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Genetic diversity and phylogenetic analysis of native mountain ponies of Britain and Ireland reveals a novel rare population

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Introduction

An understanding of the genetic characteristics of a population is paramount in order to inform and implement conservation strategies that preserve and maintain genetic diversity (Hall and Bradley 1995). This principle applies to both domestic livestock breeds and wild species in situ. Bruford et al. (2003) highlighted the complexity of domestication and a need to better understand the population dynamics involved to guide livestock conservation and maintain biodiversity resources for the future. Domestication of livestock has resulted in the selection of certain animals according to their suitability for specific tasks, leading to post-domestic polychotomy in phenotypic appearance within species. Breed development occurs by the mixing of different populations, or by

Abstract

The conservation of unique populations of animals is critical in order to preserve valuable genetic diversity and, where populations are free-living, maintain their irreplaceable influence upon habitat ecology. An accurate assessment of genetic diversity and structure within and between populations is crucial in order to design and implement conservation strategies in natural and domesticated species. Moreover, where it is possible to identify relic populations that are related to a structured breed an ideal opportunity presents itself to model processes that reveal historical factors that have shaped genetic diversity. The origins of native UK mountain and moorland ponies are uncertain, but they may have directly descended from prehistoric populations and potentially harbour specific adaptations to the uplands of Britain and Ireland. To date, there have been no studies of population structure and genetic diversity present within a free-living group of ponies in the Carneddau mountain range of North Wales. Herein, we describe the use of microsatellites and SNPs together with analysis of the mitochondrial control region to quantify the extent and magnitude of genetic diversity present in the feral Carneddau pony and relate this to several recognised British and Irish pony breeds. Our results establish that the feral Carneddau ponies represent a unique and distinctive population that merits recognition as a defined population and conservation priority. We discuss the implications for conservation of this population as a unique pool of genetic diversity adapted to the British uplands and potentially of particular value in maintaining the biodiversity of these habitats.

selecting from a sub-population within an existing breed, to create a group of animals that contain a defined set of desired traits (Goodwin et al. 2008).

Several definitions of what constitutes a breed exist, and the term is therefore flexible and potentially confusing dependent upon the context in which it is used. Clutton-Brock (1987) defines a breed as “a group of animals that has been selected by humans to possess uniform appearance that is inheritable and distinguishes it from other groups of animals within the same species.” Lush and Crow (1994) argues that the term “breed” was created by breeders of livestock prior to the recognition of genetic selection and is commonly used in lay-situations according to popular consensus. In practice, certain breed societies will not register an individual within a breed stud book without a pedigree demonstrating several

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generations of closed matings (Welsh Pony and Cob Society). Other societies register animals according to both pedigree and morphological assessment of phenotype based upon agreed guidelines (Connemara Society). Nonetheless, the issue of whether or not a group of individuals can be designated as a certain breed should not detract from the recognition that a particular population, with a distinct genetic profile following substantial isolation, may be worthy of conservation.

A better understanding of population origin and history of development gained through molecular genetic analysis may provide information on relic populations. Putative genetic links may then be made to ancestral groups from which modern breeds have developed following periods of population management (Hall and Bradley 1995). Molecular approaches have begun to be applied to the genetic examination of feral populations of horses around the world in comparison to managed registered breeds that show phenotypic and/or geographical similarities (Vega-Pla et al. 2006; Plante et al. 2007; Rendo et al. 2012). The primary aims of these studies were to identify conservation priorities and guide conservation management schemes. In Britain, there are only a limited number of free-living populations of domesticated livestock species (Hall and Moore 1986; Goulding 2001; Wilson 2003; Burthe et al. 2011), and to-date no molecular genetic analysis has been conducted upon semi-feral British native pony breeds with a view to better understand the structure of genetic diversity.

A free-living equine population known as Carneddau ponies exist in Snowdonia National Park (Wales) that have been suggested to be direct descendants of the ancestral Welsh Mountain Pony (Fig. 1). The Carneddau ponies are anecdotally thought to have existed during the Roman era and have been implicated as representative of the historical foundation of a modern, registered breed, the Welsh Section A pony. Carneddau ponies are one of the last remaining collections of free-living ponies in Britain. Compared to other equine populations maintained on hills or moors, Carneddau ponies are less intensively managed in terms of reproduction, with intervention being limited to late autumn removal of a proportion of offspring for sale. Access to grazing, water and shelter, social interactions and general maintenance of these mares, stallions and residual young stock are unhindered by human intervention. The geographically nearest group of registered, traditionally managed animals to the Carneddau population is the Welsh Pony and Cob, a breed indigenous to Wales. The Welsh Pony and Cob is subdivided into four distinct sections whose defining criteria are the animal's adult height and conformation characteristics: Section A is a Welsh Mountain Pony, <121.9 cm; Section B is a Welsh Riding Pony, of fine-built



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Figure 1. The free-living Carneddau pony in its natural environment in north Wales. Photo courtesy: ©2012 Osian Rees.

morphology, <137.2 cm; Section C is a Welsh Pony of Cob Type, of stocky morphology, <137.2 cm and Section D, a Welsh Cob and >137.2 cm (Davies 1993). The term “Cob” refers to a generically bred animal of stocky morphology, although in the case of the “Welsh Cob” it refers to registered animals of a defined phenotype (according to Welsh Pony and Cob Society guidelines). All four sections show phenotypic similarities and are assumed to have arisen from a common ancestral stock similar to the modern Section A. The Welsh Pony and Cob Society (WPCS) administer a stud book for pedigree documentation of all four sections, which has been closed since 1950, meaning that offspring resulting from breeding with unregistered animals thereafter may not themselves be registered (Davies 2001).

No previous studies have examined Carneddau ponies in terms of effective population size, degree of inbreeding or their molecular relationship to Welsh and other UK and Ireland native pony breeds. The topography of Wales creates natural environmental barriers to gene flow and population migration in the form of mountain ranges and expanses of water. More specifically, the Carneddau ponies are limited to the Carneddau mountain range and are believed to have maintained a relatively stable population of around 300 breeding animals. Thus, the Carneddau may have evolved or maintained adaptations specific to their upland habitat that have been lost from the related ponies during breed formation. We present a comprehensive analysis of the genetic diversity within the Carneddau ponies relative to other UK native pony breeds from Wales, Ireland and Scotland. The aim is to establish to what extent they represent a genetically unique subpopulation formed as a result of long-term geographical isolation within Wales or whether this population is a more recent separation from the existing Section A pony breed.

Materials and Methods

Sample collection and extraction of genomic DNA

Studies were conducted using animals representative of different bloodlines for each of five British-Irish native pony populations. Samples were collected from Welsh Section A and Section D animals, Connemara ponies (native to Ireland) and Highland ponies (native to Scotland) from individuals registered with their respective breed society stud book (see Table 1 for sample numbers and population identifier abbreviations). In each case, collection from siblings was avoided. Samples were also collected from feral Carneddau ponies during a single annual herd round-up in 2009, although the relationship between these source animals was not known. All samples were collected as tail hairs or buccal swabs. Total DNA extraction from hair was performed using the Qiagen DNeasy[®] Blood and Tissue extraction kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Total DNA from buccal samples were extracted using the BuccalAmp[™] DNA Extraction Kit (EPICENTRE[®] Biotechnologies, Cambio Ltd., UK).

SSR genotyping and data analysis

Microsatellite (Simple Sequence Repeat; SSR) amplification by PCR was performed using the StockMarks[®] equine genotyping kit (Applied Biosystems, Warrington, UK) comprising 17 multiplexed fluorescent dye-labelled primers for the microsatellite markers, recommended by the International Society of Animal Genetics. PCR amplification used 10 ng of template genomic DNA with reagents at concentrations as provided; 2.5 μ L Stockmarks PCR Buffer, 4 μ L dNTP mix, 0.5 μ L AmpliTaq Gold[®] DNA Polymerase, 4 μ L multiplexed primer mix and 3 μ L water. DNA was amplified in a G-Storm GS-1 thermal cycler (Gene Technologies Ltd., Essex, UK) and reactions incubated at 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec, followed by final extension at 72°C for 60 min. PCR products were diluted 1:20 with molecular grade water and subjected to capillary electrophoresis using an ABI 3730 DNA Analyzer (Applied Biosystems). Alleles were sized using the internal size standard GeneScan[™]-500LIZ. Size analyses of separated DNA fragments were performed with the GeneMapper software version 3.7 (Applied Biosystems). Alleles were scored based on PCR product size and recorded manually. Allele frequencies were examined for significant deviation from Hardy-Weinberg expectations using the exact test in Arlequin 3.5 (Excoffier et al. 2005; Excoffier and Lischer 2010), with Markov chain

Table 1. Summary statistics for MTCR, SSR and SNP genotyping for each of the five sample groups.

| Population (Identifier) | N (MTCR/SSR/SNP) | H | h \pm SD | π \pm SD | MNA (SSR/SNP) | Ho (SSR/SNP) | He (SSR/SNP) | F _{IS} (SSR/SNP) | No. Markers (SSR/SNP) |
|-------------------------|------------------|----|--------------------|---------------------|---------------|--------------|--------------|---------------------------|-----------------------|
| Section A (A) | 47/48/12 | 17 | 0.932 \pm 0.017 | 0.0179 \pm 0.0093 | 7.47/1.86 | 0.677/0.276 | 0.726/0.275 | 0.0536/−0.001 | 17/48,703 |
| Section D (D) | 46/46/12 | 26 | 0.950 \pm 0.025 | 0.0190 \pm 0.0077 | 6.65/1.82 | 0.637/0.266 | 0.702/0.266 | 0.1015**/−0.003 | 17/48,715 |
| Carneddau (S) | 46/46/12 | 8 | 0.788 \pm 0.0361 | 0.0146 \pm 0.0077 | 6.94/1.84 | 0.659/0.282 | 0.733/0.272 | 0.0868**/−0.037 | 17/48,715 |
| Connemara (N) | 46/46/12 | 19 | 0.878 \pm 0.032 | 0.0137 \pm 0.0072 | 6.65/1.91 | 0.672/0.303 | 0.748/0.295 | 0.0922**/−0.029 | 17/48,716 |
| Highland (H) | 45/39/12 | 14 | 0.916 \pm 0.019 | 0.0142 \pm 0.0075 | 6.41/1.80 | 0.650/0.263 | 0.723/0.262 | 0.0320/−0.004 | 17/48,710 |
| Total | 225/233/60 | 53 | 0.932 \pm 0.0171 | 0.018 \pm 0.0090 | 9.53/1.84 | 0.659/0.278 | 0.778/0.304 | 0.0749**/0.086 | 17/48,327 |

N = sample number, MTCR = mitochondrial control region, SSR = simple sequence repeat, SNP = single nucleotide polymorphism, H = number of MTCR haplotypes, h = MTCR haplotype diversity, π = MTCR nucleotide diversity, MNA = mean number of alleles, Ho = observed heterozygosity, He = expected heterozygosity, He = inbreeding coefficient, SD = standard deviation, No. Markers = number of (SSR/SNP) markers used in Ho, He and F_{IS} calculations for each population.

*Significant deviation from HWE at $P < 0.05$.

**Significant deviation from HWE at $P < 0.01$.

length set to 100,000 following 10,000 dememorization steps. Markers that demonstrated highly significant deviations in more than one population and/or contained large amounts of missing data values were removed to limit the influence of allele drop out. Loci with missing data unevenly distributed across the populations (>5% per breed) were also excluded to limit ascertainment bias. According to these criteria, five loci were removed from the analysis, including two that resulted in null alleles in other pony breeds (Rendo et al. 2012). Individual samples with poor quality DNA with calls missing from >3 loci were also excluded. To elucidate the relationships between the five populations and provide fine quantification of the different ancestral contributions, an unbiased Bayesian approach using Markov chain Monte Carlo (MCMC) clustering of samples was conducted via the STRUCTURE v2.2.3 software (Pritchard et al. 2000) using both SSR and SNP loci. Allele calls were loaded into STRUCTURE as diploid data for each individual and assessed for prior values of K ranging from 2 to 9. Burn-in and MCMC iteration settings were 50,000 and 10,000, respectively. Allele frequencies were treated as correlated. For each value of K , three replicate simulations were conducted. The ΔK statistic (the second order rate of change in log probability between successive values of K) was calculated using STRUCTURE Harvester v0.6.7 (http://taylor0.biol.og.ucla.edu/struct_harvest/) as per Evanno et al. (2005). Results from replicate runs at the optimal K were combined in CLUMPP (Jakobsson and Rosenberg 2007) and the average Q-table exported to DISTRUCT (Rosenberg 2004) for graphical presentation. Population summary statistics and analysis of molecular variance (AMOVA) were calculated using Arlequin 3.5 (Excoffier and Lischer 2010).

Single Nucleotide Polymorphism (SNP) genotyping and data analysis

A subset of 12 individuals from each group was selected and 200 ng of genomic DNA was supplied to Central Biotechnology Services (Cardiff University) for genotyping using the Illumina Equine SNP50 assay. Data were returned as binary allele calls and reformatted for STRUCTURE. Data were filtered for quality control purposes by removing any loci with >10% missing data and/or loci with extreme deviation from HW expectations ($P < 0.001$) (determined using PLINK <http://pngu.mgh.harvard.edu/purcell/plink/>). After filtering observed heterozygosity (H_o), expected heterozygosity (H_e) and F_{IS} values were calculated using PLINK, using between 48,703 and 48,710 SNPs per population (see Table 1). The proportion of shared alleles identical by state (IBS) between individuals was calculated in PLINK and the resultant

similarity matrix used to establish the relationships between the horses using UPGMA clustering in MEGA4 and by multidimensional scaling within PLINK. To further investigate any higher order patterns within the SNP data, STRUCTURE analysis was performed on three random subsamples of 5000 markers each analysed for values of K ranging from 2 to 10 (five iterations per K). As Alpha values stabilised rapidly, burnin and MCMC values were set at 25,000 each to improve computational speed. Data from each subsample analysis were combined and ΔK statistics calculated using STRUCTURE Harvester as for the SSR analysis. Results from replicate runs at the optimal K were combined in CLUMPP (Jakobsson and Rosenberg 2007) and the average Q-table exported to DISTRUCT (Rosenberg 2004) for graphical presentation.

Mitochondrial DNA sequencing and analysis

Using a Welsh Cob sequence (GenBank AF072983), the primer pair (Forward - 5'-ATT TCT TCC CCT AAA CGA CAA C-3') and (Reverse - 5'-CGT TCA ATT TAA GTC CAG CTT C-3') was designed to a 606 bp amplicon of the mitochondrial control region (MTCR). PCR amplifications consisted of 10 ng template DNA, 7.5 μ L of ImmoMix (Bioline, London, UK), 1 μ mol/L forward and reverse primer and water to a final volume of 15 μ L. Amplifications were performed in a G-Storm GS-1 thermal cycler at 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec, with final extension at 72°C for 10 min. Reaction products were purified using a Multiscreen® 96-well filter plate (Millipore, Durham, UK) to remove primers and dNTPs then diluted 1:20 with molecular grade water in preparation for sequencing using an ABI 3730 DNA Analyzer (Applied Biosystems) and 1 μ mol/L of the forward primer described above. To place the current data into a wider context against the major worldwide horse mtDNA haplogroups, 83 reference horse and pony samples were downloaded from GenBank based upon a recent whole mitochondrial genome study (Achilli et al. 2012) and included within the data file. Sequence data were trimmed to a common 519 bp sequence and aligned using BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999) before being exported in phylip (phy.) format. The package NETWORK (<http://www.fluxus-engineering.com>) was employed to produce a median-joining network based on maximum parsimony to reconstruct the phylogenetic relationships among haplotypes. Individuals showing unusually long branch lengths as part of the network were examined more closely for poor sequence quality. Sequence traces were checked for nucleotide call errors and either manually rectified or removed in the case of

overall low-quality sequence. Population allelic and molecular diversities were compared using Arlequin 3.5 (Excoffier and Lischer 2010).

Results

Population genetic diversity parameters

Summary statistics for MTCR, SSR and SNP data are presented in Table 1. Analysis of MTCR sequence from 230 individuals ($n = 47$ for Section A, $n = 46$ for Section D, $n = 46$ for Connemara, $n = 45$ for Highland and $n = 46$ for Carneddau) (Table 1) identified 54 haplotypes based on 60 variable nucleotide sites. Haplotype diversity was high (0.932 ± 0.0171) with relatively low nucleotide diversity (0.018 ± 0.0091). The Carneddau possessed the lowest haplotype diversity of the five groups (but not the lowest nucleotide diversity), and also the lowest number of haplotypes (approximately half that observed in the other groups). For SSRs, 225 individuals were successfully genotyped using the StockMarks kit ($n = 48$ for Section A, $n = 46$ for Section D, $n = 46$ for Connemara, $n = 39$ for Highland and $n = 46$ for Carneddau) (Table 1). The mean number of alleles per locus (MNA) ranged from 6.4 to 7.5 for the 17 SSRs, with average expected (H_E) and observed (H_O) heterozygosity values of 0.778 and 0.659 respectively. In contrast to the MTCR results, the Carneddau showed higher expected and observed heterozygosity than both the Highland and the Section D. The Section D samples possessed the lowest number of alleles, the lowest H_E and H_O and also the highest inbreeding coefficient (F_{IS}), demonstrating a clear reduction in heterozygosity probably due to non-random mating. Section A showed the highest MNA although Connemara had equal H_O and displayed higher H_E . SNP results, based on 12 individuals per population, show roughly similar patterns of diversity with no significant differences in heterozygosity between the 5 groups, but with the Connemara ponies again showing the highest expected heterozygosity ($H_e = 0.342$) and the Carneddau the highest observed ($H_o = 0.338$). Global exact tests of HWE performed across all loci revealed all five groups deviated significantly ($P < 0.01$) from HWE indicating a significant level of structure between the groups. Molecular analysis of variation demonstrated highly significant levels of between group variation relative to within group for both SSR and MTCR results, with 9% and 85% of total variation between groups respectively. Individual HW tests indicate that consistently significant deviations were found across several of the populations for a few of the SSR markers. These loci were removed from the data set for later STRUCTURE analysis below, to exclude the

possibility of allele drop out. Similar results were obtained with SNP data again indicating that the Carneddau had relatively high heterozygosity levels and no evidence of significant inbreeding.

Population structure and gene flow

To elucidate the relationships between the five populations and provide finer quantification of the different ancestral contributions, STRUCTURE analysis was conducted using both SSR and SNP loci. Initial analysis included a small number of two other Welsh pony groups, Section B and Section C. These two categories are known to be intermediate between Section A and D ponies in terms of height and conformation and in fact the direct offspring of a Section A crossed with a Section D may be registered as a Section C. This is clearly demonstrated within the STRUCTURE plots obtained (Fig. A1). The partial assignment of most Section B/C animals to both Section A and Section D clusters confirmed the package was effectively detecting cases of known admixture. The Section B and Section C animals were excluded from the main analysis as the sample size available were much fewer than in the samples from the 5 populations whose results are presented here. STRUCTURE analysis of the log likelihood [$\ln \Pr(X/K)$] of the posterior probability of the SSR data for a given K suggested the optimal value of K was 5. The five clusters that were assigned match closely to the populations from which individuals had been sampled, though all populations contained individual animals that could not be unambiguously assigned to a single cluster (Fig. 2). Cluster analysis based on SNP data (Figs. 3, 4) also clearly demonstrates the separation of individuals into groups corresponding to their "breed". The Section A and the Carneddau clusters are generally closest in multidimensional space and in the hierarchical cluster "tree," but are consistently distinct, as demonstrated in the plot of mds dimension 1 against dimension 4 (Fig. 4C). There are a few Carneddau individuals who appear genetically distinct from the others Carneddau and cluster with the Section A animals or in an intermediate position between the two populations. Structure analysis of the SNPs favoured 6 clusters matching the five sampled populations, but with an additional split found mainly within the Section D cobs (Fig. 5A), a subdivision that is also apparent, though less clearly so, in the mds plots (Fig. 4C). All populations showed significant divergence from each other following pairwise F_{ST} analysis of SSR and SNP data (Table 2), with the closest relationship found between the Section As and Carneddau. This is also reflected in Fig. 3, where the Section A and Carneddau form a sub grouping within the tree. Interestingly,

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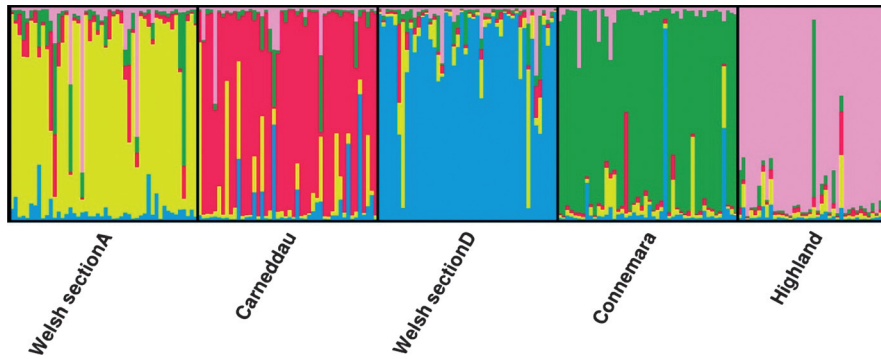


Figure 2. The results of STRUCTURE analysis for SSR loci of each population for $K = 5$. STRUCTURE Harvester analysis as per Evanno et al. (2005) suggests true value of K is 5. There is no real further subdivision beyond 5 clusters at $K > 5$.

for the SSRs, the Section As showed more divergence from the Section Ds (0.0711) compared to the relationship of this group with the other pony breeds, including the Irish Connemara (0.0535) and Scottish Highland (0.0658). This finding is also supported by the data from the SNP analysis, where it is most clearly illustrated by relative positions of the Section A and Section D animals on dimension 1 in the mds plot of dimension 1 versus dimension 4 (Fig. 4C).

Table 3 demonstrates that the maternal lineages in the Section A and Section D animals cannot be distinguished, but that the Carneddau are highly divergent both from these groups and from the Connemaras and Highlands. The Carneddau had fewer mitochondrial haplotypes present (eight haplotypes within four major haplogroups) compared to other breeds, but contained a number of unique mitochondrial haplotypes present at high frequency within the population (Fig. 6). Thus, Carneddau may have become isolated from the other groups quite some time ago.

Analyses of potential population expansion based upon Harpending's raggedness score (Harpending 1994) indicated that the Carneddau were the only population with a highly significant deviation from a unimodal pattern of molecular diversity expected from a population expansion (raggedness index = 0.121, $P < 0.001$), although the Section Ds also had a moderate disturbance (raggedness index = 0.022, $P < 0.05$).

Discussion

Population genetic diversity parameters

All groups displayed high levels of mitochondrial haplotype diversity ($>0.788 \pm 0.0361$). SSR/SNP analysis suggested that heterozygosity levels, although lower than some existing reports (Leroy et al. 2009; Prystupa

et al. 2012a), remains relatively high and are comparable to values in other European pony breeds, including semi-feral ponies (Solis et al. 2005; Luis et al. 2007; Rendo et al. 2012). Prystupa et al. (2012b) report lower numbers of mitochondrial haplotypes (approximately half) and lower haplotype and nucleotide diversity in Canadian populations of the Connemara, Highland and Welsh Pony than identified here. This perhaps reflects a sampling effect in the founding of those breeds in Canada.

Reduced MTCR haplotype numbers and significant population frequency divergence of the Carneddau could be explained by the effects of severe bottlenecks in a small, recently established but geographically isolated population. However, contrary to expectations for this scenario, mtDNA haplotype diversity remained relatively high and analyses performed to detect evidence of recent potential population expansion demonstrate the Carneddau have remained at a relatively constant size without any evidence of recent bottleneck contraction in allele diversity. Similarly nuclear diversity is not significantly reduced in the Carneddau, despite the census number of the Carneddau being much lower than the other groups. The Carneddau ponies exist as small, scattered harems across their range and have had minimal human intervention. It appears that this breeding pattern has maintained variation while the population has become genetically isolated via drift or natural selection over an extended period, rather than as a consequence of a recent founder effect. Other free-living ungulate populations have been shown to avoid inbreeding, even in relatively small populations, with a social structure of female philopatry and male-biased dispersal patterns similar to the Carneddau (Coltman et al. 2003; Lawson Handley et al. 2007). In more intensively-managed breeds, popular stallions have artificially large harem sizes, resulting in high variance in male reproductive success, as is apparent in equine

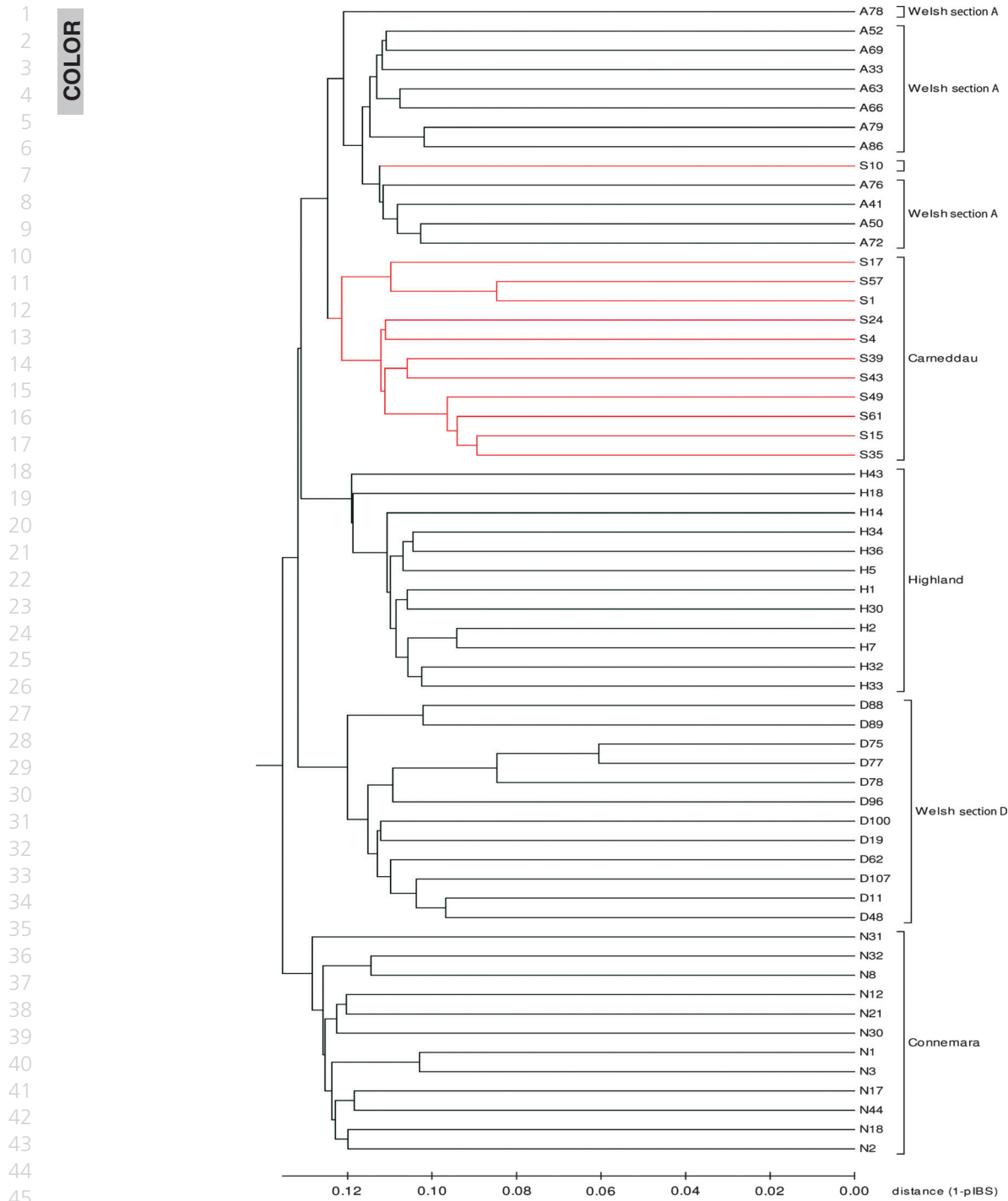


Figure 3. Clustering of genetic distance between individuals based on 1-proportion of alleles identical by state. The phylogeny was inferred using the UPGMA method (Sneath and Sokal 1973). The optimal tree with the sum of branch length = 6.66,442,597 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The identity of individual horses and the groups they belong to are shown on the right.

pedigree records. Population genetic theory predicts high reproductive variance will dramatically reduce the effective breeding size (N_e) of a population and the similarity

in diversity in the Carneddau and other breeds, despite the apparent differences in census numbers, may reflect this effect.

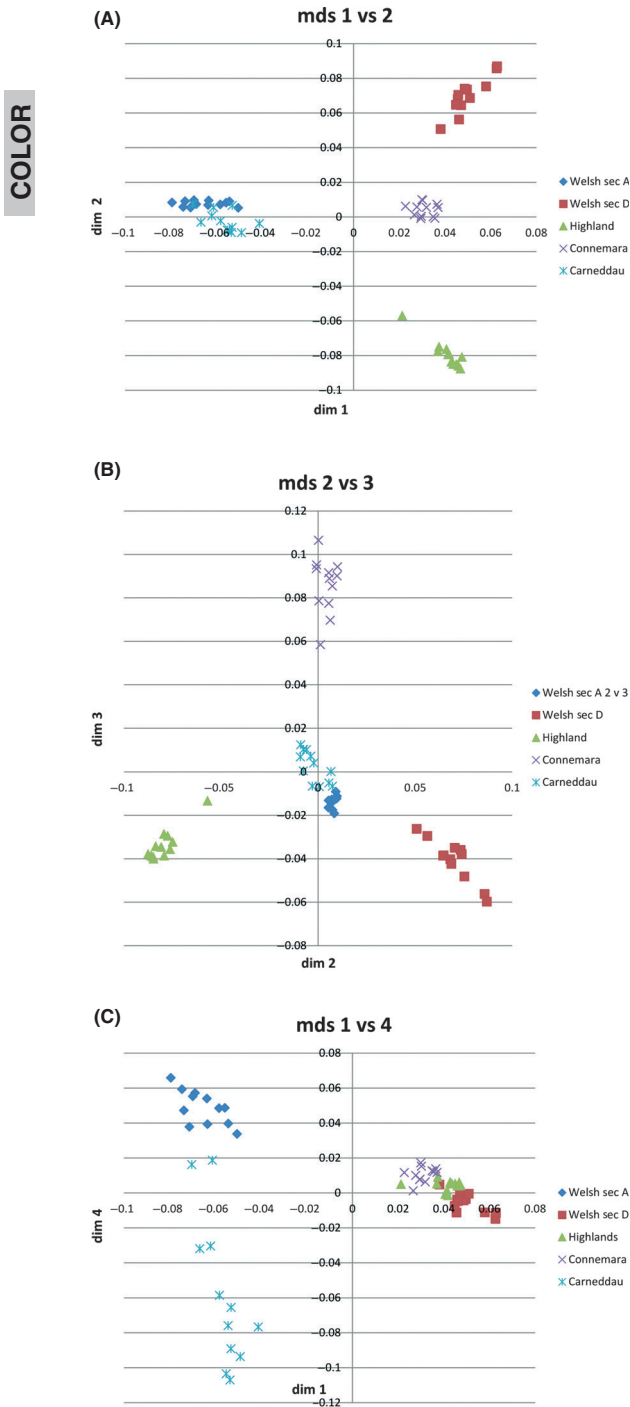


Figure 4. A–C. Identity by State similarity scores were calculated in PLINK from 48,721 diallelic markers. The resultant similarity between individuals was subjected to classical metric multidimensional scaling and the first four most significant dimensions extracted and used to plot the three graphs shown above. Dimension 1 splits Section A/ Carneddau from the rest, Dimension 2 separates Section D and Highlands from the rest, Dimension 3 separates Connemaras from the rest and Dimension 4 separates Carneddau and to a lesser extent Section A from the rest.

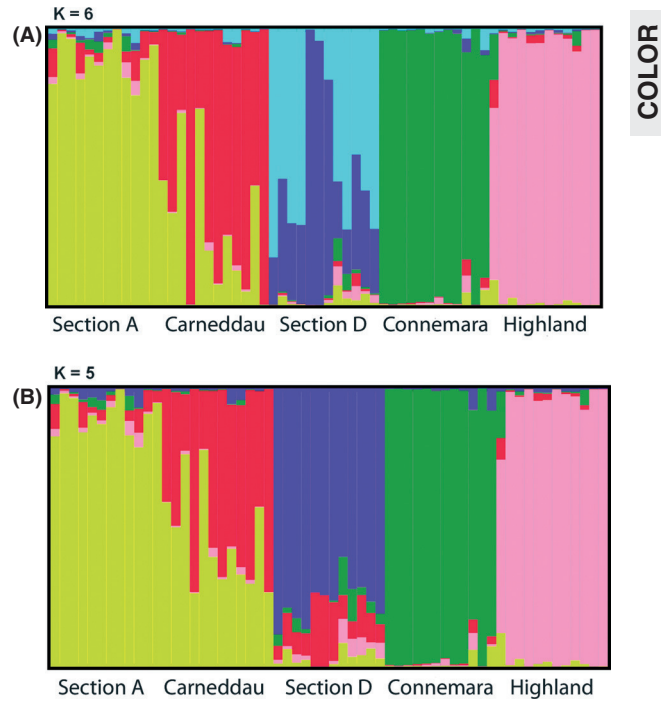


Figure 5. The results of STRUCTURE analysis for values of $K = 6$ (Fig. 5A) and $K = 5$ (Fig. 5B), averaged over three independent runs of 5000 SNPs and 12 ponies per group. STRUCTURE Harvester analysis suggests optimal cluster value of K is 6.

Table 2. H (on the diagonal for SSRs), Pairwise F_{ST} comparisons and effective migration rates between groups based on SSR and SNP data.

| | Section A | Section D | Connemara | Carneddau | Highland |
|-------------|--------------|--------------|--------------|--------------|--------------|
| SSRs | | | | | |
| Section A | 2.013 | 3.27 | 4.42 | 5.44 | 3.55 |
| Section D | 0.0711 | 1.891 | 2.76 | 2.81 | 1.89 |
| Connemara | 0.0535 | 0.0831 | 2.150 | 2.68 | 3.26 |
| Carneddau | 0.0439 | 0.0817 | 0.0852 | 2.055 | 2.47 |
| Highland | 0.0658 | 0.1166 | 0.0711 | 0.0920 | 1.996 |
| SNPs | | | | | |
| Section A | * | 2.44 | 2.87 | 4.59 | 2.35 |
| Section D | 0.0931 | * | 2.69 | 2.47 | 2.18 |
| Connemara | 0.0802 | 0.0851 | * | 3.01 | 2.60 |
| Carneddau | 0.0517 | 0.0919 | 0.0766 | * | 2.47 |
| Highland | 0.0960 | 0.1030 | 0.0877 | 0.0918 | * |

Below diagonal: Pairwise F_{ST} based on number of different alleles. Above diagonal: effective number of migrants (N_m) per generation, based on Slatkin 1995. Theta H estimates $4N_e\mu = (1/(1-H_e)^2) - 1$. All F_{ST} values significant at $P < 0.01$.

Population structure and gene flow

The majority of genetic variation between domestic horses and ponies can be attributed to differences between

Table 3. MtDNA population average pairwise differences.

| | Section A | Section D | Carneddau | Connemara | Highland |
|-----------|-----------|-----------|-----------|-----------|-----------|
| Section A | 9.145 | 9.436 | 9.977*** | 8.712*** | 8.714** |
| Section D | -0.072 | 9.871 | 10.588*** | 8.874** | 9.027 |
| Carneddau | 1.540*** | 1.788*** | 7.729 | 10.810*** | 10.986*** |
| Connemara | 0.672*** | 0.471** | 3.478*** | 6.934 | 7.377* |
| Highland | 0.499** | 0.450** | 3.480*** | 0.268* | 7.284 |

Above diagonal: Average number of pairwise differences between populations (π_{XY}).

Diagonal elements: Average number of pairwise differences within population (π_X).

Below diagonal: Corrected average pairwise difference ($\pi_X - (\pi_X + \pi_Y)/2$).

Probability of result: * <0.05 , ** <0.01 , *** <0.001 .

individuals within breeds, where only approximately 8% of variation results from breed differences (Canon et al. 2000; and also found here in this study). However, despite evidence of admixture between these breeds, analysis of population structure using SSR data demonstrated that each population clustered individually according to distinct genotype at the optimal value of $K = 5$. A few Carneddau individuals showed approximately 25%, 50% and 75% assignment to the Section As suggesting recent admixture. This may have occurred if these animals were the first and second generation progeny of three Section A stallions released onto the mountain in 2007–2008, rather than originating from retained similarity to the Section A animals from a common ancestral population. Graziers released Section A stallions at this time to

POOR QUALITY COLOR FIG

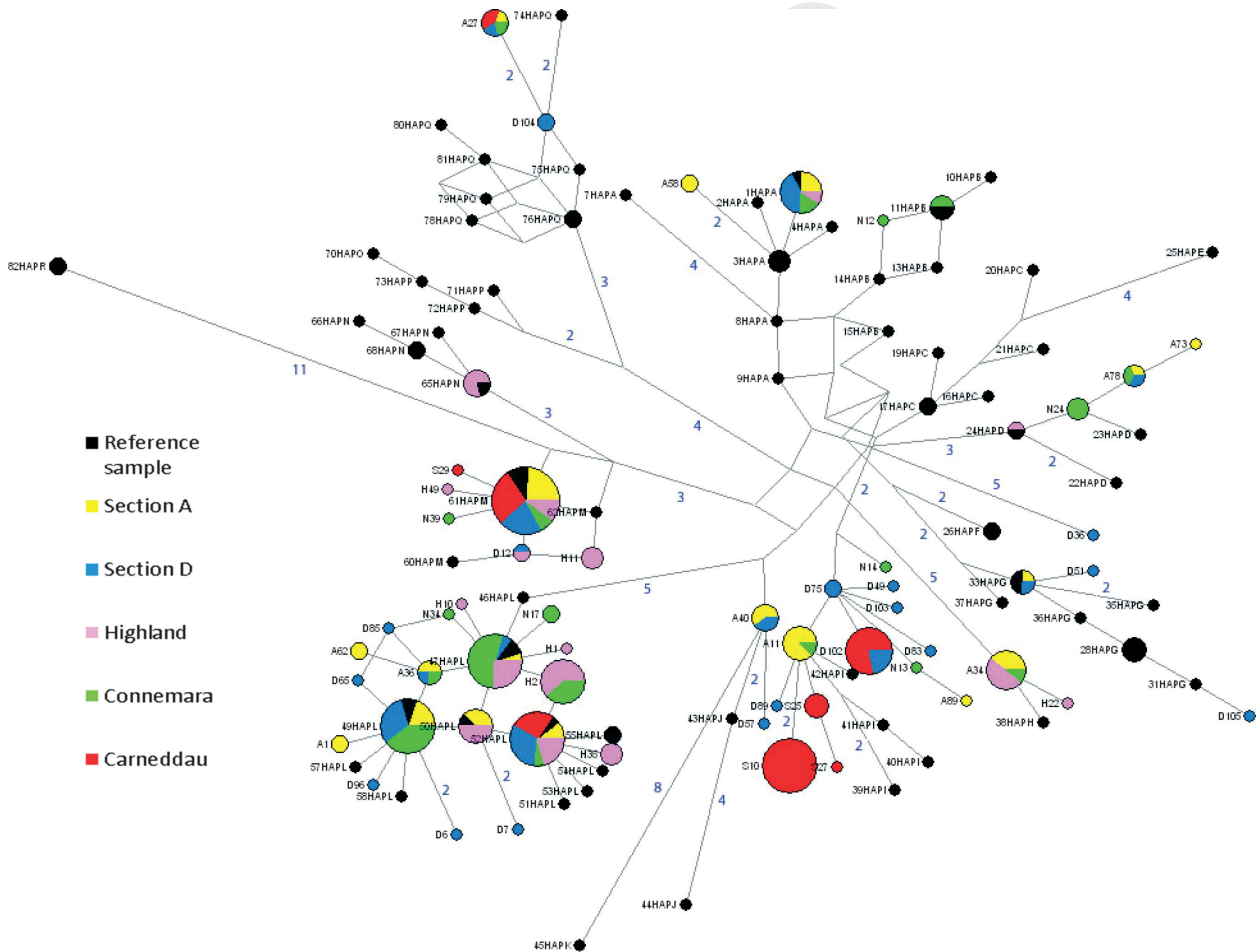


Figure 6. A median-joining network for mtDNA haplotypes based on shared allele frequencies. Node size represents overall haplotype frequency with pie charts within nodes showing frequency of that haplotype by population. Carneddau samples are displayed in red, Section A in yellow, Section D in blue, Connemara in green and Highland in pink. Reference samples representing examples of each of the major haplogroups identified by Achilli et al. (2012) are displayed in black and labelled according to sample number and haplogroup e.g. “1HAPa” etc. Numerical values indicate the number of nucleotide changes (>1 mutation) between primary nodes.

1 attempt to maintain diversity, because of concerns about
2 the small number of animals. Individuals S17 and S10 in
3 particular cluster with the Section A horses, but do not
4 show a raised level of identity by state with each other, as
5 would be expected if they were siblings by a single stallion.
6 Indeed, despite a lack of knowledge of relationships
7 prior to sampling, the Carneddau do not demonstrate a
8 higher degree of allele sharing than the individuals from
9 the other breeds where siblings had been excluded
10 (Fig. 3). The central position of both the Section A and
11 Carneddau animals in the plot of dimension 2 versus
12 dimension 3 (Fig. 4B) would be consistent with the
13 assumptions that these breeds have retained many ancestral
14 allele frequencies. In contrast the other groups have
15 diverged in different directions as a result of differential
16 selection and specialisation during breed formation. An
17 assessment of the SSR data revealed the presence of an
18 apparently private allele at locus HTG10 in the Carneddau
19 (unique alleles were also observed in the other groups).
20 The 97 bp allele was present in 26% of the Carneddau
21 samples, suggesting that lack of this allele in the other
22 groups is unlikely to be due to under-sampling. Several
23 mitochondrial haplotypes were found to be unique to the
24 Carneddau population, including one predominant within
25 this population. The diversity pattern of the Carneddau
26 showing restricted matriline combined with more comparable
27 levels of nuclear diversity is unusual. It is possible that
28 the recent admixture of the Section A stallions released
29 into a small population of isolated Carneddau mares could
30 also create the observed diversity patterns. However, the
31 significant differences in allele frequencies required to
32 generate the SNP plots, and unique mitochondrial and SSR
33 alleles present, suggests this is not the case, or at least
34 not the major cause. Therefore, the Carneddau have most
35 likely been isolated for a relatively long period, with
36 effective random breeding and low levels of variability
37 in reproductive success maintaining allelic diversity while
38 allowing change in allele frequencies. As such, the
39 Carneddau appear to be a distinct population that shares
40 an origin with the Welsh ponies in general and more
41 particularly with Section A animals. However, they have
42 been segregated for considerable time to have acquired
43 both allele frequency and mutational changes that are
44 unique and perhaps adapted to their mountain habitat.
45 The recent practice of introducing outside stallions
46 potentially dilutes this distinctive genetic signature.

48 Mitochondrial sequencing shows extensive sharing of
49 haplotypes across all the populations analysed here and
50 with those from other studies (Achilli et al. 2012; Prystupa
51 et al. 2012b). This is a likely legacy of extensive
52 crossbreeding occurring historically between populations
53 of horses and ponies prior to recognised “closed” breed

formation (Vila et al. 2001; McGahern et al. 2006a).
However, breed-specific haplogroups were also found
(such as haplogroup B in Connemaras and haplogroup N
in Highlands). The predominance of haplogroup I in the
Carneddau (76% of the population) associated with high
autosomal diversity is particularly noteworthy. This
haplogroup has not been found above a frequency of 20%
in other populations (Achilli et al. 2012) (Fig. 7). Similar
over-representation of a particular mitochondrial haplo-
group and reduction in haplotype diversity have been
demonstrated for the native Irish Kerry Bog Pony (30–
50% of population contain Achilli et al. (2012) haplo-
group D/Jansen et al. (2002) haplogroup E) where this
pattern was taken to indicate the archaic origin of the
breed (McGahern et al. 2006b; Prystupa et al. 2012b).
The Kerry Bog also shows relatively high nuclear diversity
($H_O = 0.770$, Prystupa et al. 2012a). However, the American
population of this breed demonstrated low F_{IS} values,
combined with the lack of any breed-specific clustering
following Bayesian analysis. This suggests an effect of
significant recent outbreeding and admixture with other
pony breeds (supported by historical records, McGahern
et al. 2006b). This situation contrasts with the largely
population-specific SSR clustering pattern of the Carneddau,
demonstrating that the recent introduction of Section
A stallions has not yet resulted in a predominant
signature of admixture.

For the mitochondrial data, the Carneddau are the only
group consistently significantly different from the other
populations, either due to long isolation, or perhaps
because they have not been exposed to the non-native
mares introduced to “improve” the other breeds. The
striking lack of distinction between the Section A and
Section D populations in matriline diversity suggests
that they originate from the same ancestral population
of mares. The alternative explanation of extensive
interchange of mares is excluded by the clear contrast
in the nuclear data for these breeds; the distinct
differentiation in the SSR and SNP plots in combination
with similarity in mtDNA implies extensive male-biased
line-breeding. The Highland and Connemara
matriline were consistently more closely related to
each other than to the Welsh ponies. This is again
in contrast to the SSR results and dimension 3 of the
SNP analysis, which suggest significant gene flow
between the Section As and Connemaras: nearly as
great as between the Section As and Carneddau.
Taken together these results suggest that the maternal
and paternal histories of the British upland breeds
studied here are quite distinct.

The division within the Section Ds may be consequent
on two historic breeding events and subsequent stud
management decisions. In the 12th Century Spanish
(Iberian) stallions were imported and used to breed with

| | A | B | C | D | E | F | G | H | I | J-K | L | M | N | O'P | Q | R |
|--------------------------|-------|-------|------|-------|------|------|-------|-------|-------|-------|-------|-------|------|-------|-------|------|
| Section A | 10.64 | | | 4.26 | | | 2.13 | 8.51 | 17.02 | 6.38 | 34.04 | 14.89 | | | 2.13 | |
| Section D | 10.87 | | | 4.35 | | | 6.52 | | 19.57 | 6.52 | 41.30 | 15.22 | | | 6.52 | |
| Highland | 2.22 | | | 2.22 | | | | 13.33 | | | 57.78 | 17.78 | 8.89 | | | |
| Connemara | 4.35 | 6.52 | | 8.70 | | | | | 2.17 | 6.52 | 63.04 | 6.52 | | | | 2.17 |
| Carneddau | | | | | | | | | | 76.09 | 10.87 | 17.39 | | | | 4.35 |
| European* | 4.49 | 9.38 | 0.32 | 4.57 | 0.48 | | 8.73 | 1.04 | 8.01 | 0.56 | 38.06 | 7.29 | 8.49 | 1.36 | 3.85 | 2.24 |
| Middle Eastern* | 7.81 | 10.94 | 3.13 | 2.08 | 0.52 | | 9.09 | 3.03 | 15.15 | | 24.24 | 3.03 | 3.03 | 9.09 | 9.09 | |
| Asian* | 11.93 | 1.7 | 3.58 | 2.9 | 2.21 | 3.07 | 16.35 | 1.36 | 6.13 | 6.47 | 13.46 | 4.09 | 2.9 | 6.64 | 13.8 | 1.87 |
| Ancient European* | 11.43 | | | 1.43 | | | 10 | 2.86 | 8.57 | 2.86 | 21.43 | 17.14 | 10 | 2.86 | 4.29 | 7.14 |
| Ancient Asian* | 8.82 | | | 10.29 | | 1.47 | 22.06 | 5.88 | 2.94 | 2.94 | 10.29 | 2.94 | 4.41 | 10.29 | 10.29 | 7.35 |

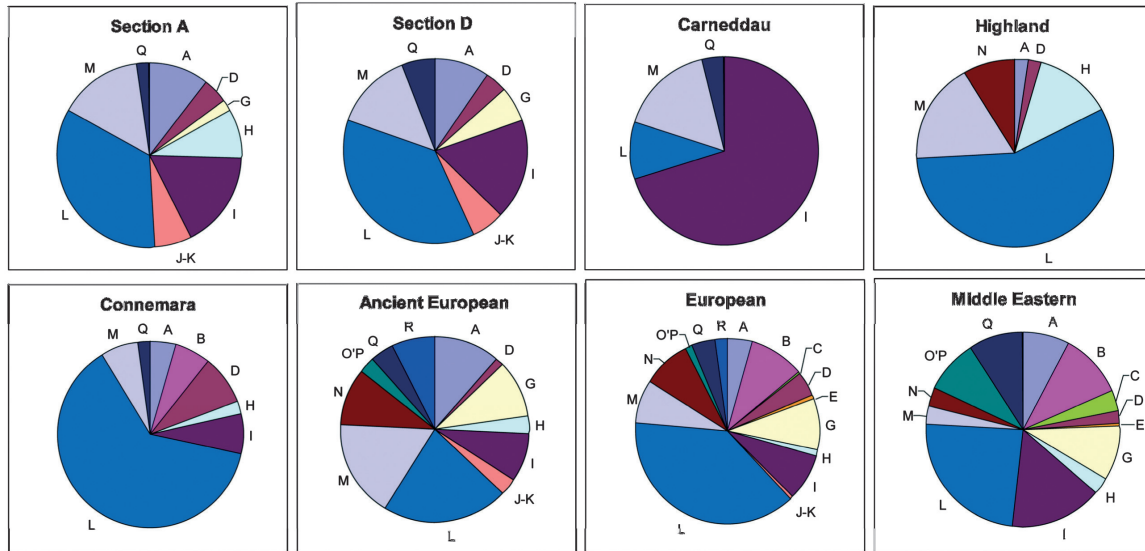


Figure 7. Haplogroup frequency (%) according to breed. The first row identifiers “A” to “R” represent the major worldwide mtDNA haplogroups in horses: haplogroup classification is based upon control region motifs, as described by Achilli et al. (2012). Geographic regions* are according to sequence data collated by Achilli et al. (2012) and comprises of 2401 reference sequences, including ancient (fossil) DNA data.

small mountain mares (Davies 1993, 1999). These crosses produced the larger “Powys Horse” which may form one of the founding types of the Section D ponies. In the 19th century livestock drovers brought fashionable trotters/Hackneys from the East of England to breed with local Welsh mares (Welsh Pony and Cob Society 2002). Regional studs within Wales may have acquired different levels of the incoming genes from these two periods and divergence in breeding target between studs maintained the genetic dichotomy within the Welsh Section Ds, even after the closure of the stud book.

Implications for conservation of Carneddau ponies

Although clearly related to the Section A pony, the Carneddau represent a genetically distinct population. For many populations of domestic livestock the definition of uniqueness is relatively subjective and differences between two populations may only be a function of the relative frequencies of a few genes, often associated with a single physical character or small group of specific characters (Henson 1992; Groenvelde et al. 2010). Any population

that is historically, geographically or reproductively isolated (has had little genetic influence from other breeds for a long time period) could be considered to be a unique population (Henson 1992). The Carneddau ponies may therefore represent an important source of uncharacterized genetic diversity and adaptations restricted to less intensively managed upland populations. In this study we have detected more than 10 times the number of SNP loci expected with extreme deviations from HW equilibrium after Bonferroni correction for the number of tested loci. These thereby form a potential set of markers for determining the genetic differentiation between the groups as a consequence of drift or selection, and as a future target for understanding adaptations to the upland environment. Specific adaptations have been found in upland and moorland populations of feral or free-living species previously, including parasite load tolerance (Coltman et al. 1999) and coat hair physiology (Wilson 2011). The importance of preserving feral/wild populations as valuable sources of genetic diversity for domestic relatives has been highlighted in other species (Schoen and Brown 1993; Giovambattista et al. 2001; Tapio et al. 2005; Taberlet et al. 2008; Medugorac et al. 2009). The value of hardy

1 native pony breeds for encouraging biodiversity and
2 repopulation of rare species has also been recognized
3 within conservation grazing schemes (Mitchell and Kirby
4 1990; Kohyani et al. 2008). The semi-feral Carneddau
5 ponies play a vital role within the mountain ecology of
6 the Snowdonia National Park, as they are involved in a
7 grazing scheme maintaining preferred habitat for an
8 endangered bird, the red-billed chough (*Pyrrhocorax pyr-
9 rhocorax*) (Murray 2006; Johnstone et al. 2010). Limited
10 funding was provided for the land owners to manage a
11 subgroup of ponies upon the open mountain; however
12 the project has now ended.

13 The Carneddau population is believed to be ≤ 300
14 individuals and at risk due to economic pressures on the
15 land they graze. A derogation has been granted so that
16 only ponies sold at auction have to be passported and mi-
17 crochipped, rather than requiring that all new-born Car-
18 neddaus are issued with a passport (in accordance with
19 UK law for other equids). However, because the ponies
20 are unregistered their sales value is very low (typically a
21 meat value), and so the expense of microchipping and
22 passporting is unviable for their owners. Animals which
23 are sold have limited use; they have no pedigree and are
24 not registered as a recognised breed, preventing them
25 from competing in many show classes. From the perspec-
26 tive of population conservation, the removal of young fil-
27 lies for auction may compound the issue of reduced
28 mitochondrial haplotype numbers. Other human selection
29 practices are minimal in these animals, making them a
30 particularly important group. In the New Forest, Dart-
31 moor and the Brecon Beacons, specially selected individ-
32 ual stallions are released every year into known, limited
33 geographical areas frequented by free-living mares and re-
34 captured at the end of the breeding season. With the
35 exception of three stallions released (but only one since
36 removed) in 2007–2008, this does not occur with the Car-
37 neddau population.

38 It is widely acknowledged that domestic pony and
39 horse breeds have undergone varying degrees of admix-
40 ture and gene flow prior to establishment of defined,
41 “closed” pedigrees (Thirstrup et al. 2008; Groenveld et al.
42 2010; Prystupa et al. 2012a). This is clearly evident in the
43 genetic data presented here, for all of the populations
44 considered. However, in terms of *relative* levels of admix-
45 ture, the Carneddau appear to show similar degrees to
46 that found between the recognised breeds included in this
47 analysis. In addition to this, the distinct genetic signatures
48 (mtDNA haplotypes, SSR frequencies, potential SNP
49 adaptations) suggest that the Carneddau ponies are a
50 defined, long-established population worthy of conserva-
51 tion. However, whilst the WPCS register Section A ponies
52 that are free-living on the mountains of south Wales, this
53 semi-feral grouping does not include the Carneddau.

Consequently, the Carneddau are excluded from current
Rare Breeds Survival Trust conservation management
schemes and are not considered for use within nationwide
conservation grazing projects utilising ponies such as
Dartmoor, Exmoor and imported Polish Koniks. The cur-
rent lack of recognition surrounding the Carneddau
ponies contributes to the risk of losing a potentially use-
ful, and distinct, native British breed.

Conclusions

The genetic relationship of Carneddau ponies in relation
to other native breeds of the UK and Ireland was revealed
by the integrated results of each genetic analysis. The
Carneddau ponies have a shared ancestry with the recog-
nized Section A pony yet have remained in relative isola-
tion, subjected to natural selective pressures for a
significant period of time. This has been followed by
more recent genetic introgression by Section A stallions
and round up practices. The genetic impact of human
activities, particularly the selective use of stallion crossing
upon the pony breeds, are evident in the comparison of
the different genetic systems; both more recently in the
effect upon SSR frequencies within the Carneddau and
historically in the SNP profile of the Section D cobs.
Despite the recent management practices, the Carneddau
ponies maintain a distinct genetic signature that should
be conserved. Given the restricted geographic distribution
and the low numbers of the Carneddau, in order to pro-
vide some conservation protection measures, these ani-
mals should be classified as a rare pony population at
critical extinction status. This study illustrates the rele-
vance and potential power of combining autosomal and
maternally transmitted polymorphic assays to unravel
genetic structure and relatedness in feral and intensively
managed populations. Herein we provide new informa-
tion and knowledge to support conservation strategies of
endangered breeds.

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within this manuscript.

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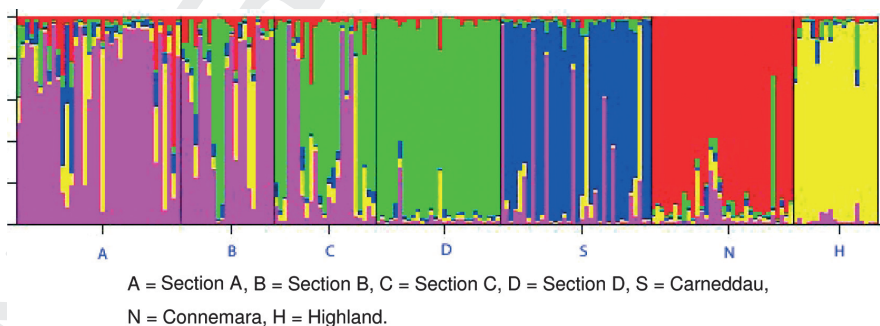
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Appendix

Figure A1. The results of STRUCTURE analysis for SSR loci of each population (including admixed Section B and Section C) for $K = 5$. Populations are labelled as follows: A = Section A, B = Section B, C = Section C, D = Section D, S = Carneddau, N = Connemara and H = Highland.



Graphical Abstract

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Herein, we describe the use of microsatellites and SNPs together with analysis of the mitochondrial control region to quantify the extent and magnitude of genetic diversity present in the feral Carneddau pony and relate this to several recognised British and Irish pony breeds. Our results establish that the feral Carneddau ponies represent a unique and distinctive population that merits recognition as a defined population and conservation priority. We discuss the implications for conservation of this population as a unique pool of genetic diversity adapted to the British uplands and potentially of particular value in maintaining the biodiversity of these habitats.

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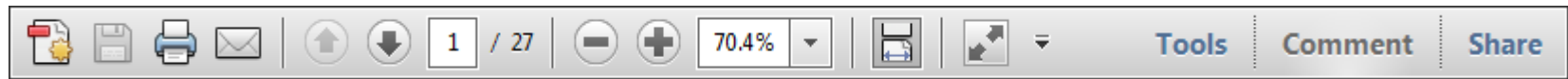
| Query reference | Query | Remarks |
|-----------------|---|---------|
| 1 | AUTHOR: Please give manufacturer information for this GeneScan™-500LIZ: company name, town, state (if USA), and country. | |
| 2 | AUTHOR: Please provide the page range for reference Henson (1992). | |
| 3 | AUTHOR: Please provide the page range for reference Leroy et al. (2009). | |
| 4 | AUTHOR: Please provide the publisher location for reference Lush and Crow (1994). | |
| 5 | AUTHOR: If there are fewer than 7 authors for all et al references, please supply all of their names. If there are 7 or more authors, please supply the first 6 author names then et al. Please check and update all such references found in the list. | |
| 6 | AUTHOR: Figure 6 is of poor quality. Please check required artwork specifications at http://authorservices.wiley.com/bauthor/illustration.asp | |
| 7 | AUTHOR: Figure 7 is of poor quality. Please check required artwork specifications at http://authorservices.wiley.com/bauthor/illustration.asp | |
| 8 | AUTHOR: Figure A1 is of poor quality. Please check required artwork specifications at http://authorservices.wiley.com/bauthor/illustration.asp | |

USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

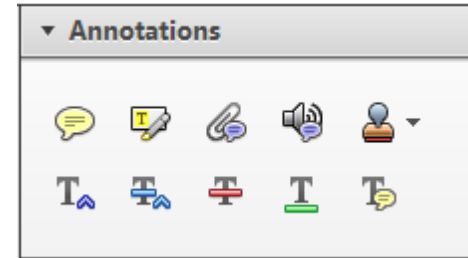
Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: <http://get.adobe.com/uk/reader/>

Once you have Acrobat Reader open on your computer, click on the [Comment](#) tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the [Annotations](#) section, pictured opposite. We've picked out some of these tools below:



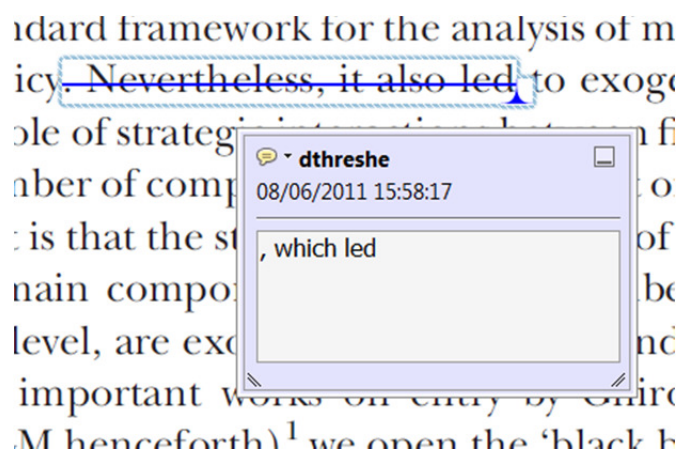
1. Replace (Ins) Tool – for replacing text.



Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the [Replace \(Ins\)](#) icon in the Annotations section.
- Type the replacement text into the blue box that appears.



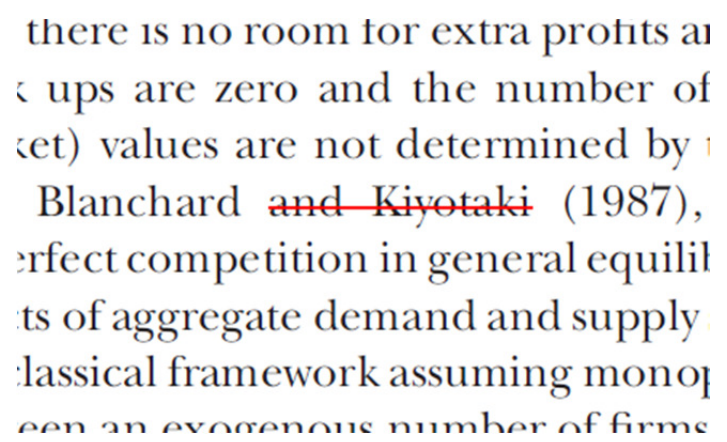
2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.



3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

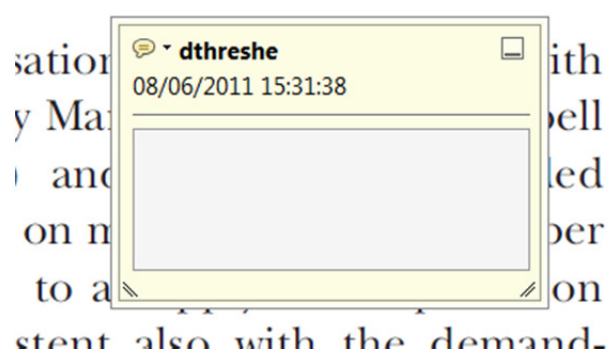


Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

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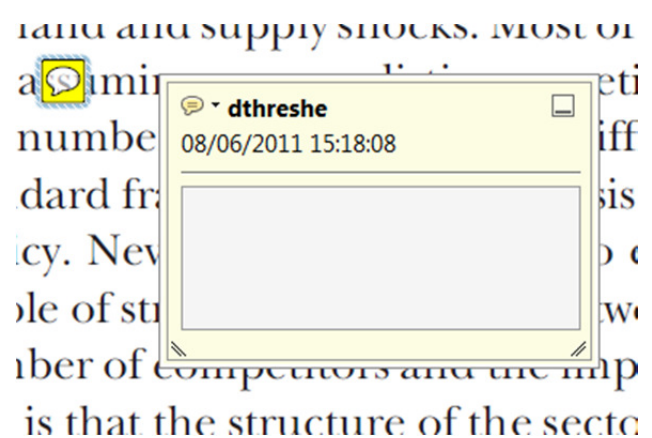
4. Add sticky note Tool – for making notes at specific points in the text.



Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the [Add sticky note](#) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

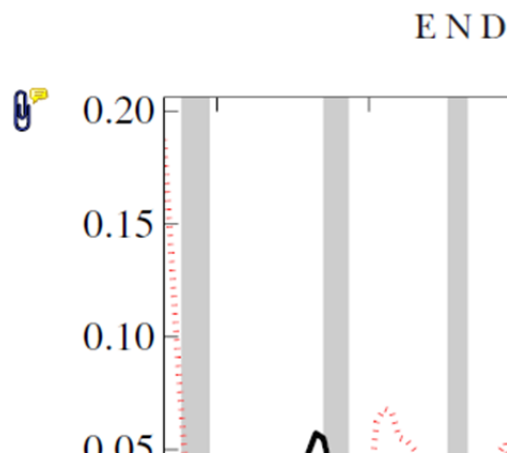
5. Attach File Tool – for inserting large amounts of text or replacement figures.



Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it

- Click on the [Attach File](#) icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



6. Add stamp Tool – for approving a proof if no corrections are required.

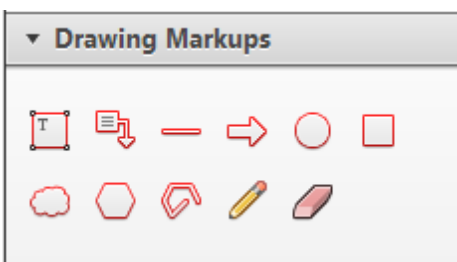


Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the [Add stamp](#) icon in the Annotations section.
- Select the stamp you want to use. (The [Approved](#) stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

of the business cycle, starting with the
 on perfect competition, constant return
 production. In this environment goods
 extra profits and the market
 he market. The New-Key
 otaki (1987), has introduced product
 general equilibrium models with nomin
 ed and supply shocks. Most of this literat

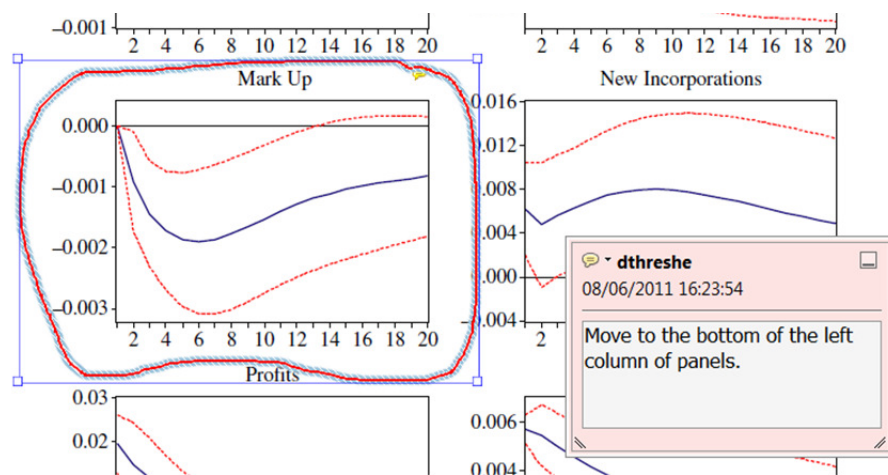


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the [Drawing Markups](#) section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the [Help](#) menu to reveal a list of further options:

