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AN ASSOCIATION OF MITOCHONDRIAL HAPLOTYPE WITH SHELL SHAPE IN THE
INTERTIDAL GASTROPOD *LITTORINA SAXATILIS*

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ASSOCIATION OF HAPLOTYPE WITH SHELL SHAPE

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ABSTRACT

The high rates of substitution in mitochondrial genes and assumed neutrality of their polymorphisms encourage the use of mtDNA in phylogenetic and evolutionary studies. However, the true nature of the mitochondrial genome and its influence upon processes of adaptation and speciation are not fully understood, particularly where associations appear to exist between 'neutral' mitochondrial haplotypes and characteristics that are under direct selection. In snails, one such characteristic is shell shape. In this study a mitochondrial haplogroup is found to be confined towards one end of the spectrum of shell variation in an ecotype of the rough periwinkle *Littorina saxatilis* on a shore in the British Isles. We speculate that this may signal hitherto unexpected selection, and perhaps even some reproductive isolation between this group and the rest of the population, and we propose intergenomic coadaptation as a possible mechanism through which this occurs.

INTRODUCTION

Evolution in mitochondrial DNA (mtDNA) happens quickly and, in the time scale required for selection to act upon nuclear genes, considerable variation in mtDNA can occur. One consequence of this is that mitochondrial haplotypes associate with morphology when evolution of the mitochondrial genome occurs in tandem with changes in morphological characteristics that are under direct selection. An example in marine bivalves is the link between amino acid sequence divergence encoded by the mitochondrial cytochrome c oxidase I (CO1) gene and egg size (Marko & Moran, 2002) in populations that have been physically isolated since the uplift of the Isthmus of Panama.

Should populations re-establish contact following periods of allopatry, introgression of mitochondrial haplotypes might be expected to mask any previous associations, assuming there is free interbreeding. However, mtDNA is involved in coding proteins responsible for key metabolic processes, and some studies (Burton, Byrne & Rawson, 2007; Gershoni, Templeton & Mishmar, 2009; Ellison & Burton, 2010) have suggested that co-adaptation between mitochondrial- and nuclear-encoded protein subunits of the mitochondrial electron transport chain can result in reduced F2 viability when populations that have diverged during periods of allopatry subsequently hybridize on secondary contact. While not all studies are in emphatic agreement (Pichaud *et al.*, 2012), this

nonetheless offers a mechanism whereby reproductive isolation could be maintained despite the opportunity for introgression, leading to the persistence of diverged populations.

The rough periwinkle *Littorina saxatilis* (Olivi, 1792) is a small intertidal snail widely distributed across the North Atlantic, which shows several characteristics making it suitable for the investigation of these processes. Shell shape is highly variable and the species is known to differentiate into at least two distinct forms on many shores in the British Isles: a highshore H form which has a thin shell and large aperture allowing for a larger foot and greater adhesion to the substrate in the face of increased wave action, and a midshore M form with a larger, more robust shell and smaller aperture offering increased protection against predation by the shore crab *Carcinus maenas* (Raffaelli, 1982). A partial reproductive barrier exists between the two forms, and there is increased incidence of aborting embryos in intermediate females (Hull, Grahame & Mill, 1996). Furthermore, a substantial amount of variation occurs within H alone, which shows a considerable range of shell shapes. These forms are often referred to as morphs or ecotypes; here we will use the latter word. In addition to this morphological diversity, *L. saxatilis* also exhibits high diversity in mtDNA haplotypes (Wilding, Grahame & Mill, 2000). Haplotype diversity across the North Atlantic has been described by Doellman *et al.* (2011), who found that *L. saxatilis* can be grouped into two distinct lineages (I and II), which are believed to have diverged in separate refugia during Pleistocene glaciations. Within each of these lineages a number of distinct haplogroups (groups of similar haplotypes sharing a common ancestor) have been identified, of which haplogroup F is particularly common on many British shores (Doellman *et al.*, 2011).

While associations have been found of littorinid shell shape with AFLP genotype (Grahame, Wilding & Butlin, 2006; Galindo, Moran & Rolan-Alvarez, 2009), allozymes (e.g. Janson & Ward, 1984) and a microsatellite repeat (Wilding, Grahame & Mill, 2002), less is known about links

between morphology and mitochondrial haplotype. One exception is the shore at Ninian's Cave in Port Castle Bay, Scotland, where RFLP analysis showed a cline in mtDNA corresponding with the environmental gradient along which the two ecotypes lie (Wilding, Grahame & Mill, 2001). In the present study we relate this cline to the lineages of *L. saxatilis* as described by Doellman *et al.* (2011). By combining analyses of shell shape and mtDNA polymorphisms we present evidence for the confinement of a mitochondrial haplogroup within a restricted portion of the shape space occupied by the H ecotype at the same location. We speculate that this may be indicative of reproductive isolation between snails with this haplogroup and the rest of the H population, and suggest interactions between the mitochondrial and nuclear genomes as a possible isolating mechanism.

MATERIAL AND METHODS

Samples were collected from a number of rocky shores in the UK between 2001 and 2012. mtDNA analysis was carried out on animals collected from Thornwick Bay (UK grid reference TA233724) and Old Peak (NZ984021), Port St Mary (SC213680) and Ninian's Cave (NW417364) (Fig. 3). All these sites are on open rocky shores with at least a moderate exposure to wave action. Only adult female specimens with a clearly defined brood pouch were used here, since juveniles and male *L. saxatilis* cannot be reliably distinguished from the closely related *L. arcana* Hannaford Ellis, 1978. A total of 162 individuals from all shores were analysed for haplotype diversity and population structure across both H and M ecotypes, while a further 49 from Ninian's Cave were used for comparisons between haplotype and shell shape (obtained from a separate small-scale sampling effort of H ecotypes at Ninian's Cave).

DNA samples were prepared using CTAB protocols (see Wilding *et al.*, 2001; Doellman *et al.*, 2011 for details) and a 395-bp fragment of mtDNA including partial *NADH1* was amplified through PCR using the protocol of Doellman *et al.* (2011). PCR products were sequenced by GATC Biotech in the forward direction using the primer LsaxMt_F, 5'-CTG ATG CCG CAA AAC TTC TT-3'. Sequences were manually trimmed and edited using UGENE v. 1.10 (Okonechnikov, Golosova & Fursov, 2012) and aligned using MUSCLE (Edgar, 2004) (GenBank accession nos KJ151947–KJ151954). Haplotype networks were generated using TCS v. 1.21 (Clement, Posada & Crandall, 2000).

In the small scale, within-H part of the study at Ninian's Cave, shell dimensions were calculated as distances between landmarks placed on shell images using tpsDIG2 (Rohlf, 1996; see Fig. 1). After scaling the linear measures by their geometric mean to suppress the influence of size as such (Jungers, Falsetti & Wall, 1995), multivariate analyses were carried out on the \log_{10} -transformed scaled variables. We used principal component analysis (PCA) to determine the important axes of variation, and multivariate analysis of variance to examine the possible association of haplotype and phenotype as indicated by shape. Such a Euclidean distance matrix analysis approach has the property that it retains information about relative variation of landmark points, which is lost in Procrustes superimposition methods; the merits of the various approaches have been further discussed by Lawing & Polly (2010).

RESULTS

Based upon the initial sampling of 64 individuals from the shore at Ninian's Cave, mtDNA lineages showed a strong association with ecotype ($\chi^2 = 20.122$, d.f. = 1, $P \leq 0.0001$), with lineage I

haplogroup B appearing predominantly in the M ecotype and lineage II haplogroup F in the H ecotype (Fig. 2A). Figure 2B shows the distribution of the ecotypes at the sample sites at Ninian's Cave. All of the H ecotype snails were found high on the shore on the solid bedrock; a few M ecotype snails were found here, but most of the M snails were found in the midshore boulder field. Within haplogroup F itself, 13 individuals (44.8%) fell within a sub-haplogroup (F^+) (represented by only a single individual in the smaller sample of Doellman *et al.*, 2011). Of these 13, 12 specimens were H ecotypes and only one an M ecotype (found on the high shore). On the three other shores in this study, no relationship was found between ecotype and mitochondrial haplotype. Figure 2C shows the overall haplotype network for all the individuals in this study. Figure 2D shows that, while haplogroup F was common at all other locations sampled (which, including the F^+ subgroup, comprised 69.7% of all samples), the F^+ subgroup was found only on the shore at Ninian's Cave. Overall molecular diversity was found to be highest at Ninian's Cave (Table 1).

Analysis of shell shape of H ecotype animals obtained during the second sampling at Ninian's Cave showed haplotype to associate with shell shape (MANOVA, $P = 0.015$) and, among individual dependent variables, both aperture length (al; Walker & Grahame, 2011) and the width of the shell just below the spire (whorl width 2, ww2; Walker & Grahame, 2011) were significant (al: $P = 0.002$; ww2: $P = 0.02$). So also was the relationship with apical angle ($P = 0.002$). In a PCA the first component accounted for 39% of the variation, with an eigenvector of 1.76. Of the 49 individuals involved in shell shape analysis, 15 belonged to the F^+ subgroup of which 11 were positive on PC1. This component is most associated with apical angle (coefficient -0.51) and aperture length (coefficient -0.42) and contrasted with whorl width 2 (coefficient 0.43). To take this further we focus on the two linear variables (al and ww2), because this will allow insights into the possible influence of allometry. Plotting the \log_{10} -transformed values of these variables on one

another, and differentiating between those shells that are positive and negative on the principal component, illustrates the association of F^+ with shape variation (Fig. 3). Treating the data for positive (relatively high-spired) and negative (relatively low-spired) shells on component 1 as two sets for regression and analysis of covariance, we find that the slopes are both slightly greater than 1 (b negative = 1.241, ~ 1 , $P = 0.071$; b positive = 1.157, just different from 1, $P = 0.049$), but the slopes are the same in ANCOVA (P different slopes = 0.561). Thus, while there is evidence of slight positive allometry, this is substantially the same in both groups, and shapes of larger shells are not generated by allometric growth from smaller ones. Considering the distribution of F^+ versus other haplotypes and whether these are positive or negative on the principal component of the phenotype, this can be treated as a 2 x 2 contingency table ($\chi^2 = 4.62$, $P = 0.032$, Fisher's Exact Test $P = 0.028$). This is consistent with the MANOVA reported above and supports the inference of heterogeneity, and a degree of association of F^+ with the high-spired shell phenotype.

DISCUSSION

Wilding *et al.* (2001) showed that along four transects down the shore at a site they referred to as St Ninian's Cave, in Port Castle Bay on Burrow Head, Galloway, Scotland, there was an association of mitochondrial haplotype with morphology in the ecotypes H and M of *Littorina saxatilis*. Fieldwork was carried out in 1997 and 1998, and the association appeared in both years. Yet 6 km northeast of this location, at Back Bay, a single transect did not show the association found at Ninian's Cave. Likewise on a shore at Mumbles in South Wales, at Old Peak on the Yorkshire coast, and at Ballynahown on the west coast of Ireland, there was no association of haplotype and morphology (Wilding *et al.*, 2001). But, against this, small samples from Ursholmen, Sweden did show such an

association (the analogous ecotypes here are referred to as E and S). Wilding *et al.* (2001) concluded that such an idiosyncratic pattern of ecotype/genotype association was perhaps to be expected in a young genus with much recent diversification.

Less is known about the possible functional significance of variation in mitochondrial DNA compared with nuclear DNA. Indeed, mtDNA variations are often assumed to be neutral, hence their use in broad-scale phylogeographic studies, including those on *Littorina* (Doellman *et al.*, 2011; Panova *et al.*, 2011). Yet as just reviewed, there is evidence—admittedly patchy—of some association between mtDNA haplotype variation and morphology, on a background of ecotypes between which it is now understood that there is partial reproductive isolation (Grahame *et al.*, 2006; Panova, Hollander & Johannesson, 2006; Johannesson, Rolan-Alvarez & Ekendahl, 1995). Given the evidence to date, we sought in this study to revisit the shore at Ninian's Cave in 2011–2012 to discover if after 13 years the apparent cline in mtDNA described by Wilding *et al.* (2001) was still evident, and to make comparison with three other shores.

Our findings corroborate those earlier observations of a distinct cline in mtDNA associated with the ecotypes found along the environmental gradient between the highshore and midshore at Ninian's Cave (Wilding *et al.*, 2001), suggesting a significant degree of reproductive isolation between ecotypes like that described by Grahame *et al.* (2006). Moreover, lineages I and II (Doellman *et al.*, 2011) associate predominantly with the M and H ecotypes respectively; we did not find such association on the other three shores investigated. Indeed lineage I is rare at Port St Mary on the Isle of Man and absent at Old Peak on the Yorkshire coast, while M ecotype animals are abundant on both these shores. At Thornwick Bay (41 km away from Old Peak) where H and M ecotypes are abundant, both lineages are found (represented by haplotypes B and F), but with no association of haplotype with ecotype.

This geographically localized differentiation of populations with respect to mitochondrial haplotypes is puzzling. Lineages I and II are understood to have differentiated early in the history of the species (Doellman *et al.*, 2011); that they are associated with ecotype differentiation at Ninian's Cave but not at Thornwick Bay cannot be easily explained by the available facts. It appears that the current variation between populations represents a mosaic of variation, itself derived from a different mosaic due to the impact of glaciation-driven changes in distribution. What is apparent is that the clearly divergent H and M ecotypes do not map consistently onto the distribution of mitochondrial haplotypes. We speculate that perhaps the strength of the reproductive barrier between the ecotypes differs from shore to shore, or even along relatively long stretches of coast. If there were locations where the barrier was unusually strong, this might lead to loss of one or another haplotype from a segment of the population due to genetic drift through the reduction of effective population size. In turn this could mean that it is unlikely that the H and M ecotypes can be attributed to historically divergent populations. Rather, it would support the idea that the ecotypes diverged or are diverging independently on separate shores with a population structure peculiar to each shore (Wilding *et al.*, 2001).

This brings us to the extent and implications of variation within ecotypes, to which less attention has hitherto been given (but see Wilding *et al.*, 2001). At Ninian's Cave, variation in shell shape within the H ecotype itself appears on a continuum, while the restriction of the F⁺ subgroup within one portion of the ecotype's morphological space at the locality suggests the existence of discrete groups within this space. Firstly, this may reveal fine-scale niche partitioning and microhabitat differentiation within the area of the shore occupied by the H ecotype, with the F⁺ subgroup occupying only one part of the available habitat. Certainly, habitat heterogeneity is high in the portion of the shore occupied by the H ecotype at Ninian's Cave, animals being found both

on the rock surface and in cracks and crevices, and such micro-habitat differentiation could favour different shell shapes. Unfortunately our sampling was not planned so as to allow us to investigate this, and our finding suggests that future work might usefully take account of truly microhabitat differences—say, between open rock and crevices on the scale of a few cm.

Secondly, it raises questions regarding the extent to which gene flow is occurring within populations of the H ecotype, hinting at a degree of reproductive isolation between the F⁺ subgroup and the remainder of the population. Such isolation could be a function of the reproductive strategy of *L. saxatilis*. Unlike closely related species, *L. saxatilis* is ovoviviparous, rearing live young within a brood pouch and releasing juveniles directly into the parental environment with no pelagic dispersal phase (Reid, 1996). Since both juvenile and adult dispersal are low, family groups are likely to exhibit high site fidelity and, if a particular haplotype were to be found within a particular microhabitat, it might remain there due to limits of dispersal alone.

The haplotype F⁺ has been found only at Ninian's Cave. Although the sampling effort was greatest there, it seems likely that the restriction of F⁺ to this site (among those sampled) is a real finding. We speculate whether a direct genetic link could exist between F⁺ and the associated shell shape. The increased molecular diversity seen at Ninian's Cave (if not a sampling artefact) could be an indication that this shore is a site where previously allopatric populations met after changes in sea level at the beginning of the current interglacial. The F⁺ subgroup could be the remnant of a population isolated in a glacial refuge where the shell shape with which it associates was under positive selection. If sufficient divergence in mtDNA had occurred for coadaptation between the mitochondrial and nuclear genomes to take place, and this was followed by reconnection with conspecific populations during postglacial range expansion, intergenomic conflict might result in a fitness penalty in hybridizing populations. This could potentially lead to the situation seen here.

Although hybridization may well occur between F^+ and the rest of the population, decreased fitness of the F_2 generation could mean that the haplogroup does not introgress fully into the entire range of variation normally seen in the H ecotype. At the same time, because the F^+ haplotype is generated by what appears to be a noncoding single-nucleotide polymorphism (SNP) it cannot itself be involved in selection. Since mtDNA behaves as a single locus, this SNP may be in full linkage disequilibrium with other untyped nonsynonymous SNP(s) in the mitochondrial genome. Further whole-mitochondrial genome sequencing would be needed to resolve this.

The mechanisms involved in the sequestration of F^+ may depend on the interplay of several factors. While definitive explanations are beyond us here, the association of mitochondrial haplotype with shell shape—and the implications that this may have for the processes governing adaptation and speciation—offers an accessible and attractive example for the study of intergenomic conflict in evolution.

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Figure captions

Figure 1. The shell dimensions of *Littorina saxatilis* measured in this study. Abbreviations: aa, apical angle; al, aperture length; aw, aperture width; cl, columella length; ll, lip length; ww0, 1, 2, whorl widths 0, 1 and 2 respectively. The dimension cl measures 14.4 mm in this shell.

Figure 2. Haplogroups and ecotypes within *Littorina saxatilis*. **A.** Map of shore at Ninian's Cave, Port Castle Bay, Scotland, showing distributions of mitochondrial haplogroups. Solid line indicates mean high tide level, dividing land (above) from shore (below). Sites 1, 1a, and 1b were the highest sites sampled on the shore; 2 and 2a were lower on the shore but still on solid bedrock; 3, 3a, 4 and 4a were in the mid-shore boulder field. Lineage I haplogroup B is shown in purple and Lineage II haplogroup F and the F⁺ sub-haplogroup in orange and red respectively. Total numbers of specimens sequenced from each sample site: site 1, $n = 5$; site 2, $n = 9$; site 3, $n = 12$; site 4, $n = 9$; site 1a, $n = 9$; site 2a, $n = 7$; site 3a, $n = 5$; site 4a, $n = 4$; site 1b, $n = 4$. **B.** Numbers of M and H ecotype snails from the site samples. **C, D.** Unrooted parsimony networks for mtDNA haplotypes of NADH1 in *Littorina saxatilis*, collectively for all sampled sites (**C**) and on individual shores (**D**). Haplotypes associating with haplogroup B (lineage I) are shown in purple and haplotypes associating with haplogroup F (lineage II) are shown in orange. Other lineage II haplotypes are shown in red (F⁺) and yellow. Bars are theoretical intermediates and do not represent actual specimens. All group sizes are to scale (see Table 1 for sample sizes).

Figure 3. Plot of log whorl width 2 against log aperture length (see Fig. 1) for shells of H ecotype from Ninian's Cave. These variables are among those defining PC1 (see text). Individuals with the F⁺ haplotype are plotted as triangles, solid if the PC1 score is positive (11 individuals), open if it is negative (four individuals). Individuals with F and B haplotypes are plotted as squares (PC1 score positive) or circles (PC1 score negative). The shells for two datum points are shown, ww2 is 79% of al for the shell with the positive score and only 58% of al for the shell with the negative score. Scale bars = 5 mm.

Table 1. Characteristics of molecular variability of mtDNA haplotypes within H and M ecotypes of *Littorina saxatilis* on all sampled shores.

Population	Haplotype								Segregating sites, S	Haplotype diversity, Hd	Nucleotide diversity, Pi	n
	1 ^{tt}	2 ^{tt}	3 ^{tt}	4 ^t	5	6 ^t	7 [*]	8				
Ninian's Cave	1	1	11	16	35	0	0	0	7	0.62452	0.00653	64
H	1	1	10	11	7							
M			1	5	28							
Port St Mary	0	0	0	24	2	1	0	0	5	0.21083	0.00174	27
H				10								
M				14	2	1						
Old Peak	0	0	0	34	0	0	1	0	2	0.05714	0.00031	35
H				10								
M				14			1					
Thornwick Bay	0	0	0	25	10	0	0	1	5	0.45238	0.00486	36
H				12	4							
M				13	6		1					
Total	1	0	0	99	47	1	1	1	10	0.54571	0.00511	162

t, haplogroup F; tt, subgroup F⁺; *, haplogroup J; all others haplogroup B.

Figure 1.

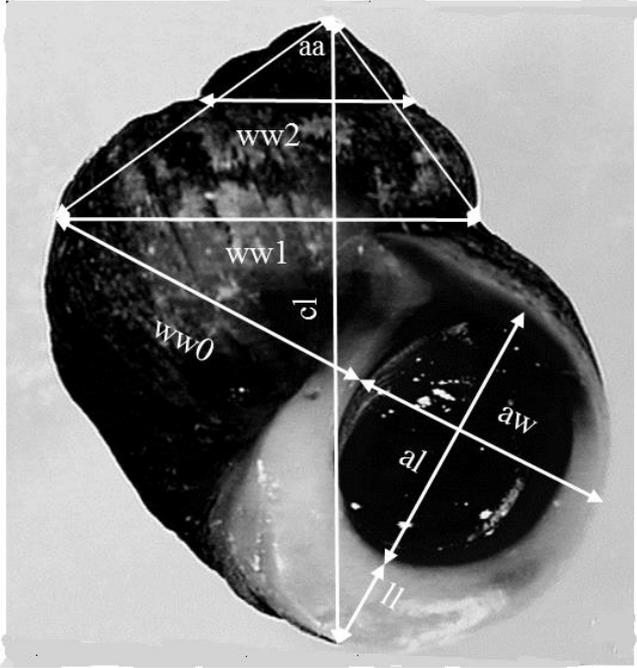


Figure 2A.

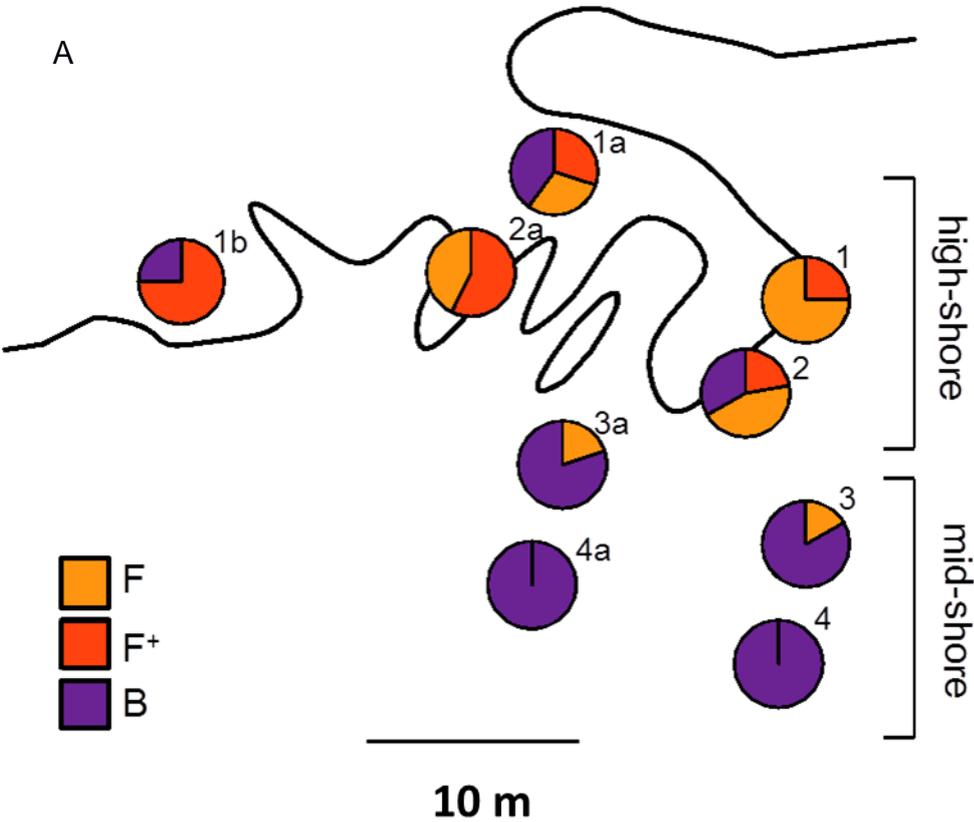


Figure 2B.

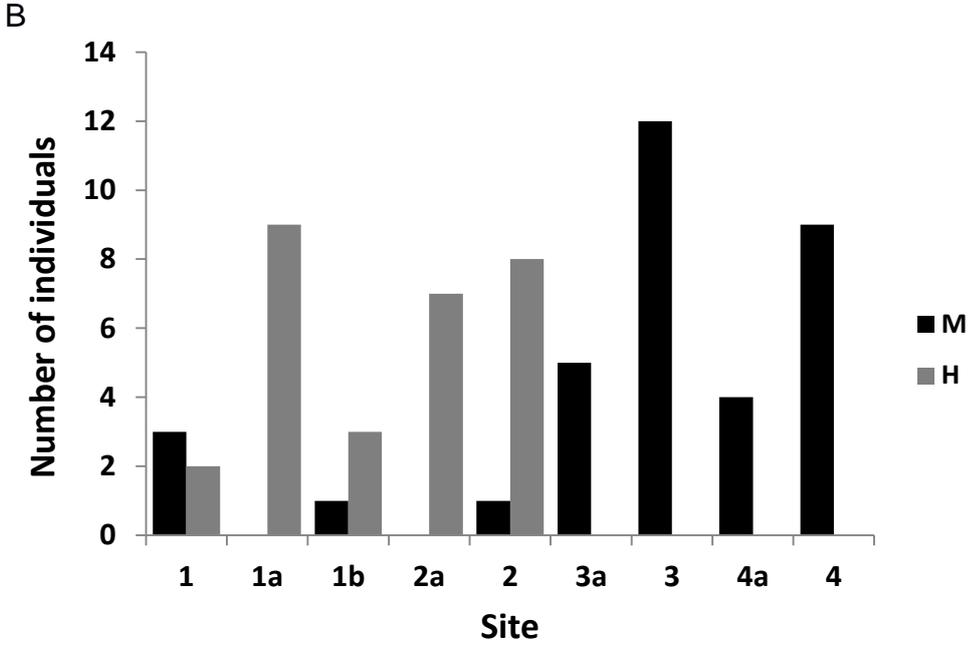


Figure 2 C, D.

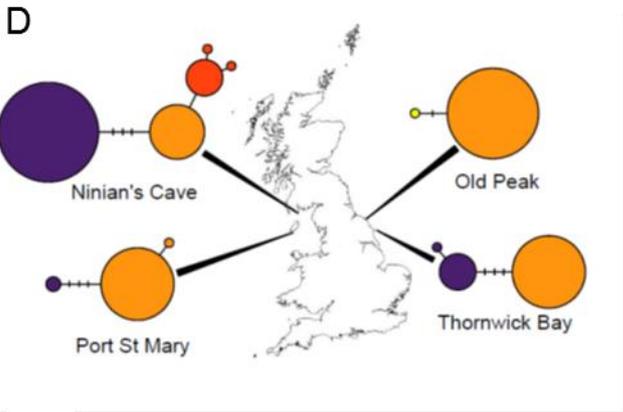
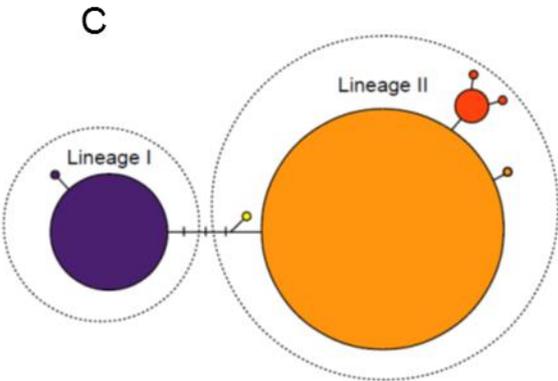


Figure 3.

