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The paradox of invasion: Reeves' muntjac deer invade the British Isles from a limited number of founding females

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Abstract

High levels of genetic diversity and high propagule pressure are favoured by conservation biologists as the basis for successful re-introductions and ensuring the persistence of populations. However, invasion ecologists recognise the "paradox of invasion", as successful species introductions may often be characterised by limited numbers of individuals and associated genetic bottlenecks. In the present study, we used a combination of high-resolution nuclear and mitochondrial genetic markers to investigate the invasion history of Reeves' muntjac deer in the British Isles. This invasion has caused severe economic and ecological damage, with secondary spread currently a concern throughout Europe and potentially globally. Microsatellite analysis based on eight loci grouped all 176 introduced individuals studied from across the species' range in the UK into one genetic cluster, and seven mitochondrial D-loop haplotypes were recovered, two of which were present at very low frequency and were related to more common haplotypes. Our results indicate that the entire invasion can be traced to a single founding event involving a low number of females. These findings highlight the fact that even small releases of species may, if ignored, result in irreversible and costly invasion, regardless of initial genetic diversity or continual genetic influx.

Introduction

Conservation biologists have long appreciated the importance of high levels of genetic diversity and high propagule pressure as the basis for the successful introduction and enhanced persistence of populations (Frankham et al. 2002). The parallel case of species invasions, however, is often characterised by limited numbers of individuals and associated genetic bottlenecks, a so-called "paradox of invasion" (Sax & Brown 2000; Dlugosch & Parker 2008a). Although there are several well-known cases of invasive species that exhibit extremely low levels of genetic variation (Hollingsworth & Bailey 2000), many successful invasions have been facilitated by multiple introductions, resulting in high levels of genetic diversity in the invasive range (Lockwood et al. 2005, 2009; Lavergne & Molofsky 2007; Roman & Darling 2007; Simberloff 2009). In particular, we need to know if particularly damaging species are likely to establish and spread from small founder events or if high propagule pressure is required (Sax & Brown 2000; Lockwood et al. 2005, 2009; Simberloff 2009). The use of genetic studies to gain insight into various aspects of the invasion process, such as the mode and frequency of introduction, can help predict the potential likelihood and impacts of further invasion (e.g. Provan et al. 2008; Xavier et al. 2009) and also offer information helpful for management and control (Allendorf & Lundquist 2003).

The impacts of invasive species are of global concern (Lowe et al. 2000; Simberloff et al. 2013). Introduced deer species represent one such problem, with more than a quarter of deer species having been introduced outside of their native range (Dolman & Waeber 2008). Over-abundant deer negatively impact biodiversity (Cote et al. 2004), commercial land use (Putman & Moore1998), and human health and well-being through potentially fatal deer-vehicle collisions (Bruiderink & Hazebroek 1996). The arrival of non-native deer species can impose ecological pressure on woodland ecosystems, with new species moving into

unoccupied niches detrimentally impacting native flora and fauna (White et al. 2008). With deer introductions and secondary spread continuing, such as the arrival and establishment of muntjac throughout Ireland (Dick et al. 2010, 2012), knowledge of invasion history is critical to assess risks of future ecological and economic damage through population expansion.

Outside of their native range of south-east China and Taiwan, Reeves's Chinese muntjac (*Muntiacus reevesi*, Ogilby, 1839) have been introduced to France, Japan and the British Isles, though only the latter two countries now have established populations (Lever 2009; Ohdachi et al. 2009). The post-introduction natural range expansion of muntjac in the British Isles has been around 1km per year, similar to other introduced deer (Chapman et al. 1994). However, their full range expansion has been supplemented by secondary innocula via human-mediated dispersal (Smith-Jones 2004). Their invasive success is further facilitated by year-round breeding, rapid reproductive maturity (Chapman et al. 1997), and an ability to inhabit anthropogenically modified habitats (Dansie, 1983). Indeed, the Game and Wildlife Conservancy Trust reported a 1,756% increase in the numbers of muntjac shot between 1961-2009 (Aebische et al, 2011), with the national population estimated very conservatively at 52,250 individuals by 2008 (Harris and Yalden, 2008). This rapid dispersal and increase in abundance in such a relatively short time has resulted in considerable ecological and economic damage (Cooke & Farrell 2001; Dolman and Waeber 2008; Mayle 2002). Browsing and grazing pressure by muntjac has had major impacts on woodland ground flora diversity and tree regeneration (Joys et al. 2004) and, as a result, there have been cascade effects on other taxa, including rare butterflies such as the heath fritillary (*Melitaea athalia)* and wood white (*Leptidea sinapis*) (Tabor 1998). The removal of ground cover by muntjac reduces nesting sites for woodland songbirds (Holt et al. 2011) and is also thought to be responsible for the reduction in woodland small mammal populations (Flowerdew & Elwood 2001).

Historical information on the sourcing, supply and release of muntjac in the British Isles is highly confusing (Chapman et al, 1994). Records indicate that a pair of Reeves' muntjac were presented to the Zoological Society of London in 1838 by John Russell Reeves, possibly sourced from Guangdong (Canton) Province, South China. In 1867, the Zoo purchased a replacement male obtained by Robert Swinhoe, possibly sourced from Formosa (also known as Chinese Taipei, or more commonly now, Taiwan, which has its own subspecies known as Taiwanese Reeves's muntjac *Muntiacus reevesi micrurus*). The female died shortly after giving birth and was replaced in 1873 with a female from Ningpo. The species is known to have bred at the London Zoo during this period. In November 1874, another pair from Formosa (also known as Chinese Taipei, or more commonly now, Taiwan, which has its own subspecies known as Taiwanese Reeves's muntjac *Muntiacus reevesi micrurus*) were presented to the Zoological Society of London and, in the same year, a further female was sent from Ningpo and a male from Hong Kong, with the parents of the latter believed to have originated from Formosa (Sclater, 1875). Out of the 14 births that were recorded at London Zoo between 1874 and 1881, nine were sold to Tring Park, England, and Jardin des Plants, France, amongst other possible locations and between 1890 and 1928 no records of muntjac in London Zoo exist (Chapman et al 1994).

Woburn Abbey had an initial import of three pairs of muntjac from a dealer on three separate occasions in 1893 (Chapman et al. 1994). These individuals may have come direct from wild caught animals in China, but it is likely that they were captive-bred individuals, as, by this time, at least six other collections were known to keep and trade muntjac in Europe, including Rambouillet, France (Dansie, 1983) and Berlin Zoo, Germany (Chapman et al. 1994). The 11th Duke of Bedford was also well known for travelling around Europe collecting deer for his park in Woburn Abbey (Dansie, 1983). What is known is that he received a male and two females, most likely of the London Zoo descendents, from Jardin

94 des Plants in 1902 (Chapman et al 1994). In total between 1894 and 1906, 13 males and 15
95 females were brought into Woburn Park and records show that eleven individuals were
96 released from there in 1901 (Chapman 1993).
97 Until 2009, it was widely assumed that muntjac had not reached Scotland or Ireland
98 (Lever 2009). Recent deer surveys, however, suggest that muntjac have spread westward into
99 Wales and south-west England as well as northward up the eastern half of England to the
100 Scottish Borders (Ward et al. 2008), and most recently have been discovered in Ireland
101 (Hailstone 2012). The first confirmed sighting of muntjac in the wild in the Republic of
102 Ireland was a culled animal in Co. Wicklow (Carden et al. 2011), swiftly followed by a
103 carcass from a deer-vehicle collision in Co. Down, Northern Ireland (Dick et al. 2010). The
104 question remains, however, as to the size of the actual founding 'propagule' that led to this
105 dramatic and continuing invasion of the British Isles. Such information is important to help
106 undertstand and predict invasion success in general and with regards to the likelihood of
107 further invasions by non-native deer globally.
108 In the only population genetic analysis of muntjac in Britain carried out to date, it was
109 suggested that there were at least eight maternal lineages, and most likely more, based on
110 restriction fragment length polymorphism (RFLP) analysis of the mitochondrial genome
111 (Williams et al. 1993). However, mitochondrial markers only give an indication of female-
112 mediated gene flow, and analysis of high-resolution nuclear microsatellite markers offers a
113 more complete picture of the invasion history of a species (Guillemaud et al. 2010).
114 Consequently, in the present study we carried out a combined analysis using microsatellites
115 and sequencing of the mitochondrial D-loop region to resolve the invasion history of the
116 species in the British Isles, specifically the likely number of founding females.

Materials and methods

Sampling and DNA extraction

Tissue samples were collected from 176 muntjac across the majority of their known distribution in the British Isles (Figure 1; Table 1; Appendix 1). Samples were collected as part of ongoing control programme by deer managers from the British Deer Society (BDS). Tissue samples, mostly tongue or ear clippings (1 cm^3), were collected and stored in absolute ethanol. In addition, five DNA samples of the Taiwanese subspecies *Muntiacus reevesi micrurus* were obtained directly from Taiwan. DNA was extracted from tissue samples using a high salt extraction technique (Aljanabi & Martinez 1997).

Genotyping

All samples were genotyped for eight microsatellite loci originally developed for *M. crinifrons* (Wu et al. 2008): Mcr-3, Mcr-4, Mcr-5, Mcr-6, Mcr-7, Mcr-13, Mcr-14 and Mcr-19. The remaining three loci reported by Wu et al. (2008) could not be amplified reliably. PCR was carried out in a total volume of 10 µl containing 100 ng genomic DNA, 5 pmol of 6-FAM-labelled M13 primer, 0.5 pmol of M13-tailed forward primer, 5 pmol reverse primer, 1x PCR reaction buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.25 U GoTaq Flexi DNA polymerase (Promega, Sunnyvale, CA, USA). PCR was carried out on a MWG Primus thermal cycler (Ebersberg, Germany) using the following conditions: initial denaturation at 94 ^oC for 3 min followed by 40 cycles of denaturation at 94 ^oC for 30 s, annealing at 55 ^oC for 30 s, extension at 72 ^oC for 30 s and a final extension at 72 ^oC for 5 min. Genotyping was carried out on an AB3730xl capillary genotyping system. (Life Technologies; Carlsbad, California, USA). Allele sizes were scored using the GENEMAPPER software package (v4.1;

Applied Biosystems) using LIZ-500 size standards, and were checked by comparison with previously sized control samples. All chromatograms were inspected visually.

Mitochondrial D-loop sequencing

The complete mitochondrial D-loop region was amplified using a pair of primers designed from the complete mitochondrial genome of *M. reevesi* (GenBank accession number AF527537): trn-Pro-F 5'-TCAACACCCAAAGCTGAAGTT-3 and trn-Phe-R 5'-TCAGTGCCTTGCTTTATTGC-3. PCR was carried out in a total volume of 20 µl containing 200 ng genomic DNA, 10 pmol of each primer, 1x PCR reaction buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.5 U GoTaq Flexi DNA polymerase (Promega, Sunnyvale, CA). PCR was carried out on a MWG Primus thermal cycler (Ebersberg, Germany) using the following parameters: initial denaturation at 94 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. Five µl PCR product were resolved on 1.5% agarose gels and visualised by ethidium bromide staining, and the remaining 15 µl were EXO-SAP purified and sequenced in both direction using the BigDye sequencing kit (V3.1; Applied Biosystems) using the primers Munt-DLOOP-IN-F 5'-ATCCTTGTCAACATGCGTATC-3' and Munt-DLOOP-IN-R 5'-TTATGTGTGAGCATGGGCTG-3' and run on an AB 3730XL DNA analyser (Life Technologies; Carlsbad, California, USA).

Data analysis

GENEPOP (V3.4; Raymond and Rousset, 1995) was used to test for linkage disequilibrium between nuclear microsatellite loci. MICRO-CHECKER (van Oosterhout et al. 2004) was used to check for the possible occurrence of null alleles. To estimate genetic diversity within populations containing six or more individuals (Table 1), levels of observed (H_O) and

166 expected (H_E) heterozygosity, levels of allelic richness (A_R) and fixation indices (F_{IS}) were
167 calculated using the FSTAT software package (V2.9.3.2; Goudet, 2001). Significance of F_{IS}
168 was determined by 10,000 randomisation steps. Levels of genetic diversity (H) based on
169 mtDNA D-loop haplotype frequencies were calculated using the ARLEQUIN software package
170 (V3.5.1.2; Excoffier and Lischer, 2010).

171 The overall level of genetic differentiation between populations was estimated using Φ_{ST},
172 which gives an analogue of F_{ST} (Weir and Cockerham, 1984) calculated within the analysis
173 of molecular variance (AMOVA) framework (Excoffier et al. 1992) using ARLEQUIN. To
174 further identify possible patterns of genetic structuring, the software package BAPS (V5;
175 Corander et al. 2003) was used to identify clusters of genetically similar populations based on
176 the complete microsatellite data set (181 individuals) using a Bayesian approach. Ten
177 replicates were run for all possible values of the maximum number of clusters (K) up to K =
178 40, with a burn-in period of 10,000 iterations followed by 100,000 iterations. Multiple
179 independent runs always gave the same outcome. To further identify possible spatial patterns
180 of gene flow, a principal coordinate analysis (PCA) was carried out on the population-level
181 data set (105 individuals; Table 1) in GENALEX (V6.1; Peakall & Smouse 2006). Inter-
182 individual genetic distances were calculated as described in Smouse & Peakall 1999, and the
183 PCA was carried out using the standard covariance approach.

184 To test for the occurrence of a genetic bottleneck, the Wilcoxon test for heterozygote
185 excess was performed under the infinite alleles model (IAM), the stepwise mutation model
186 (SMM) and a two-phase model (TPM) incorporating 90% single-stepwise mutations using
187 the program BOTTLENECK (V1.2; Piry *et al*. 1999). The Wilcoxon test was used as it is
188 recommended for less than 20 microsatellite loci.

Results

No evidence of linkage disequilibrium was detected between any of the eight nuclear microsatellites analysed. Between eight (Mcr-3) and 16 (Mcr-5) alleles were detected per locus, with a total of 98 (mean = 12.25 per locus). Within populations for which a minimum of six individuals were sampled, levels of allelic richness (A_R) averaged over loci ranged from 3.723 (Kineton) to 5.120 (Sandlings), with a mean value of 4.136 (Table 2). Levels of observed (H_O) and expected (H_E) heterozygosity ranged from 0.450 (Welford) to 0.663 (Wytham Wood; mean = 0.558), and from 0.675 (Kineton) to 0.855 (Sandlings; mean = 0.755) respectively. The heterozygote deficit observed in the majority of the populations gave rise to F_{IS} values which were significantly different from zero in all of the populations studied, ranging from 0.140 (Wytham Wood) to 0.420 (Welford; mean = 0.270), which is consistent with the departure from Hardy-Weinberg equilibrium generally associated with invasive species. MICRO-CHECKER indicated the possibility of null alleles at four of the eight loci (Mcr-5, Mcr-7, Mcr-13 and Mcr-14), which could at least in part explain this heterozygosity deficit.

Complete D-loop sequences were obtained for 121 individuals, with an alignment length of 815 bp. A total of 23 substitution mutations gave rise to seven haplotypes (Figure 2). No indels were observed, which can often make the alignment of D-loop sequences difficult. There was a notable east-west cline in the frequency of haplotypes (Figure 1). Haplotype diversity values for populations for which a minimum of six individuals were sampled ranged from zero (Ickworth Park, for which only two complete sequences were obtained) to 0.800 (Sennowe Park; mean = 0.430). As the mitochondrial D-loop region exhibits an extremely fast mutation rate, and thus provides a high-resolution marker for female lineages, since the mitochondrial genome is maternally inherited in mammals (Harrison 1989), the results

indicate a low number of founding females. Two of the seven haplotypes occurred at very low frequency. All seven haplotypes were split into four very divergent groups, each separated by at least seven mutations. It is possible that Haplotype H6 evolved from Haplotype H2 after the introduction, since the two differ by a single mutation, although it is difficult to be more certain in the absence of an accurate mutation rate for this region. Likewise, Haplotype H7 is only found in a single individual and is one mutation removed from Haplotype H5, suggesting very recent divergence.

The overall level of differentiation estimated by nuclear microsatellites was low (Φ_{ST} = 0.050, $P < 0.001$), whilst the level based on mitochondrial D-loop sequences was much higher ($\Phi_{ST} = 0.470$, $P < 0.001$; Table 2). The BAPS analysis indicated that all the individuals analysed from Britain and Ireland were grouped into a single genetic cluster (100% probability), separate from the Taiwanese subspecies *Muntiacus reevesi*. This was reflected in the PCA, which showed no evidence of geographical structuring of individual multilocus genotypes (Figure 3). Finally, the Wilcoxon test for heterozygote excess suggested a bottleneck under the IAM ($P = 0.006$), but not under the SMM or the TPM, although it should be borne in mind that the number of loci used (eight) may be insufficient to detect the latter (see Discussion).

Discussion

The probability of invasion success generally increases with propagule pressure, in terms of a high number of viable founding individuals and repeated introductions (Simberloff 2009). However, we also know that some invasions appear to establish from small founding events, including the well-known case of the green seaweed *Caulerpa taxifolia*, which spread throughout the Mediterranean rapidly following a single aquarium release (Jousson et al. 1998), and the suggestion that a single pair of squirrels would have a greater than 50% chance of establishing a new population (Bertolino 2009). However, the invasion history of many alien species is unknown. Resolving the size of founding propagules of major invasions could help predict future invasions. Here, the use of high-resolution microsatellite markers suggested that the invasion of the British Isles by Chinese Reeve's muntjac resulted from a very small founding population. Indeed, our analysis is not only consistent with the known introduction history of muntjac, but implies, through combined nuclear and mtDNA data, that the current population was founded by a single group including a low number of females. From Chapman et al. (1994), it seems probable to suggest those females are descendents of some of the original five females imported to London Zoo amongst others imported elsewhere.

The distribution of mtDNA haplotypes across southeast England is consistent with separate escapes and releases from Woburn and other captive collections, since Haplotypes H2 and H3, represented in yellow and blue respectively, tend to be primarily found west of Woburn, which lies just northwest of Site 4 in Figure 1, and indeed are the only two haplotypes found in this area, consistent with an extreme maternal founder effect. Likewise, Haplotypes H1 and H4, shown in red and green, are not found west of Woburn, and are the dominant haplotypes in sites to the east. The only previous genetic study on muntjac in the

256 UK suggested “at least eight maternal lineages in the UK” (Williams et al. 1993). This was
257 based on RFLP analysis of the same D-loop region analysed in the present study, but this
258 approach, unlike the sequencing analysis carried out here, cannot elucidate the genetic
259 relationships between haplotypes. Three of the eight haplotypes detected by Williams et al.
260 (1993) occurred at frequencies of 1.7%, 0.5% and 0.25% and could, as in the present study,
261 represent recent, post-introduction mutations. However, the lack of information on the
262 genealogical relationships between the RFLP haplotypes meant that this aspect could not be
263 addressed in the earlier study, but their identification of four or five haplotypes at relatively
264 high frequency (5% or above) is consistent with our findings, particularly when considering
265 that they analysed over three times as many samples. It is difficult to quantify accurately the
266 number of females involved in the introduction, since many of the original mitochondrial
267 lineages may have become extinct since the original founding event, but both studies indicate
268 a low number of individuals. The additional use of high-resolution microsatellite markers in
269 the present study suggests a single introduction of muntjac into the Britain Isles, since levels
270 of genetic differentiation were very low. As invasion events, single or multiple, are generally
271 characterised by founder effects, the random nature of these episodes means that multiple
272 events tend to involve separate gene pools from the original source population (Provan et al.
273 2005). This is contrary to the assignment of all individuals sampled from Britain and Ireland
274 in the present study to a single genetic cluster in the BAPS analysis, and the lack of any
275 geographical structuring in the PCA. The results of the BOTTLENECK analysis were
276 somewhat inconclusive, with a genetic bottleneck suggested under the IAM but not under the
277 other two models. This could be due to the fact that the two models that assume stepwise
278 mutation, particularly the SMM, are more conservative than the IAM (Cornuet and Luikart
279 1996; Luikart and Cornuet 1998). Alternatively, it may be that the short generation time of
280 muntjac, which can start breeding at around 36 weeks (Chapman et al. 1997), means that

281 sufficient generations have passed since introduction to mask the signature of any genetic
282 bottleneck.
283 Our findings highlight the risk of directly equating propagule pressure with the success of
284 an invasion, since, despite a potentially high number of released individuals across multiple
285 sites, the data here show that the invasion is descended from the same import source which
286 consisted of a limited number of founders. Given that invasive deer species have been found
287 to have earlier weaning and sexual maturity age (Fautley et al. 2012), the success of muntjac
288 invasion undoubtedly appears to be due their high fecundity and rate of increase in the initial
289 stages, as well as secondary introductions at multiple locations over a sustained period of
290 time. However, instead of offering a chance for an increase in genetic variation, due to the
291 restricted original gene pool, the sole advantage of these multiple release sites appears to be
292 demographic, by offering additional mates if a nearby population begins to fail. Invasion
293 success, in spite of population bottlenecks, has previously been reported in species once kept
294 as exotic pets or decorative plants (Le Page et al. 2000; Goodman et al. 2001; Dlugosch &
295 Parker 2008b). It is possible that human-mediated species introductions such as those from
296 ornamental and/or pet species have an increased chance of invasion due to *ex-situ* breeding,
297 despite low genetic diversity and limited primary introductions from the native range.
298 More optimistically, this finding also has implications for captive management of
299 conservation reintroductions. Though low genetic diversity is not considered ideal in species
300 reintroduction programmes (Frankham et al. 2002), this study supports the idea that a
301 successful introduction can result from a low number of individuals with limited genetic
302 variation. Many examples of successful reintroductions from low number of founding
303 individuals exist. Taylor et al. (2005) found that the number of released New Zealand
304 saddlebacks (*Philesturnus carunculatus*) and robins (*Petroica australis*) on different islands
305 did not affect the success of introductions. The alpine ibex (*Capra ibex ibex*), carefully bred

306 in captivity from a very low number of founding individuals, was reintroduced successfully
307 on several occasions (Stüwe & Nievergelt 1991). Most notably, the mílù (*Elaphurus*
308 *davidianus*), also known as Père David's deer, was successfully reintroduced to China having
309 been rescued from just two females and a single male after careful captive breeding by the
310 11th Duke of Bedford (Zeng et al. 2007). In all cases, success was based on a combination of
311 selective breeding to increase the effective captive population size and multiple releases.
312 Conversely, our paper offers an insight for legislative policy in invasive species
313 management. Our data are consistent with four or five females leading to a major geographic
314 invasive species problem. This should serve as a warning for future muntjac invasions, such
315 as those unfolding in Ireland currently (Dick et al. 2010; Freeman et al. 2011; Dick et al.
316 2012; Hogg et al. 2014) and a suspected introduction in Belgium (T. Adrianens, pers.
317 comm.). It appears quite plausible that, in both cases, muntjac could colonise a large area,
318 giving rise to large numbers of individuals, from relatively few founding individuals. We
319 raise the point to caution against complacency if invasive species such as muntjac are
320 suspected in a new area and we would advocate heightened biosecurity and a need to react to
321 sightings rapidly (Caffrey et al. 2014). Indeed, under recent EU legislation, inclusion of
322 muntjac on the list of high-risk species would restrict the movement and release of the
323 species (Genovesi et al. 2014). The present study is an example of the value of genetics to
324 invasion ecology, and also helps to illuminate the origin of muntjac deer populations in the
325 British Isles. Vigilance should be exercised even with small releases of species such as
326 muntjac, which may, if ignored, result in an irreversible invasion, regardless of initial genetic
327 diversity or continuous genetic influx.

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Table 1 Details of populations studied where six or more individuals were analyzed with nuclear microsatellites. N – number of individuals analysed; A_R – allelic richness; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient; H1-H7 – frequency of mitochondrial haplotypes; H – haplotype diversity.

No	Name	Lat	Long	Nuclear microsatellites					Mitochondrial D-loop								
		(N)	(E)	*N*	A_R	H_O	H_E	F_{IS}	*N*	H1	H2	H3	H4	H5	H6	H7	*H*
1	Kineton, Warwickshire	52.136	-1.470	12	3.723	0.563	0.675	0.173**	12	-	11	1	-	-	-	-	0.167
2	Welford, Berkshire	51.478	-1.407	6	3.933	0.450	0.746	0.420***	4	-	2	2	-	-	-	-	0.667
3	Wytham Wood, Oxfordshire	51.769	-1.334	10	4.099	0.663	0.764	0.140*	10	-	7	3	-	-	-	-	0.467
4	Hexton, Bedfordshire	51.938	0.359	8	4.056	0.627	0.778	0.205**	8	-	-	1	7	-	-	-	0.250
5	Ickworth Park, Suffolk	52.218	0.649	6	4.223	0.583	0.753	0.242**	2	-	-	-	2	-	-	-	-
6	Stanta, Suffolk	52.494	0.720	9	3.768	0.500	0.700	0.298***	8	5	-	-	-	3	-	-	0.536
7	Shadwell Estate, Norfolk	53.294	0.837	14	3.826	0.537	0.718	0.259***	11	9	-	1	1	-	-	-	0.346
8	Sennowe Park, Norfolk	52.773	0.912	14	4.218	0.499	0.774	0.364***	6	2	2	2	-	-	-	-	0.800
9	Sandlings, Suffolk	52.097	1.426	12	5.120	0.626	0.855	0.277***	8	5	-	-	3	-	-	-	0.536
10	Sotterley, Suffolk	52.404	1.625	14	4.389	0.536	0.785	0.326***	10	1	-	1	7	1	-	-	0.533

Table 2 Analysis of molecular variance (AMOVA).

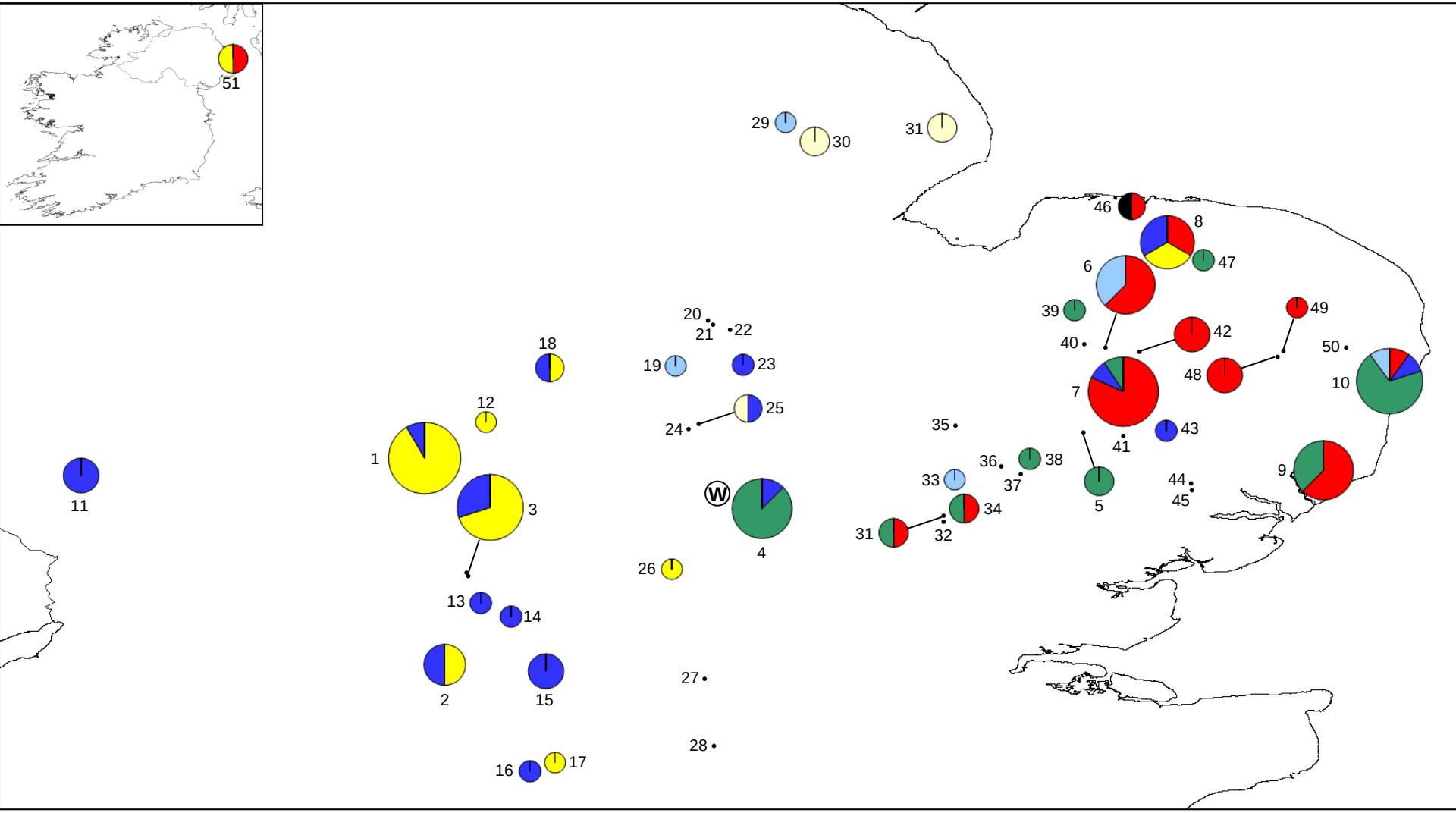
Markers	Source of variation	Sum of squares	Variance	% variation	*P*
Nuclear microsatellites	Among populations	55.584	0.155	5.01	$P < 0.001$
	Within populations	588.655	2.943	94.99	
Mitochondrial D-loop	Among populations	15.206	0.190	47.00	$P < 0.001$
	Within populations	14.769	0.214	53.00	

Figure Legends

Figure 1 Map showing the distribution of mitochondrial D-loop haplotypes in south-eastern England. Inset shows Ireland. Colours refer to haplotypes in Figure 2. Circle size is proportional to the number of samples, with the largest circle representing *N* = 12 and the smallest *N* = 1. Dots indicate sites for which only microsatellite data were obtained. Numbers refer to Table 1 and Appendix 1. "W" indicates the location of Woburn Abbey.

Figure 2 Median-joining network showing relationships between the seven haplotypes detected by sequencing the mtDNA D-loop region. Circle sizes are approximately proportional to haplotype frequency: smallest circle represents a single individual, largest circle represents 22 individuals. Each dash between haplotypes represents a single mutation. The diamond represents an unsampled ancestral haplotype.

Figure 3 Results of the PCA. The first three axes accounted for 23.17%, 20.03% and 17.59% respectively of the total variation (60.78%).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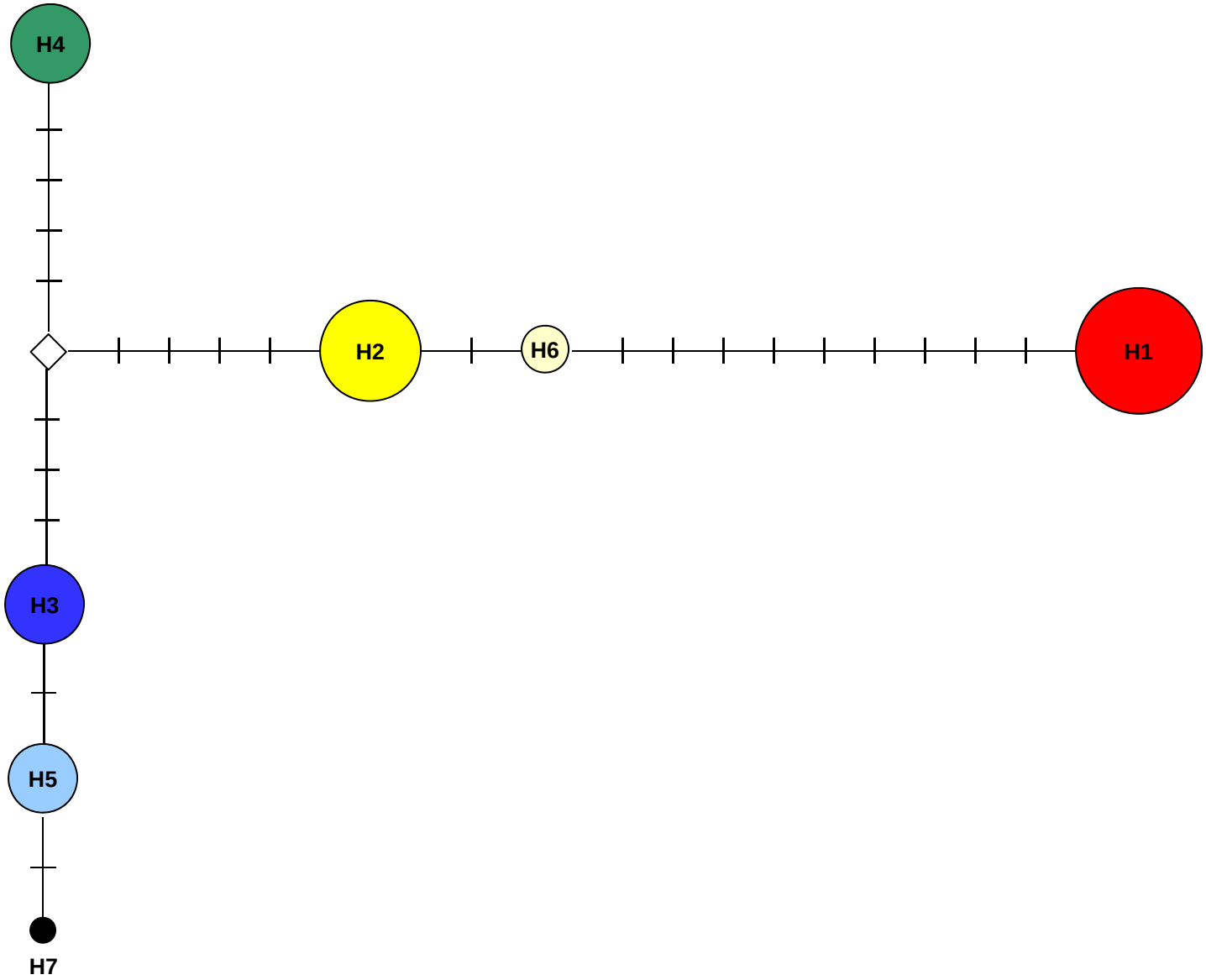
H4
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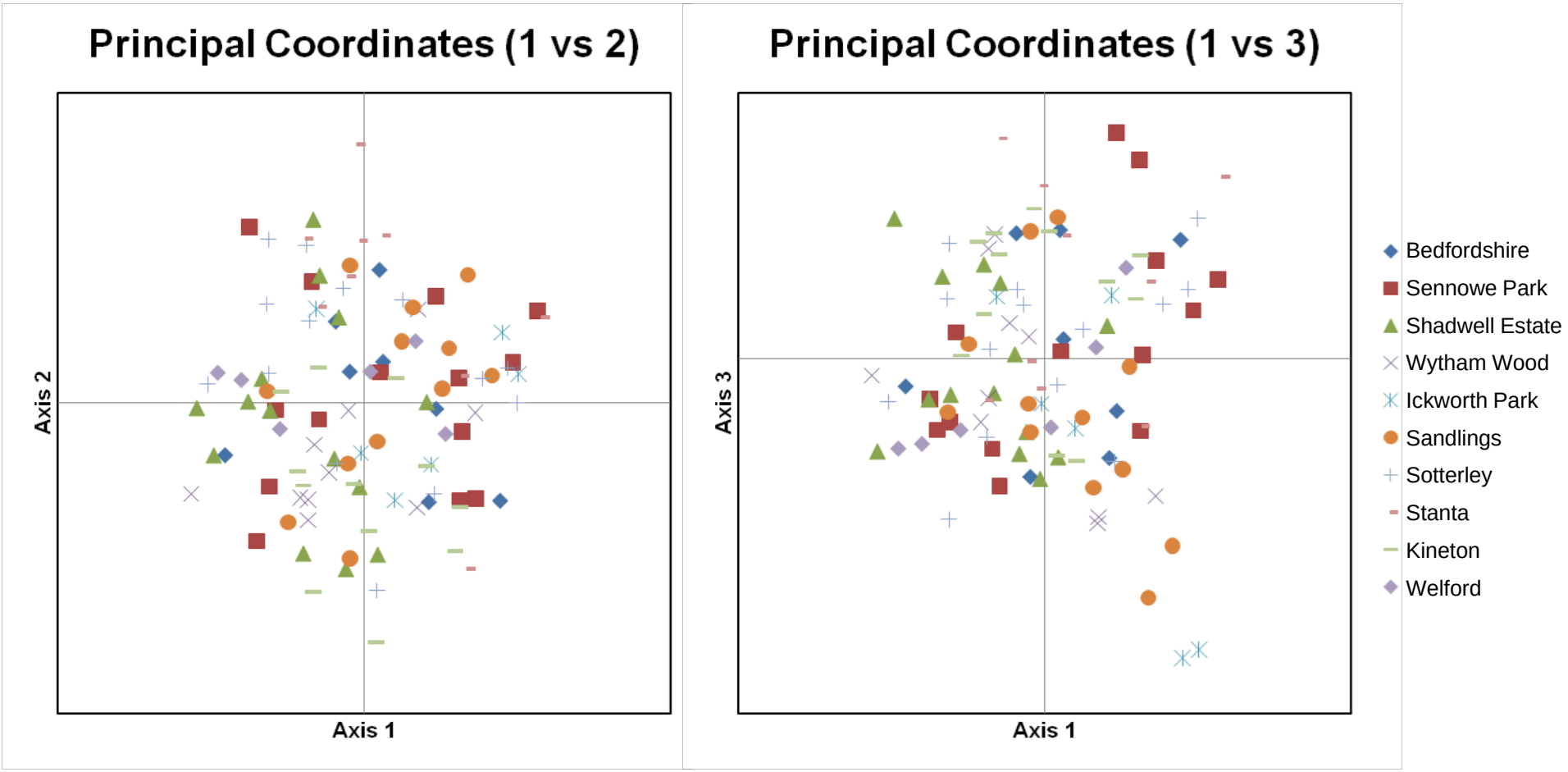
Principal Coordinates (1 vs 2)
Axis 2
Axis 1
Principal Coordinates (1 vs 3)
Axis 3
Axis 1
Bedfordshire
Sennowe Park
Shadwell Estate
Wytham Wood
Ickworth Park
Sandlings
Sotterley
Stanta
Kineton
Welford

Appendix 1 Samples collected with less than six individuals at a site.

No	Location	Lat (N)	Long (E)	N_{nuc}	N_{mt}
11	Yarkhill, Hertfordshire	52.082	-2.573	3	3
12	Shuckburgh Estate, Warwickshire	52.254	-1.274	2	1
13	Cothill, Oxfordshire	51.670	-1.288	1	1
14	Wittenham Clumps, Oxfordshire	51.631	-1.189	1	1
15	Horsemoor Wood, Oxfordshire	51.456	-1.078	3	3
16	Lower Ianham, Hampshire	51.128	-1.131	1	1
17	Alton, Hampshire	51.157	-1.051	1	1
18	Old Elvendon, Oxfordshire	52.426	-1.068	2	2
19	Broughton Wood, Northamptonshire	52.432	-0.661	3	1
20	Finesmade, Northamptonshire	52.581	-0.557	1	-
21	Duddington, Northamptonshire	52.569	-0.542	1	-
22	Apethorpe, Northamptonshire	52.551	-0.487	2	-
23	Fothoringhay, Northamptonshire	52.437	-0.444	1	1
24	Forty Acres Wood, Bedfordshire	52.230	-0.620	1	-
25	Great Hayes Wood, Bedfordshire	52.246	-0.588	4	2
26	Englefield Estate, Berkshire	51.430	-0.569	1	-
27	Chilworth, Surrey	51.214	-0.539	1	-
28	Bardney, Lincolnshire	53.215	-0.308	1	1
29	Woodhall Spa, Lincolnshire	53.158	-0.215	2	2
30	Welton Wood, Lincolnshire	53.202	0.197	2	2
31	Quendon, Essex	51.953	0.201	2	2
32	Ugley, Essex	51.931	0.198	1	-
33	Little Walden, Essex	52.067	0.239	1	1
34	Hamperden End, Essex	51.980	0.263	2	2
35	Lode, Cambridgeshire	52.241	0.239	1	-
36	Thurlow, Suffolk	52.108	0.384	1	-
37	Great Thurlew, Suffolk	52.084	0.447	1	-
38	Thurlow, Suffolk	52.137	0.480	1	1
39	Beachamwell, Norfolk	52.610	0.622	1	1
40	Thetford Forest, Norfolk	52.506	0.651	3	-
41	Rushbrooke, Suffolk	52.208	0.779	2	-
42	East Wretham, Norfolk	52.481	0.830	3	3
43	Haughley Park, Suffolk	52.222	0.920	1	1
44	Wolves' Wood, Suffolk	52.054	0.995	1	-
45	Tom's Wood, Suffolk	52.033	0.997	1	-
46	Holkham, Norfolk	52.950	0.804	4	2
47	Foxley Wood, Norfolk	52.765	1.036	2	1
48	Shelton, Norfolk	52.464	1.274	5	3
49	Sundy Green, Norfolk	52.484	1.293	1	1
50	Kirby Cane, Norfolk	52.493	1.493	2	-
51	Mount Stewart, Co. Down	54.557	-5.625	2	2