAGTCCAA-CATCGAGGTCAC-3′) amplify a small, approximately 150 bp, fragment in which the variation is easily assayed by SSCP analysis (see below). PCR was carried out under standard conditions, including 0.4 U Thermoprime^{PLUS} polymerase and 4.5 mM magnesium chloride in a 50 µl final volume. The cycling parameters were 96°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec.

The haplotype of each individual was examined by electrophoresis through an SSCP (single strand conformational polymorphism) polyacrylamide gel (Orita *et al.*, 1989). For this procedure, each PCR product was precipitated with isopropanol, resuspended in a formamide/bromophenol blue loading buffer, and denatured at 98°C for 2 minutes. It was then placed on ice to minimize DNA reannealing. The PCR products were loaded on to a 0.5 × MDE (FMC Bioproducts) vertical gel (20 × 20 cm) and electrophoresed at 6°C for 15 hours at 180 V. The gels were stained in five steps: fixing (50% methanol/10% acetic acid, 10 minutes), two washes (10% ethanol/0.5% acetic acid, 3 minutes each), soaking in silver nitrate solution (0.1% w/v, 10 minutes), and development (1.5% sodium hydroxide/0.4% w/v formaldehyde/0.2% w/v sodium borohydride), until bands were visible. Gels were neutralized in 0.75% sodium carbonate and dessicated in a gel drier at 80°C for 2 hours.

A pair of control amplifications from A and N haplotype mitochondria was included on each polyacrylamide gel. All new SSCP haplotypes (i.e. non-A, non-N) were sequenced, as well as a random selection of A and N haplotypes from different populations.

DNA sequencing

PCR products were purified by passage through a QIAquick PCR purification column (Qiagen) before sequencing. The sequencing was carried out in both directions using the dRhodamine cycle sequencing kit (Applied Biosystems), and including one of the PCR primers for polymerase synthesis.

Phylogenetic analysis

Sequences were aligned by eye in GDE (Smith *et al.*, 1994). Insertions and deletions were then removed, and a Kimura 2-parameter distance matrix was created using the Dnadist program of PHYLIP (Felsenstein, 1993). A phylogenetic

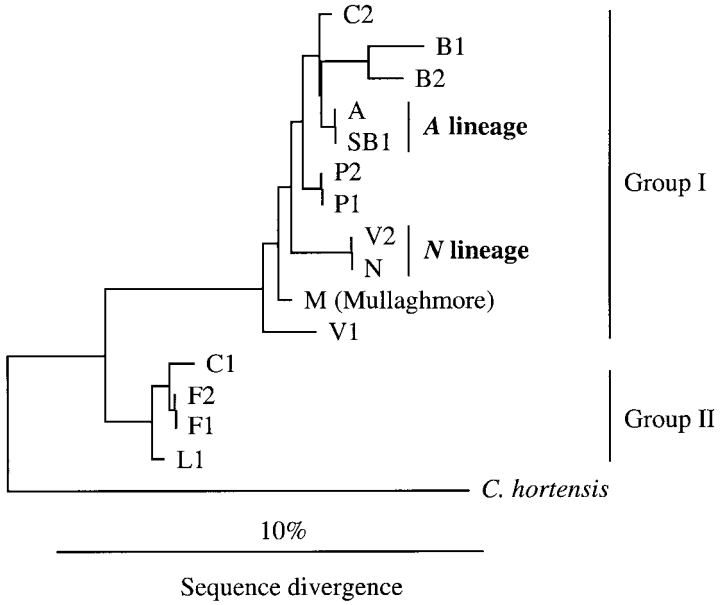

Figure 1. Neighbour-joining tree (using Kimura 2-parameter distances) of all the *C. nemoralis* 16S rRNA haplotypes, rooted using *C. hortensis* as an outgroup (Thomaz *et al.* 1996). The tree divides into two main groups, with many sub-lineages within the groups. Note the new Irish sequence (Mullaghmore). Excluding some insertions/deletions, no other new sequences were detected in the survey. The sequences have GenBank reference numbers AF146572 to AF146587.

tree was produced using the NEIGHBOR program of PHYLIP, and displayed in TREETOOL (Maidak *et al.*, 1994).

RESULTS

Thomaz *et al.* (1996) sequenced a fragment of the mitochondrial 16S rRNA gene from 18 different European populations of *C. nemoralis* (Fig. 1). Two widely divergent lineages were found, one of which (Group I in Fig. 1) was divided into three or more lesser lineages. Only two haplotypes were found in Britain, A and N, which differ by about 6% over the sequenced region (both in Group I; Fig. 1).

I assayed a part of the same 16S rRNA fragment studied by Thomaz *et al.* (1996) in a further 423 individuals from 36 British and Irish populations, by a combination of SSCP and DNA sequencing. Over this region, A and N differ by only about 2%. As I was mainly interested in the distribution of the more divergent lineages across Britain, the lower phylogenetic resolution did not matter for this study. It meant, however, that I was unable to distinguish between the closely related haplotypes A and SB1, or N and V2 (Fig. 1), because they are identical over the region that was amplified. Nevertheless, the overall structure of the tree was the same as that produced using the larger fragments, and there was no problem in distinguishing the two main divergent lineages present in Britain (*A* and *N*).

Including the populations already described by Thomaz *et al.* (1996), the vast majority of mitochondrial types were from either the *A* or *N* lineages (537 out of

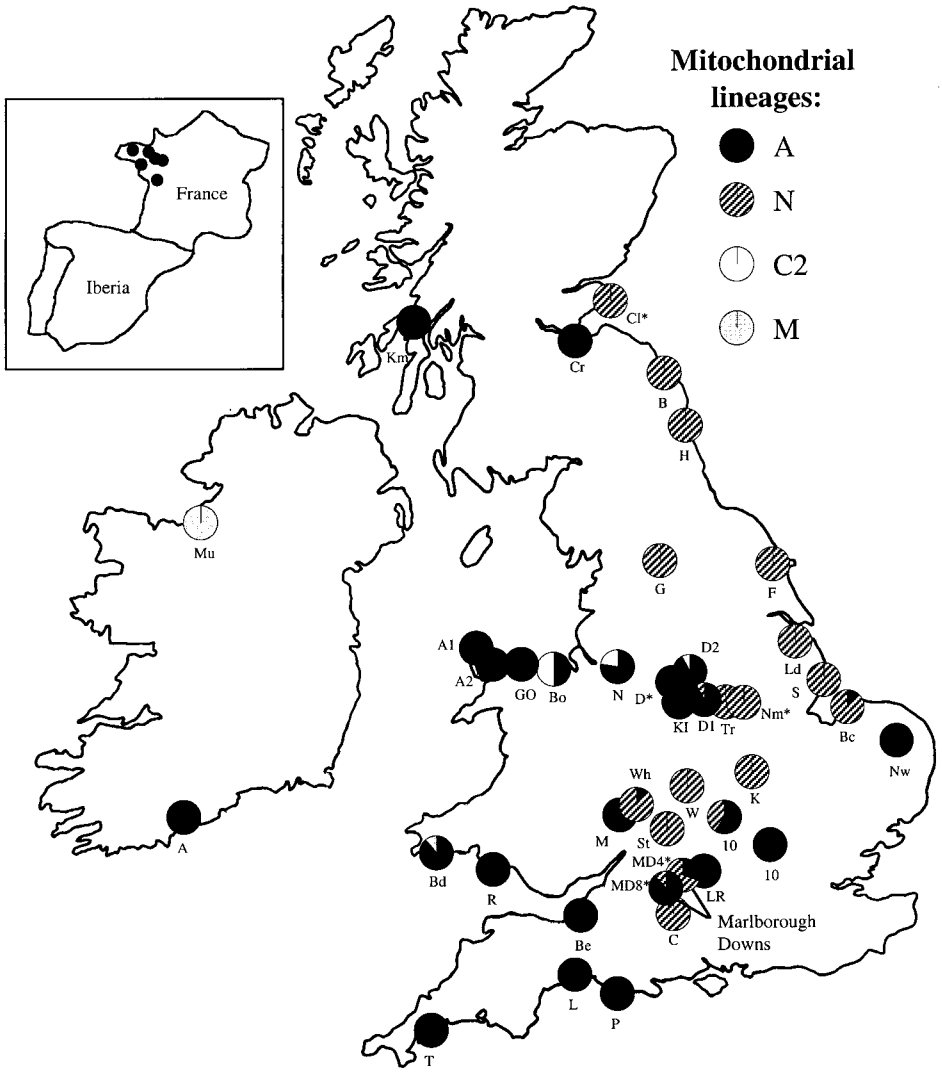

Figure 2. The distribution of *C. nemoralis* mitochondrial types across Britain and Ireland, including the sample site code. Two main lineages were found, *A* and *N*, which show a strong East–West distribution pattern. The names of the types are the same as in Thomaz *et al.* (1996); asterisks show samples from that study. Inset: sites in France where the A haplotype was identified by Thomaz *et al.* (1996).

578; Fig. 2). The next commonest type was the C2 lineage (as defined by Thomaz *et al.*, 1996), found at Bodfari, Dovedale-2, and Northwich. Four other haplotypes were found only at single sites: Kettering (4 out of 10 individuals), Mullaghmore (15/15), Norwich (4/8) and Trowell (10/10). The Kettering and Trowell haplotypes differed from the *N* lineage by a single insertion/deletion, and the Norwich type differed from the *A* lineage also by a single insertion/deletion. Disregarding insertions and deletions, only the Mullaghmore haplotype was a new addition to the phylogenetic tree of European variation, first modelled by Thomaz *et al.* (1996; shown in Fig. 1 with the new sequence). The Mullaghmore sequence differed from *A* and *N*

by approximately 1.4% and 2% respectively. However, the phylogenetic placement of the clades, with the exception of the major division into groups I and II, was unreliable because most of the variation was in insertion/deletions, and the sequences were short. Bootstrap values were very low and are not shown.

The geographical distribution of the mitochondrial lineages is very striking (Fig. 2). The *A* lineage appears to be confined to Wales, western and central England, and Scotland, while the other (*N*) is found mainly in eastern and central England. The two lineages meet in a zone of overlap, in the range of ten to a hundred kilometres; the exact width may vary in different parts of the country. There are a few exceptions to the East–West pattern. For instance, the *A* lineage was found at Brancaster and Norwich in East Anglia, as well as Edinburgh (Craiglockart).

DISCUSSION

The most likely explanation for the distribution of mitochondrial types is that it reflects separate routes of colonization within Britain after the last ice age (the Würm II glaciation; see Hewitt, 1996). It has already been remarked that *Cepaea* is an efficient colonizer of vacant habitats with a leptokurtic pattern of dispersal. Perhaps the first suitable habitats were in lowland regions near the coasts. One genetic type reached the East or West coast first because Britain was joined to mainland Europe via a southern land bridge. Thereafter, some snails spread more widely and the two types met. Since only a limited flow of genes occurs between established populations, the original pattern remains to this day.

This type of colonization has been modelled by Ibrahim, Nichols & Hewitt (1996). Rare long-distance migration leads to the establishment of populations in advance of the main front of expansion. This generates a geographical patchwork of genotypes that is most pronounced when dispersal is leptokurtic. Because *Cepaea* has colonized most of Britain since the melting of the ice, much of the colonization must have taken place by 'rare' long distance events. If we were to assume that dispersal was limited to that in the Wrightian neighborhood (i.e. about 20 m per generation), then in the approximately 2500 generations since the retreat of the ice, *Cepaea* could only have moved 50 km northwards.

There are other possible explanations of the geographical patterns. Mitochondria are energy-producing organelles, and some haplotypes may be more or less efficient depending upon local environmental conditions. The eastern and western sectors of Britain differ in several climatic factors, notably rainfall (more in the West) and winter temperature (colder in the East). There is also more limestone in the East than the West, and calcium is very important in pulmonate metabolism. It is possible that the first snails to colonize were by chance better adapted to the East or West. Different mitochondria could then have spread by hitchhiking with these genetic backgrounds. It is also possible that a degree of nuclear-mitochondrial coadaptation occurred prior to contact, so that 'internal' selection could counteract introgression between the two types.

An earlier study measured enzyme differentiation in both *C. nemoralis* and *C. hortensis* across Britain and Europe (Ochman, Jones & Selander, 1987). Both species showed extensive local and regional differentiation, but in contrast to the present results, few correlations were found between single loci and environmental factors.

In Britain, the major component of enzyme allele frequency change was a North–South cline, rather than East–West.

Of the two main mitochondrial types in Britain, one (A) was also found in France (Fig. 2). The other (N) has not yet been found outside Britain, but the very closely related type V2 was found at three sites in France (Thomaz *et al.*, 1996). However, much of Western Europe remains unsampled for *C. nemoralis*, and it is likely that different lineages colonized from distinct southern refugia, as in other species (Hewitt, 1999). The N haplotype may occur undiscovered in continental Europe, or conceiveably, it arose in Britain by mutation from a related haplotype shortly after the founding event. A few other species in Britain (oaks, pipistrelle bats, polecats, shrews) show genetic differentiation with an East–West pattern, although the causes may be unrelated (Barratt *et al.*, 1997; Davison *et al.*, 1999; Ferris *et al.*, 1995; Searle & Wilkinson, 1987).

Although only two Irish sites were investigated, the presence of the unique Mullaghmore haplotype suggests that Ireland was, at least in part, colonized independently of Britain. Fossil evidence supports this conclusion. Both *C. nemoralis* and *C. hortensis* arrived in Britain in the late Holocene, approximately 10 000 radiocarbon years before the present (Kerney, Preece & Turner, 1980), but did not colonize Ireland until about 1000 years later, after the disappearance of the land bridge between Britain and Ireland (Preece, Coxon & Robinson, 1986).

Finally, the distribution of the mitochondrial types has interesting implications for the existence of 'area effects' in regions such as the Marlborough Downs in Britain (see Fig. 2 for the location of the two sampling sites in the Marlborough Downs). An 'area effect' refers to puzzling geographical patterns in the frequencies of shell colours and banding (Cain & Currey, 1963). In lowland areas, there is a clear correlation between the frequencies of the various snail shell types, the type of habitat in which they live, and the activities of predators, notably the song-thrush (Cain & Sheppard, 1954). However, on chalk downlands, such as the Marlborough Downs in Wiltshire and other upland regions where predatory song-thrushes are rarer, particular colour and banding alleles predominate over large areas, apparently regardless of the habitat in which the snails live. Groups of populations are separated from each other by steep gradients of gene frequencies, over just a few hundred metres, and without any obvious change in habitat. The origins and maintenance of area effects, in *Cepaea* and other species, have been matters of contention (Cain & Currey, 1963; Goodhart, 1963; Clarke, 1966; Cameron & Dillon, 1984; Cook, 1998).

If the mitochondrial patterns are indicative of two distinct genetic 'races' of *C. nemoralis*, then the regions where they meet could be viewed as hybrid zones. The Marlborough Downs represent a meeting point of the two types, but a simple explanation for the area effects in terms of mitochondrial haplotypes would appear to be ruled out because the zone is wider than the region over which the area effects are found. Whether more subtle associations exist is the subject of another paper, where I use newly developed microsatellite markers (Davison, 1999) to investigate the origins of area effects (Davison & Clarke, 2000).

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