The establishment of a hybrid zone between red and sika deer (genus *Cervus*)

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Abstract

Japanese sika deer (*Cervus nippon nippon*) were introduced to Scotland around 80 years (20 generations) ago. The sika phenotype is expanding its range and hybridizing extensively with native red deer (*Cervus elaphus*) leading to the establishment of a hybrid zone. This zone is currently moving and cannot be considered to be at equilibrium. Cervid genotypes and mitochondrial haplotypes were mapped across the sika phenotype range, using diagnostic protein isozymes, microsatellite nuclear DNA markers and RFLPs in mtDNA. These were analysed to estimate heterozygote deficits and nuclear linkage disequilibria and cytonuclear disequilibria in relation to gene frequencies and time since contact. Introgression was found in both taxa and strong linkage disequilibria and heterozygote deficits characterize the populations longest exposed to hybridization. Populations further from the introduction site, where hybridization is facilitated by the dispersal of sika-like stags, show low values for linkage disequilibria and heterozygote deficit. The observed patterns in genotype are explained in terms of assortative mating and a selective advantage of the sika genotype. The genetic integrity of the Scottish mainland red deer is shown to be at risk from the invasion of sika.

Keywords: *Cervus*, hybridization, introduction, introgression , red deer, sika deer

Received 15 February 1994; revision accepted 18 May 1994

Introduction

Sika deer (*Cervus nippon* Temminck 1838) were brought to Britain over 100 years ago (Powerscourt 1884), and have since become part of the naturalised fauna (Lever 1977), though little is known of their impact on the native wildlife (Ratcliffe 1987a). Sika are congeneric with the native red deer (*Cervus elaphus* L. 1758) and members of this genus have been known to hybridize in many cases (Caughley 1971; Harrington 1973, 1982; Fennessey *et al*. 1990) as have other cervid species (Wishart 1980; Stubblefield *et al*. 1986; Carr *et al*. 1986; Cronin 1991). Japanese sika are smaller than Scottish red deer with grey, spotted coats rather than brown–red pelage. They are characterized by pale brow markings, rounded, black-edged ears and white hair in the metatarsal gland and caudal disc (Ratcliffe 1991). Red deer have no brow marking, longer ears and cream or buff coloured caudal discs (Staines

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1991). The metatarsal gland is often undifferentiated in colour from the rest of the coat. Sika stags have thin antlers with a maximum of eight points where red males commonly have 12 points, or more, with thicker beams (Whitehead 1960). In both species stags are larger than hinds, sika stags being of similar body weight to red hinds (Whitehead 1960).
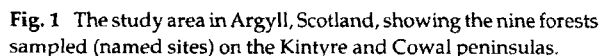
Immediately after their introduction the chance of hybridization with red deer was thought to be low as body sizes were considerably different (Powerscourt 1884). This view persisted until the latter half of this century when hybrids were reported in many areas (Whitehead 1950, 1964; Delap 1967; McNally 1969; Lowe & Gardiner 1975). Concern over the possible threat to the genetic integrity of Scottish red deer was still slow to arise, despite reports of rapid and complete introgression in populations of red and sika in Ireland (Harrington 1982) and England (Lowe & Gardiner 1975). Ratcliffe (1987a) reviewing the status of sika deer in Britain reported many cases of putative hybrids in Scotland and then voiced serious concern over the genetic impact of sika on red deer.

Most reports of hybrids were from the Argyll region of south-west Scotland (Fig. 1), where a 'small number' of sika (probably less than 20) are reported to have escaped from Carradale estate in the early 1900s (Whitehead 1950, 1964; Ratcliffe 1987a). Initial observations were made in Argyll in 1990–91 to assess the distribution of sika and their hybrids. Sika-like animals were present up to 400 km from Carradale, though they were rare beyond 200 km away.

The introduction of a hybridizing species and the parameters affecting the establishment of a hybrid zone have rarely been documented (Hewitt 1988), however, *Cervus* in Scotland provide an opportunity for such a study. This hybrid zone is moving; the sika have been present only around 20 generations, are expanding their range and hybrids continue to be reported from new locations, further from the introduction site (Ratcliffe 1987a). As a species invades the range of another and hybridizes with it the hybrid zone may move across that range as a wave front (Fisher 1937; Levin 1986; Andow *et al.* 1990). The population behind this initial wave, or cline, will contain alleles originating from both parental populations and moving toward equilibrium at differing frequencies, dependent on selection strengths and associations within the genome (Fisher 1937). Possible outcomes range from total fusion of the taxa when some genes originating in each population are universally advantaged, to the formation of a stable hybrid zone between taxa which remain genetically differentiated as genic associations within the two parental genomes, coupled with disadvantage of hybrids create a barrier to gene flow (Barton 1979; Barton & Hewitt 1985, 1989). Associations between genes can be maintained in a hybrid zone by selection against heterozygote or recombinant individuals (Key 1968; Bazykin 1969) coupled with constant influx of parental types (Barton & Hewitt 1985), by assortative mating between the parental types (Moore 1979, 1981) by selection for certain genotypes against an environmental background (Moore 1977; Harrison & Rand 1989) or by differential dispersal of the genotypes or sexes (Mallet *et al.* 1990). In a moving zone habitat-genotype associations are unlikely to be strong, and in a population resulting from a small founder event, the influx of parental types must be limited. A moving zone will, however, be strongly influenced by dispersal rates of the genotypes and sexes. The effects of assortative mating and selection on hybrids are also likely to be important in maintaining the genetic architecture of this establishing hybrid zone.

This paper maps gene frequencies at four nuclear loci and mitochondrial DNA haplotypes across the sika phenotype range in Argyll. Cline widths and concordance are calculated to demonstrate the extent of sika allele introgression in populations along a 400-km transect from the introduction site. Linkage disequilibria, cytonu-

clear disequilibria and within-locus heterozygote deficits are analysed to examine the contributions of selection, dispersal and mating patterns to the genetic architecture of a moving and establishing hybrid zone (e.g. Barton & Hewitt 1985; Barton & Gale 1993; Asmussen *et al.* 1987, 1989). The limitations of disequilibrium analyses applied to a moving zone are discussed.

Methods

Study sites

The study area chosen covered the range of phenotypically sika-like deer in Argyll, Scotland, within the range of resident red deer (Fig. 1). The area consists of two roughly parallel peninsulas running north-south and joined at their northern ends. This northern area is bounded to the north by Loch Awe and to the east by Loch Lomond. Four sika deer (three females, one male) were introduced at Carradale at the southern end of the Kintyre peninsula in the late 1800s and their descendants escaped around 1914 (Whitehead 1964). Sika-like deer are now seen throughout the area (personal observation). As the topography of the region essentially limits the possible range extension to one direction, it greatly simplifies interpretation of data relating to population spread. The history of the introduction of sika to the region is reasonably well-documented (Ratcliffe 1987a, for review).

Sampling

Within the large study area, nine sampling sites were chosen in forest blocks managed by the Forestry Commission (Fig. 1). Kidney tissue samples from culling operations

Fig. 1 The study area in Argyll, Scotland, showing the nine forests sampled (named sites) on the Kintyre and Cowal peninsulas.

were provided by the Forestry Commission. Approximately 1 cm^3 of kidney tissue was removed at the time of death and frozen to –20 °C in field freezers within 24 h. Samples were collected weekly and frozen to –70 °C. At each site samples were collected from all animals culled from December 1991 to March 1992. Approximately equal numbers of each sex were culled each week at each site. Culling was carried out on a daily basis at each site, in early morning and late afternoon, and covered all forest age classes from planting to felling. Stalkers did not select animals by phenotype.

Censuses

Censuses from vantage points were made at each tissue sampling site in order to assess the proportions of each population that were phenotypically sika-like. Sika-like animals were classed as those possessing morphological characters of general body size and shape, antler form, ear shape, facial markings and pelage that were predominantly sika-like rather than red-like (see above). Animals were counted from a vantage point in prethicket forest blocks and numbers averaged over four, three-hour counts on two to four consecutive days. Counting methods follow those of Ratcliffe (1987b).

Each animal was recorded as sika-like or red-like, dependent on the majority of the phenotypic characters above. No animal possessed equal amounts of sika-like and red-like characters. Identification of complex hybrids in the field is deemed unreliable as there is large variation in some characters (coat colour, antler size) within the red population. Harrington's (1973, 1982) work on captive breeding showed no predictable phenotype combinations in hybrid offspring. Clearly this type of census will give only a coarse estimate (and probably an underestimate) of sika phenotype range. In this context this is sufficient, though further work should aim to improve the accuracy of censusing quantitative traits

Allozymes

Allozymes found in previous work to be likely to have fixed differences between red and sika (Feldhammer *et al.* 1982; Herzog 1988; Linnell & Cross 1991; Emerson & Tate

1993) were screened on a test panel to check their applicability to the Argyll population. The test panel consisted of three Japanese sika from a zoo population, six from the present Carradale herd and 12 red deer from Rhum and Mull. The two allozymes found to be diagnostic were 6-phosphogluconate dehydrogenase (*6Pgdh*) and Superoxide dismutase-1 (*Sod-1*), although three other loci were originally screened.

Alleles were scored using horizontal starch gel electrophoresis of kidney tissue homogenate (500 mg of tissue in 0.5 mL of dH$_2$O) stained by agar overlay. Both systems were run simultaneously on a single gel which was then sliced and stained separately for each system. *6Pgdh* ran anodally and *Sod 1* cathodally. Running conditions were a Tris-citrate electrode buffer at pH 8.0 diluted at 1:20 in the gel, running through a 11% hydrolysed starch gel (200 × 160 × 8 m). Gels were loaded using Whatman No. 4 filter paper wicks soaked in homogenate and inserted along a sliced edge at an origin, 10 cm from the cathode. Running time was approximately 6 h at 4 °C, 300 V, 20 mA.

Gels were stained following the methods given in Harris & Hopkinson (1976). Stains were each prepared as a 5-mL volume per gel and added to an equal quantity of warm 2% agar immediately before application to the gel. Bands developed at 37 °C in around 10 min, *6Pgdh* in the dark and *Sod-1* under a light source.

Microsatellites

The microsatellites (variable number tandem repeat sequences) were developed in other ungulates, but were screened across test panels of red and sika as above to assay diagnostic red and sika alleles. The microsatellites used were 'BOVIRBP' and 'OarFCB193'. Sequences and suppliers are given in Table 1. Alleles found in red deer were consistent in Rhum (J. Pemberton personal communication) and Mull red deer and different from those found in sika. The alleles found in the sika test panel differed from the nearest red allele by six repeats at the BOVIRBP locus and 10 repeats at the OarFCB193 locus. The differences at the BOVIRBP locus have since been independently assessed as species-specific (M. Bruford, personal communication). A radiolabelled polymerase

Table 1 Primer sequences and suppliers

Locus	Sequence	Reference/ supplier
ORF 381	5′-ACC CCG CCT GTT TAC CAA AAA CAT-3′	Georgiadis & Patton, unpublished.
ORF 583	5′-GGT ATG AGC CCG ATA GCT TA-3′	Cited in Hall & Nawrocki 1994
BOVIRBP A	5′-TGT ATG ATC ACC TTC TAT GCT TC-3′	D. MacHugh, Trinity College, Dublin.
BOVIRBP B	5′-GCT TTA GGT AAT CAT CAG ATA GC-3′	Modified from Moore *et al.* 1991
OarFCB193 A	5′-TTC ATC TCA GAC TGG GAT TCA GAA AGG C-3′	Buchanan & Crawford 1993
OarFCB193 B	5′-GCT TGG AAA TAA CCC TCC TGC ATC CC-3′	Genbank accession no. LO1533.

chain reaction (PCR) was used to amplify fragments of genomic DNA and the PCR products visualised by autoradiographic exposure following polyacrylamide gel electrophoresis. PCR conditions were identical to those of Bancroft, Pemberton & King (1994).

Mitochondrial haplotypes

Haplotypes were derived by PCR amplification of a 2-kb section of the 16s and ND1 region of the NADH complex in the mitochondrial genome, followed by digestion of the product with single restriction endonucleases. Primer sequences and suppliers are given in Table 1. The 25-μL PCR reaction mixture contained 1 μL genomic DNA, 1 μL each of 10 pM/ μL primers ORF381 and ORF583, 4 μL of deoxynucleotide triphosphates (dNTPs) at 1.25 mM each nucleotide, 0.125 μL of Taq polymerase, 2.5 μL of relevant Taq buffer and 15 μL of dH_2O. The thermocycling regime involved a 2-min denaturing step at 95 °C followed by 30 cycles of 60 s at 55 °C, 90 s at 72 °C and 30 s at 94 °C with a final extension of 10 min at 72 °C (Hall & Nawrocki 1994). Banding patterns were visualised by horizontal agarose gel electrophoresis, followed by ethidium bromide staining and UV illumination. Diagnostic haplotypes were obtained from digestion by the endonucleases *MspI*, *HinfI* and *HaeIII*. These were assessed on the test panel of known red and sika as above. No intra-individual heterogeneity was found in the haplotypes and individuals were thus assigned without question to a species matriline. MtDNA frequencies are given as those for a single locus.

Analysis

Within-locus heterozygosities were compared to Hardy–Weinberg expectations using the χ^2 distribution of Wright's 'inbreeding coefficient' or F-statistic, F_{is} (Wright 1951) to quantify deviations from expected. Positive deviations indicate heterozygote deficit, negative ones heterozygote surplus.

Levels of association between loci were assessed by pairwise linkage disequilibria and deviations from random associations statistically estimated by maximum likelihood (using the 'ANALYSE' programme in PASCAL for Macintosh available from N. Barton, Edinburgh University). It was assumed that the linkage disequilibria were due to associations between loci within gametes, rather than to associations between loci derived from different parents, though the inheritance of double heterozygotes cannot be determined from genotypic data (Weir 1979, 1991). The estimate of linkage disequilibria will be the sum of within and cross-locus disequilibria (Weir 1979; Barton & Gale 1993). As a deficit of heterozygotes is apparent from the F-statistics, the number of homozygous gametes available for recombination in the model was re-

duced by a proportion analogous to that deficit. A consistent lack of heterozygotes would falsely inflate disequilibrium measures. The correction incorporates known observations of population process potentially contributing to linkage disequilibria without requiring prior knowledge of the mechanism (non-random mating, dispersal or genotypic selection) by which they occur. The value R is a pairwise measure of the association between alleles at different loci, corrected for allele frequencies and heterozygote deficit (Barton & Gale 1993).

Cytonuclear disequilibria were measured as (i) deviations from random associations between alleles at each nuclear locus and the mtDNA type (D) and (ii) deviations from the expected frequency of each mtDNA type within heterozygote class at the nuclear locus (d) (Asmussen *et al.* 1987, 1989). Both measures were estimated by maximum likelihood (Asmussen *et al.* 1987) and 4 models of interaction between the F statistic, D and d were fitted to the data by likelihood ratio. The models were:

$F = 0, D = 0, d = 0$ no disequilibria or het. deficit

$F \neq 0, D = 0, d = 0$ no disequilibria, het. deficit

$F \neq 0, D \neq 0, d = 0$ gametic but not genotypic disequilibria, het. deficit

$F \neq 0, D \neq 0, d \neq 0$ gametic and genotypic disequilibria, het. deficit.

Results

Censuses

Frequencies of all sika-like animals in the nine populations are given in Table 2. A disparity between the ranges of sika-like males and females was found. No sika-like females were seen beyond Birdfield forest (Fig. 1), though sika males were seen on the Cowal peninsula. At very low frequencies the census method is unlikely to be accurate and will most often underestimate numbers of infrequent types (Ratcliffe 1987b). It is likely that this is the case for sika-males on the Cowal peninsula. It may also be the case that sika-like females persist a short distance beyond Birdfield at low frequency, but it is less likely that they will occur further than this, given the short dispersal distance of females (Davidson 1979).

Allele frequencies from nuclear loci

For all loci (except *OarFCB193* where two sika alleles were present) sika showed a single allele variant. For the allozymes this was also true of the red deer, but at the microsatellite loci several red alleles were found. The lack of polymorphism in the sika population could be a result of the small number of founder animals. Animals were

Table 2 Census returns for the nine forest sites. Distances given are from the introduction site. Values given are proportions of the total cervid population at each forest. All animals were classed as 'red' or 'sika' according to the majority of their phenotypic characters, as complex hybrids were not always reliably identifiable by phenotype in the field

Forest	Distance from introduction (km)	N	Sika-like females	Sika-like males	Red-like females	Red-like males
Carradale	0	44	0.52	0.44	0.02	0.02
Achaglachgach	60	51	0.45	0.35	0.12	0.08
Knapdale	123	49	0.17	0.18	0.35	0.3
Kilmichael	138	35	0.24	0.25	0.23	0.28
Birdfield	188	42	0.45	0.35	0.15	0.05
N. Cowal	283	43	0.00	0.03	0.53	0.44
E. Cowal	313	40	0.00	0.00	0.54	0.46
Glendaruel	358	37	0.00	0.02	0.5	0.48
S. Cowal	386	29	0.00	0.00	0.55	0.45

scored as 'red' or 'sika' for any of the possible relevant alleles present.

A clear cline across the transect was discovered for each locus. Raw data is shown in Table 3. Allele frequencies plotted against minimum distance overland from the introduction point are shown in Fig. 2(a). Sika have been seen to swim Loch Fyne at its narrowest point (H. Gibb, personal communication) as well as dispersing overland. This may explain the relatively high sika allele frequencies in the furthest sites as it effectively brings these sites closer to Carradale.

The clines were concordant for all loci, meaning that at a given mean sika allele frequency, allele frequencies at individual loci were similar (Fig. 2b). This indicates that cline widths are approximately equal for all loci, though cline shape is not fully described as small variations between consecutive sites will reduce to the same straight line fit through all nine (Barton & Gale 1993) Cline shape could not be meaningfully fitted to only nine sites. Heterogeneity of linear slopes was tested by ANOVA (Sokal & Rohlf 1981, Ch. 17), $a = 9.73$, NS.

Mean cline width was estimated by maximum likelihood fit to a tanh curve (Sanderson *et al.* 1992). This gave a best estimate of 367 km with support limits of 341–398 km.

Heterozygosities

All loci showed similar patterns in F_{is} values across the transect. Significant heterozygote deficits were found in sites 0–123 km from the introduction and for two loci at sites up to 188 km (Table 4). At Kilmichael samples sizes were too low to calculate F_{is}. In sites close to the edge of the sika range, the trend is toward zero or weakly positive F_{is} values, indicating heterozygote surpluses. Although these are not significantly different from expected, the trend is similar across all loci.

Linkage disequilibria of nuclear loci

Patterns of linkage disequilibria were similar across all loci pairs across the transect. Strongly positive values, indicating nonrandom associations favouring parental combinations, were obtained from sites 0–138 km from Carradale Zero or weakly negative ones, indicating a trend toward random associations, or even favouring recombination, for sites beyond Birdfield (Table 5). For sites farthest from the introduction point, the red allele was fixed, and R values could not be calculated. South Cowal again seems to show characteristics consistent with a site closer to the introduction point. Heterogeneity between

Table 3 Nuclear gene and mitochondrial DNA haplotype frequencies across the transect in Argyll. Values are for the sika allele in each case. As there was no intra-animal variation across the 3 mtDNA haplotypes screened, they are scored as one locus

| Forest	Distance from introduction (km)	Sika allele frequency									
		6Pgdh	N	Sod-1	N	Bovirbp	N	Oar FCB193	N	mt DNA	N
Carradale	0	0.696	27	0.770	35	0.870	33	0.900	27	0.875	31
Achaglachgach	60	0.614	23	0.648	27	0.750	24	0.607	14	0.687	30
Knapdale	123	0.384	26	0.638	29	0.520	18	0.479	24	0.667	30
Kilmichael	138	0.350	6	0.450	9	0.350	9	0.800	5	0.500	4
Birdfield	188	0.329	41	0.205	39	0.100	32	0.114	22	0.029	33
N. Cowal	283	0.053	38	0.261	44	0.000	41	0.020	39	0.000	35
E. Cowal	313	0.000	6	0.167	12	0.000	12	0.111	3	0.100	11
Glendaruel	358	0.100	10	0.167	12	0.000	11	0.070	7	0.000	10
S. Cowal	386.	0.053	19	0.250	22	0.000	19	0.030	16	0.000	15

a.

b.
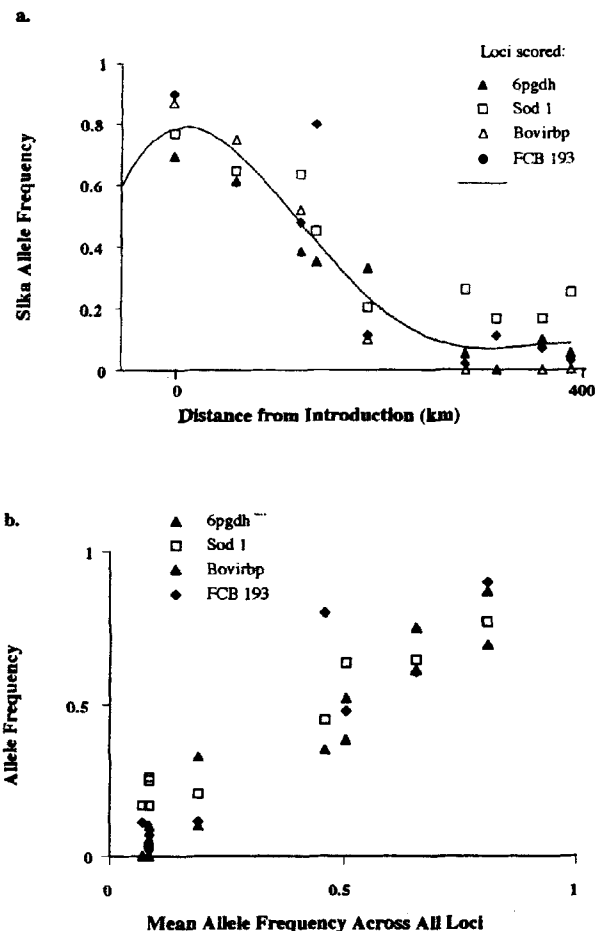
Fig. 2 Sika nuclear allele frequencies at each locus. (a) plotted against overland distance along the transect. The curve is fitted to the mean frequency. (b) plotted against mean sika allele frequency across all loci. The cline for each locus is concordant with all others. Heterogeneity of slope, a = 9.73, NS.

loci, measured by maximum likelihood (Barton & Gale 1993) gave a log likelihood of 4.11 (d.f. = 5) which is not significant, indicating that the patterns are similar across all loci pairs.

Mitochondrial DNA and cytonuclear disequilibria

Frequencies of sika mtDNA in the populations closest to the introduction site were high, though sika mtDNA was absent beyond Birdfield.

Hybrids were identified as animals possessing any combination of genotypes across all loci scored not consistently 'red' or 'sika'. A comparison of the frequency of all hybrids in the population, with those possessing sika mtDNA i.e. from a sika matriline, showed a geographical difference between the two, indicating that hybridization at the edge of the sika female range is facilitated by the dispersal of sika (or hybrid) stags into the red population (Fig. 3).

The frequency of sika mtDNA is not significantly different to the frequencies of the sika nuclear alleles in any population where sika-like females are resident (χ^2 = 1.89, d.f. = 1, NS) and sika mtDNA frequencies within hybrids are close to 0.5, giving no indication of directionality in hybridization events.

Direct comparison of nuclear and cytonuclear disequilibria values are difficult as effective allele frequencies differ between the loci. However, gametic cytonuclear disequilibrium (D) measures show a similar pattern to the disequilibria between unlinked nuclear loci in the four populations where sika mtDNA is present, giving no indication of directionality in the crosses. Genotypic disequilibria (d) show small significant values although sample sizes are so greatly reduced by the heterozygote deficit in these populations that confidence limits on the

Table 4 F_{is} values for the populations in Argyll. Sample sizes at Kilmichael were too small to calculate F_{is}. Values are compared to the χ^2 distribution to assess deviations from Hardy–Weinberg proportions using the relationship $\chi^2 = 2NF_{is}2(k - 1)$ where k = no. alleles per locus –1. Values vary between –1 and 1; large values indicate strong deviations, positive values showing a heterozygote deficit and negative ones a heterozygote surplus. Where an allele is fixed at the locus F_{is} cannot be calculated. Values differing significantly from Hardy–Weinberg expectations (P < 0.05) are marked with an asterisk

Forest	Distance from introduction (km)	Mean sika allele frequency	F_{is} 6Pgdh	Sod-1	Bovirbp	OarFCB193
Carradale	0	0.809	0.562*	0.204*	0.062	0.617
Achaglachgach	60	0.655	0.725*	0.432*	–	0.854*
Knapdale	123	0.562	0.837*	0.328*	0.668*	0.916*
Birdfield	188	0.187	0.526*	0.092	–	0.762*
North Cowal	283	0.084	–0.049	–0.119	–	–
East Cowal	313	0.069	–	–0.198	–	–
Glendaruel	358	0.084	–	0.401	–	–
South Cowal	386	0.083	–0.049	0.636*	–	–

Table 5 Linkage disequilibrium between all pairs of loci scored across the transect. *R* gives a value for the degree of association between particular alleles at a pair of loci, standardised for allele frequencies in the population and corrected for known heterozygote deficits. *R* values are calculated by maximum likelihood (using the ANALYSE programme, N. Barton, unpublished). Values significantly different from zero (> 2 units log L) are marked with an asterisk (*). Mean *R* values calculated across all loci are also given. Heterogeneity between loci is insignificant ($\Delta logL_5 = 4.11$) and mean *R* values across loci are given in the final column

Forest	Distance from introduction (km)	6Pgdh Sod-1	6Pgdh Bov	6Pgdh Fcb	Sod-1 Bov	Sod-1 Fcb	Bov Fcb	Mean R
Carradale	0	0.276*	0.175*	0.239*	0.258*	0.426*	0.164*	0.287*
Achaglachgach	60	0.363*	0.353*	0.376*	0.082	0.044	0.624	0.298*
Knapdale	123	0.432*	0.424*	0.642*	0.171*	0.430*	0.387*	0.345*
Kilmichael	138	0.282	0.587	0.874	0.984	0.412	0.786	0.338*
Birdfield	188	−0.13	−	0.114	−	0.354	−	0.019
N. Cowal	283	−0.12	−	−0.05	−	0.089	−	−0.025
E. Cowal	313	−	−	−	−	−	−	−
Glendaruel	358	0.195	−	−	−	−0.100	−	0.201
S. Cowal	386	−0.17	−	−	−	−0.100	−	−0.025
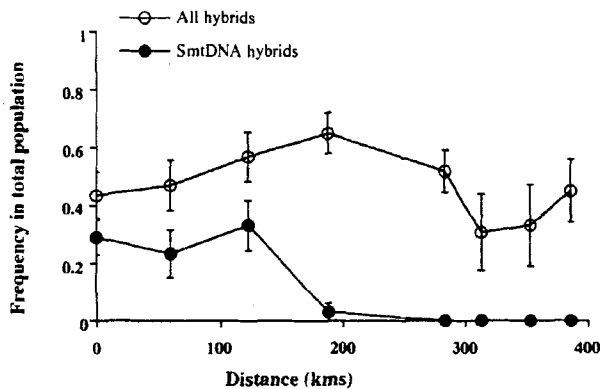
Fig. 3 Hybrids were assessed as those animals possessing alleles from both taxa or a mtDNA haplotype differing from their nuclear genotype. The proportion of hybrid animals in each population is shown by the open circles. Hybrids possessing sika mtDNA are shown in closed circles, also as a fraction of the whole population. No hybrids with sika mtDNA were found beyond the sika-like female range (138 km), indicating that in this region sika or hybrid stags mating red hinds are responsible for the hybridization events.

estimates are too large to draw robust conclusions from the patterns (Table 6). In all populations the deviation from-random expectation appeared to be through an increase in recombinant types at the expense of parental combinations.

Four hypotheses of genetic architecture applicable to the red sika populations were defined by interactions between *F*, *D* and *d* and tested by likelihood ratio. The data is generally best explained by the final model, including heterozygote deficit and positive nuclear and cytonuclear disequilibria, though in most cases no best fit was ob-

Table 6 Cytonuclear disequilibria in populations to Birdfield. Beyond Birdfield sika mtDNA is absent and disequilibria cannot be calculated. At Kilmichael sample sizes were too small to use. *D* denotes gametic disequilibria between the sika nuclear allele and sika mtDNA type, *d* denotes genotypic disequilibria between the three nuclear genotypes mtDNA. *D* values show a similar pattern to the disequilibria found in the nuclear loci and do not indicate directionality in the crosses. *d* values are generally very small. Most populations cannot be fitted to a model of mating preference (see text) as sample sizes are reduced by the heterozygote deficit in these populations. The row 'log likelihood loss' shows the decrease in log L resulting from fitting other models. The best fitting model is shown above. A decrease of < 2 units of log L is insignificant and precludes designation of a best fitting model

| Forest	Locus 6Pgdh	Sod-1	Bovirbp	OarFCB193
Carradale				
D	0.041	0.041	0.03	−0.002
d	0.0004	0.003	0.03	0.001
BFM	all > 0	none	all > 0	none
Log L loss	2.29		2.48	
Achaglachgach				
D	0.19	0.121	0.063	0.058
d	0.069	−0.05	0.021	0.016
BFM	all > 0	none	none	none
Log L loss	9.06			
Knapdale				
D	0.136	0.144	0.08	0.195
d	0.0009	0.019	0.054	0.0004
BFM	all > 0	none	all > 0	none
Log L loss	4.88		2.92	
Birdfield				
D	0.145	−0.008		
d	0.088	−0.001		
BFM	none	none		
Log L loss				

BFM = best fitted model *F, D, d*
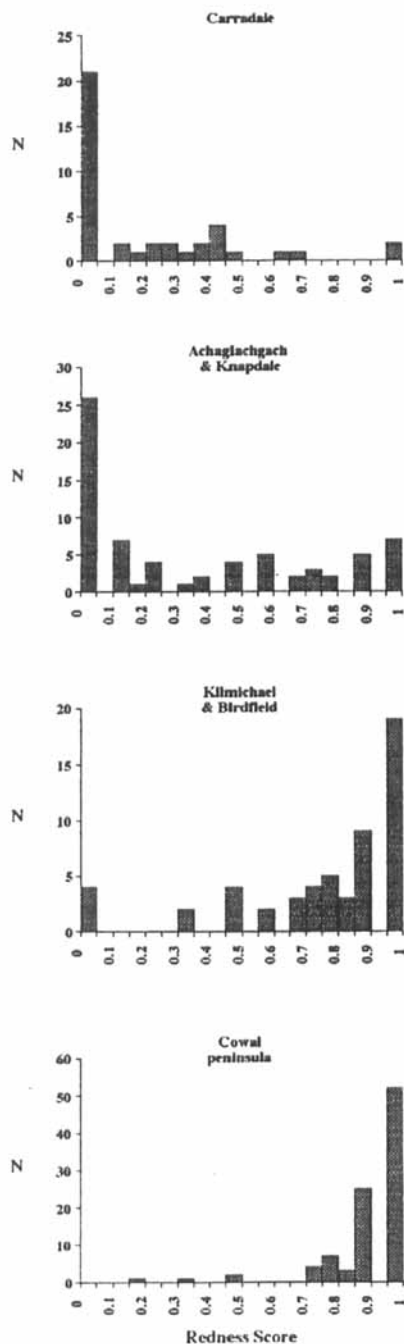
Fig. 4 The distribution of individuals across a hybrid index, described above. 'Pure' red individuals score 1, 'pure' sika individuals score 0. Scores are pooled across populations dependent on mean sika alleles frequency. From top; $P = 1.0–0.75$, $P = 0.749–0.5$, $P = 0.499–0.15$, $P = 0.149–0.0$. The only population that had a sika frequency of between 0.499 and 0.25 was Kilmichael, where only 9 individuals were scored. It was therefore pooled with the Birdfield population ($P = 0.187$) to increase the sample size. This does not alter the observed pattern in back-crossing. A shift from red to sika in the distribution of back-crosses can be seen with increasing sika allele frequency.

tained (Table 6). Cytonuclear disequilibria were tested by pairwise interactions as a maximum likelihood estimate of homogeneity proved impossible to fit reliably.

Distribution of hybrid types

Individuals were each scored on an index of 0–1, giving the red alleles as a proportion of all alleles scored across all loci, including mitochondrial haplotype. 'Pure' red individuals scored 1, 'pure' sika scored 0. The distributions of individual scores pooled over populations in four areas of the transect are shown in Fig. 4. Populations were pooled according to allele frequencies, but these divisions accorded to geographical location also. A trend from sika-like to red-like hybrids can be seen moving away from the introduction point. There is also a change in the variability of the population, the greatest variation being in the 60–138-km region where sika gene frequencies were between 0.5 and 0.749. In these populations complex hybrids and back-crosses to each parent exist.

Discussion

Concordant and coincident clines in sika allele frequencies occur at all loci studied in Argyll. Mean cline width was shown to be approximately 367 km, which covers the entire sika phenotype range. It is impossible to consider this hybrid zone simply as a band between parental types, or as a stable state. In populations close to the introduction site, where hybridization has occurred longest, strong linkage disequilibria between nuclear loci indicate a deficit of recombinant genotypes. Strong within-locus heterozygote deficits are also seen in these populations. Cytonuclear disequilibria show small but significant values for gametic and genotypic measures although sample sizes for measures of genotypic disequilibria are much reduced by the existence of heterozygote deficits, lowering confidence in the estimates. The three analyses all contradict predictions of a null hypothesis that these red sika populations are random mating and experiencing no genotypic selection. They each allow inferences about the possible roles of assortative mating and selection in altering the genetic architecture of the hybrid zone (e.g. Wright 1965; Weir 1979; Butlin, Ritchie & Hewitt 1991; Asmussen *et al.* 1987, 1989). There are several levels at which the effect of the sika introduction can be considered, the greatest divide being between that of the single locus and that of the whole organism.

Single locus analysis

At the single locus level, alleles at a consistent selective advantage in the whole cervid population would be expected to go to fixation, and others would segregate at

different frequencies in the population, according to their associations with selected loci ('hitch-hiking' – Maynard Smith and Haigh 1974; Berry *et al.* 1993). Time to fixation is dependent on the strength of selection and the population size. Neutral loci would eventually introgress through the entire population or be lost by chance. In the formation of the zone, differing levels of association would be expected between genes, altering levels of introgression of various loci (Hewitt 1988; Mallet & Barton 1989). In a moving zone these would be expressed as geographically differing cline positions and widths, selected loci introgressing faster, and with narrower clines than neutral or hitch-hiking loci (Slatkin 1982). Uniform levels of selection across several loci would produce concordant and coincident clines even if these loci were not otherwise associated (Barton & Hewitt 1985, 1989; Hewitt 1988).

Information from single loci in this data set show patterns of cline coincidence and concordance that seem best explained by selection acting with similar force on all marker loci. The randomly chosen markers are unlikely to be under direct selection, but may be hitch-hiking on selected genes (Maynard Smith & Haigh 1974). Cline position in neutral markers could be altered by linkage to selected loci, however, the relative differences in selection strength and linkage required to produce discordance so soon after introduction are not easily quantified. Simulations of the strengths of selection and the numbers of selected loci required to separate neutral clines in this short time frame will be dealt with in a subsequent analysis (Baird & Abernethy, in preparation).

An alternative hypothesis is that the increase in sika frequency could be accounted for by genetic drift, however, this alone seems insufficient to explain the data. In populations recently invaded by sika the increase of the phenotype frequency has been dramatic. Though earlier data on genotype frequencies are unknown, prior to the introduction they must have been zero. In Knapdale, the sika phenotype has increased from < 1% to 35% of the cervid population in just 30 years ($N \approx 2000$, $t \approx 7$ generations) and the mean sika allele frequency is currently 0.505. This seems unlikely to be the result of genetic drift. Overall the immense increase in frequency of sika from an introduction of around 12 individuals to a present total population of thousands in Argyll (over 80% of the Carradale population), is also unlikely to be accounted for by drift and so implies that one or more sika genes, are being selected for.

Multilocus analysis

Predicting the fate of the whole organism or more practically, the phenotype, requires analysis of the interactions between several loci rather than the behaviour of a single locus. In many ways this is a more meaningful analysis of the fate of 'red' and 'sika' deer, which are obviously defined by their multilocus genotype rather than single genes. If quantitative traits under multilocus control are under selection, then associations between these loci are unlikely to be random, generating linkage disequilibria (Lewontin & Kojima 1960). Linkage disequilibria can result from selection against recombinant types, nonrandom mating (sexual selection) or parental immigration to a hybrid zone (Barton & Hewitt 1985). In populations where these forces are acting associations may break down over time, due to the relative neutrality of some parts of the genome, but initially strong and concordant linkage disequilibria between parental allele combinations would be expected within individuals, and similar rates of introgression of the loci would be expected at the population level (Barton 1983; Barton & Bengtsson 1986). This would distinguish selection on multilocus traits from uniform selection on independent loci, which would not be expected to produce persistent, strong linkage disequilibria in hybridizing populations. Coherent and species-specific phenotypes will be likely to persist as a result of strong disequilibria.

At the multilocus level, the pattern of heterozygote and recombinant deficit behind the initial advance of sika could be produced by hybrid disadvantage, by assortative mating in the hybridizing populations or by a combination of both these forces (Barton & Hewitt 1985). As no population exists where sika alleles are fixed, there is effectively no 'parental population' of sika to provide immigrants. Parental type red deer could move into the zone, but in populations near the introduction where red frequencies are low, immigration of parents is unable to account for the pattern. The range of back-crosses to each parent, indicated by the distribution of individuals across the hybrid index, clearly argues that hybrids are breeding within the populations after the F1 event, although they could be experiencing decreased reproductive success. Harrington's (1973, 1982) work on back-crossing of captive red-sika crosses showed no apparent physiological disadvantage in the hybrids, though in a wild population they may suffer from behavioural abnormalities in the breeding rut.

As well as indicating the contribution of males in propagating the initial wave of hybridization, the mtDNA survey shows that hybrids are produced from both red and sika matrilines; the *original* parents being either a red male and a sika female or a red female and a sika male, though not necessarily both combinations. If only one parental combination is involved in the F1 event, introgression will still occur in both taxa as long as those F1 offspring will back-cross to either parental type. If mating is so strongly assortative that hybrids will only back-cross to one taxon, then introgression will be unidi-

rectional (e.g. Paige, Capman & Jennetten 1991; Sperling & Spence 1991). This is clearly not the case in Argyll. Data from mitochondrial-nuclear interactions can be useful in detecting the mechanism creating disequilibria as, by deriving information from uniparentally inherited (cytoplasmic mtDNA) and biparentally inherited (nuclear) markers, they can be used to test hypotheses about mating patterns.

Cytonuclear disequilibria analyses have been used successfully to infer directionality of hybridization and strength of assortative mating preferences in *Hyla* tree frogs (Lamb & Avise 1986; Asmussen *et al.* 1987) and North American cottonwoods (Paige *et al.* 1991) and in some cases mitochondrial data alone have been sufficient to detect strong directionality in mating preferences of hybridizing deer populations (Cronin *et al.* 1988). Maximum likelihood comparisons of the cytonuclear genotype distributions in these data show that the pattern in gametic cytonuclear disequilibrium is similar to that for unlinked nuclear loci pairs and gives no indication of directionality in the crosses (compare Tables 5 and 6). Genotypic disequilibria (*d*) values which would potentially yield information on the directionality and strength of assortative mating in the sexes (Asmussen *et al.* 1987, 1989; Arnold *et al.* 1988), appear to show a departure from random mating, but may be unreliable as heterozygote numbers are small. Comparisons of expected cytonuclear genotypic distributions with those observed show an increase in recombinant animals (red homozygotes with sika mtDNA or vice versa) at the expense of parental combinations. The interpretation of this is not obvious.

Differences in the strength of mate preference between males and females could alter cytonuclear disequilibria as could differential dispersal of the sexes. Both of these are plausible occurrences in hybridizing deer populations (Cronin 1991; Carr *et al.* 1986; Stubblefield *et al.* 1986) and differential dispersal of the sexes is demonstrated by this paper. As sika nuclear genes are known to be introduced to populations before mitochondrial DNA, cytonuclear disequilibria should be expected in newly colonized populations, however, the mating pattern may alter this in older populations and the interaction between the two factors is unclear. At present, studies that have assessed cytonuclear disequilibrium statistics have examined populations where mating has been strongly directional in the F1 cross and inferences including both nuclear and cytonuclear disequilibria have not been necessary to explain the structure of the hybrid population (Lamb & Avise 1986; Carr *et al.* 1986; Paige *et al.* 1991; Hoffman & Turelli 1988). Models of disequilibrium expectations are based on mate fidelity being independent of frequency, and immigration being solely of parental types (Asmussen *et al.* 1989). In the red–sika populations there is no clear directionality in the F1 cross but strong nuclear

linkage disequilibria and heterozygote deficits indicate likely assortment by, or selection on, nuclear genotype. Potential colonization by hybrid immigrants make interpretation of back-cross frequencies in the style of previous cytonuclear disequilibrium measures extremely difficult (Asmussen *et al.* 1989). A composite measure of nuclear and cytonuclear disequilibrium may be able to disentangle the contribution of assortative mating and genotypic selection, but differential reproductive success, migration rates and mate fidelity between the taxa and the sexes, make this analytically laborious. Subsequent work will use computer simulations to address these questions (Baird & Abernethy, in preparation).

In populations at the edge of the range of sika-like females (beyond Birdfield), linkage disequilibria become low or weakly negative and F_{is} values show a trend toward equilibrium or even weak heterozygote surpluses within loci. This seems at first incompatible with ideas of heterozygote disadvantage or species-specific mate choice above, but can be explained by the differential dispersal of the two sexes. At the end of the transect furthest from the introduction point, genetically sika females are not resident, yet hybrids are still present. The greater dispersal distance of stags (Davidson 1973, 1979) means that sika males are moving into red areas before sika females and therefore hybridizing with red females. This differential dispersal creates difficulties in applying conventional population equilibrium tests, such as Hardy–Weinberg or Wright's *F*-statistics, to populations where only sika males are present. For any particular sika allele frequency, there is no chance of producing sika homozygotes in the offspring of that generation. This creates the illusion of a heterozygote excess for the given sika allele frequency in these populations.

Although clear in theory, this effect is hard to perceive when populations are sampled across generations as in the present study, because the hybrids back-cross to the resident red deer reducing the heterozygosity in their offspring. This counteracts the effect of the original hybridization events when hybrid frequencies are assessed in the population as a whole. The weak heterozygote excesses in populations beyond the sika female range and trends toward low or negative linkage disequilibria are consistent with this explanation.

Conclusions

The possibility of hybridization between red and sika deer subsequent to the introduction of the Japanese subspecies of sika (*Cervus nippon nippon*) to Scotland, has been the subject of some debate (Powerscourt 1884; Whitehead 1964; Lowe & Gardiner 1975; Harrington 1973, 1982; Ratcliffe 1987a for review).

It is clear from the above data that Japanese sika do

hybridize with red deer in Scotland, and that genetic introgression occurs in *both* directions. This supports the conclusions of Ratcliffe (1987a). Chronologically, the first hybridization events are the result of sika or hybrid stags invading red female ranges, but thereafter hybrids are produced from either female or back-cross. Selection pressure appears to be acting on multilocus traits in the sika phenotype rather than at a few individual loci, retaining strong associations between sika alleles. Patterns of linkage disequilibria and heterozygote deficit in populations where sika females are present are probably being maintained by a combination of heterozygote disadvantage and assortative mating (Barton & Hewitt 1885; Barton & Gale 1993). These factors may have a different importance in the sexes, but conventional measures of cytonuclear associations (Asmussen *et al.* 1987, 1989; Arnold *et al.* 1988) are unable to give information on directionality of mating as the assumptions of the available models are not met.

The exact mechanisms maintaining genic associations and the numbers of selected loci involved are not yet clear, but currently the subject of further study. If it proves to be the case that a selectively advantaged sika phenotype, involving many loci, remains coherent and is able to out-compete red deer in Scottish woodlands, the ability of the red deer phenotype to persist in this habitat must be questioned.

Acknowledgements

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