The Introduction of Sika Deer (*Cervus nippon nippon*) to Scotland

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Thesis submitted for the degree of Doctor of Philosophy
University of Edinburgh
1994

Contents.

Abstract 1

Preface 2

Chapter 1: Deer in Scotland 3

1.1. Biology of *Cervus* 3
1.1.1. Descriptions 4
1.1.2. Habitat 4
1.1.3. Feeding 11
1.1.4. Social groups, dispersal and ranging behaviour 12
1.1.4.1. Females 12
1.1.4.2. Males 14
1.1.5. Breeding 15
1.1.6. Discussion 17

1.2. Red deer in Scotland 21
1.2.1. History 21
1.2.2. Current interests and concerns 23

1.3. The Sika Introductions 25
1.3.1. The arrival of sika in Britain 25
1.3.2. Translocations, escapes and introductions 25
1.3.3. Establishment of sika in Scotland 26
1.3.4. Range expansion in the Great Glen 28
1.3.5. Range expansion in Argyll 28

1.4. Hybridisation 29
1.4.1. Overview 29
1.4.2. Hybridisation of red and sika in Scotland 30

1.5. The Aims of this Study 30
Specific aims 32

Chapter 2: The Cervidae 33

2.1. Distribution 33
2.1.1. Origins of *Cervus* 33
2.1.2. Current distribution 36

2.2. Taxonomy 36
2.2.1. Debate over classification of the Cervidae 39
2.2.2. Defining the relationship between taxa:
Cervus elaphus scoticus and *C. nippon nippon* 41

Chapter 3: Hybrid Zones 46

3.1. The Definition of Species 46

3.2. The Systematic Problem of Hybrid Zones............. 59

3.3. Describing Hybrid Zones 50
3.3.1. Overview.. 50
3.3.2. Dispersal-independent clines 52
3.3.3. Tension zones 52
3.3.4. Mosaic hybrid zones... 54
3.3.5. Associations between loci: linkage disequilibrium............................ 56

3.4. Creating Hybrid Zones...................................... 58
3.4.1. Waves of advance.. 58
3.4.2. Introductions 60
3.4.3. Moving Zones.. 61

3.5. Directional Introgression 62
3.5.1. Overview.. 62
3.5.2. Cytonuclear disequilibria... 63

Chapter 4: Methods in Cline Analysis 67

4.1. Study Areas and Sites...................................... 67
4.1.1. Overview.. 67
4.1.2. Argyll.. 69
4.1.2.1. Topography 69
4.1.2.2. Climate.. 69
4.1.2.3. Vegetation and Land Use.. 72
4.1.2.4. Deer population.. 72
4.1.3. Great Glen 73
4.1.3.1. Topography 73
4.1.3.2. Climate.. 75
4.1.3.3. Vegetation and Land Use.. 75
4.1.3.4. Deer populations.. 77

4.2. Census Methods 77
4.2.1. Overview.. 77
4.2.2. Observational Methods.. 78
4.2.2.1. Team Sweep Counts 78
4.2.2.2. Vantage point counts... 78
4.2.2.3. Census routes... 79

4.3. Laboratory Methods.. 79
4.3.1. Tissue Collection and Storage.. 80
4.3.2. DNA Extraction and Storage 80

4.3.3. Amplification And Electrophoretic Separation Of Microsatellite Marker Loci.. 81
4.3.3.1. The polymerase chain reaction....................................... 81
4.3.3.2. Microsatellites... 83
4.3.3.3. Polyacrylamide gel electrophoretic separation 86
4.3.3.4. Scoring the microsatellite gels 89

4.3.4. Amplification, Restriction Digestion and Electrophoretic Separation of Mitochondrial DNA Haplotypes 89
Overview 89
4.3.4.1. The PCR amplification of the 16s + ND1 region (ND genes in the NADH complex) of the cervid mitochondrial genome. 90
4.3.4.2. The principle and application of polymorphic RFLP loci 90
4.3.4.3. Scoring mtDNA gels 94

4.3.5. Electrophoresis and Staining of Species Diagnostic Isozymes 95
4.3.5.1. Isozyme use in population genetics 95
4.3.5.2. Isozymes in this study 96
4.3.5.3. Tissue preparation 102
4.3.5.4. Starch gel electrophoresis 102
4.3.5.5. Scoring isozyme bands 102

4.4. Analytical Methods 105
4.4.1. Maximum Likelihood 105
4.4.2. Cline width 106
4.4.3. F-statistics 107
4.4.4. Linkage disequilibria of nuclear loci 108
4.4.5. Cytonuclear Disequilibria 110

Chapter 5: Description and discussion of clines in *Cervus* in Scotland 111

5.1. Phenotypic Clines: data from censuses 111
5.1.1. Argyll 111
5.1.2. Great Glen 112

5.2. Clines in Gene Frequencies:data from nuclear loci 113
5.2.1. Argyll 113
5.2.2. Great Glen 115

5.3. Population Structure: heterozygote deficits 119
5.3.1. General remarks 119
5.3.2. Argyll 119
5.3.3. Great Glen 119

5.4. Associations between loci: linkage disequilibria 121
5.4.1. General remarks 121
5.4.2. Argyll 121
5.4.3. Great Glen 123

5.5. Cytonuclear Disequilibrium 125

5.6. Mitochondrial DNA Introgression 127
5.6.1. Argyll 127
5.6.2. Great Glen 127

5.7. Distribution of Genotypes 130
5.7.1. General remarks 130

5.7.2. Argyll 130
5.7.3. Great Glen 131

5.8. Rare Alleles 131

5.9. Discussion of Cline Characteristics 134
5.9.1. Summary of results 134
5.9.2. Single locus analysis 134
5.9.2. Multi-locus analysis 135
5.9.3. Conclusions 139

Chapter 6: Ecological Significance of Genotype: a study of winter diet 140

6.1. Diet as a Functional Response of Genotype 140

6.1.1. A brief theory of herbivore diet selection 140
6.1.1.1. Body size 140
6.1.1.2. Jaw shape and body allometry 141
6.1.1.4. Social organisation 143

6.1.2. Existing knowledge of red and sika diet selection 143
6.1.2.1. Feeding ecology of red deer in Scotland 143
6.1.2.2. Feeding ecology of sika deer in Japan 144

6.1.3. Competition and selection in mixed populations 145
6.1.3.1. Three possible interactions between individuals in a mixed population 145
6.1.3.2. Predictions for behaviour of the Scottish red and sika populations 146

6.2. Methods in Diet Analysis 148
6.2.1. Sampling and storing rumen contents 148

6.3. Particle Size Distribution in the Rumens 149
6.3.1. Overview 149

6.4. Botanical Composition 150
6.4.1. The use of rumen analyses to assess diet 150
6.4.2. Volumetric regression method 152
6.4.3. Dry weight separations 153
6.4.4. Common considerations and comparisons of the two methods 155

6.5. Assessing Diet Quality 157
6.5.1. Overview 157
6.5.2. Calculation of cell components from detergent residues. 158
6.5.2.1. NDF and cell solubles 158
6.5.2.2. ADF and hemicelluloses 158
6.5.2.3. Lignin, cellulose and ash 159
6.5.2.4. Diet quality 159
6.5.3. Sequential analysis of fibre composition of the rumen contents 159

Chapter 7: Winter Diet Selection in a Scottish Red-Sika Population 161

7.1. Botanical Composition of the Diet 161
7.1.1. Diversity of the diet 161
7.1.2. Plant community designation and use 164
7.1.3. Do animal characteristics of genotype and sex explain community use? ... 165
7.1.3.1. Analysis 165
7.1.3.2. Results 166
7.1.4. Dietary niche overlap 170

7.2. Fibre Composition 172
7.2.1. Does the botanical composition of the diet affect fibre composition? 172
7.2.1.1. Analysis 172
7.2.1.2. Results 172
7.2.2. Do animal characteristics of genotype and sex affect fibre composition of the diet? 174
7.2.2.1. Analysis 174
7.2.2.2. Results 174

7.3. Particle Size Distribution 174
7.3.1. Does lignification affect particle distribution in the gut? 177
7.3.2. Do animal charcteristics affect particle size distributions? 177
7.3.3. Once botanical composition is accounted for, do animal characteristics affect particle distribution? 177

7.4. Comparisons to Prior Work 179
7.4.1. Botanical composition 179
7.4.2. Diet quality and lignification 180

7.5. Limitations of the Data 180
7.5.1. Community use 180
7.5.2. Body sizes in relation to genotype 181
7.5.3. Genotype index 181

7.6. Summary of Results 182

Chapter 8: The Interaction Between Ecology and Population Genetics 184

8.1. The Selective Pressures on Individual Fitness of Red and Sika Deer 184
8.1.1. Fitness in red deer 185
8.1.1.1. Females 185
8.1.1.2. Males 185
8.1.3. Fitness in sika deer 186
8.1.4. The interaction of sex, size and time of fecundity 186

8.2. Diet Choice as a Function of Genotype and Sex in the Argyll Population 187
8.2.1. How do genotypes and sexes segregate? 188

8.2.2. Explanations of the patterns 189
8.2.3. Comparison to the predictions made in Chapter 6 for the behaviour of mixed populations.. 190
8.2.4. The potential for a mosaic type structure in the hybrid zone................. 191

8.3. Predicting the Further Spread of Sika Genes............ 192
8.3.1. Simulating the travelling wave 192
8.3.2. Initial comparisons of *Cervus* data to the models............................ 194
8.3.3. Discussion.. 196

8.4. Conclusions.. 197

8.5. Future Management of Mixed Sika-Red Populations.. 198
8.5.1. Management for damage control.. 198
8.5.2. Conservation genetics ?.. 199

Acknowledgements.. 200

References Cited 201

Appendices.. 216
Appendices for Chapter 4.. 216
Appendices for Chapter 6.. 221
Appendices for Chapter 7.. 229
Publications arising from the Thesis.. 230

Abstract

This thesis examines the genetic and ecological consequences of the introduction of exotic Japanese sika deer (*Cervus nippon nippon* Temminck, 1838) to the range of congeneric red deer (*Cervus elaphus scoticus* L. 1758) in Scotland. Sika were introduced as result of escapes and deliberate releases of park deer around the turn of the century and have since established feral populations in several locations. Most of these populations are achieving high reproductive rates, are currently expanding their range and are hybridising with red deer. These circumstances are of practical concern as red deer have considerable economic importance in Scotland, both for the positive values of stag stalking and leisure use of the hills and through the negative effect of damage to crops and forestry. The situation is also of biological importance as it provides a rare opportunity to assess the genetic structure of a hybrid population after an introduction and to examine the functional significance of hybridisation affecting quantitative traits.

Sampling transects were established across two sympatric sika-red population ranges, in Argyll and Invernesshire. Genotypes of 235 deer from nine forests in Argyll and 136 deer from 7 forests in the Great Glen, Invernesshire, were scored at 2 isozyme, 2 microsatellite and 3 mitochondrial DNA (mtDNA) marker loci. The species-specific mtDNA markers were generated within this study. Clines in sika allele frequency were found across both transects, with sika frequency reaching 0.8 in Argyll and fixation in Invernesshire. Sika are assumed to have at least one and probably several selectively advantaged genes to account for this consistent and rapid increase in frequency. There were significant heterozygote deficits and linkage disequilibria in the centre of the clines, even though samples in the Great Glen were small. MtDNA clines were steeper than nuclear clines, indicating the role of sika or hybrid stags in propagating the advance, but patterns of cytonuclear disequilibrium were not informative about mating pattern, due to the possibility of hybrid immigration and the influence of heterozygote deficit reducing sample sizes. There was no evidence of directionality in the F1 cross, though assortative mating in the backcrosses could contribute to the linkage disequilibrium observed, as could selection against hybrids.

The cline is a moving wave, not a stable hybrid zone, and the properties of that wave are considered using computer simulations and maximum likelihood analysis to test hypotheses about how selection may be acting on the sika genome. The number of genes involved in the selective advantage of sika is crucial to the prediction of how the genome, and thus the phenotype, may be robust to recombination or may breakup to form a completely introgressed population behind the wave. The behaviour of hitch-hiking nuclear markers is investigated under models of a single selected locus, and many selected loci and the predictions compared to the data in a case study.

As sika have been observed to have a higher reproductive rate in Scottish populations than red deer, and fertility is known to be related to body condition in females, the effect of genotype on winter diet choice was investigated in an intensive study in Argyll analysing rumen contents of genotyped deer for botanical composition, particle size distribution and fibre composition. Plant community selection was found to be affected by genotype and sex. Plant community use had a significant effect on the fibre composition and diet quality, though within animals choosing a plant community, genotype and sex explained no significant further variance. Diet choice was highly variable in terms of botanical content.

The results are discussed in terms of hybrid zone theory and how hybridisation blurs the theoretical concepts usually applied to niche separation and interspecific competition for food resources. If management goals are to be established for genetic conservation of the native red deer in Scotland, it is essential to understand the way in which sika genes introgress and the functional significance of hybridisation.

Preface

This project came about primarily through common concern expressed by myself, the Forestry Commission and many others involved in wildlife management and conservation in Scotland (particularly Scottish Natural Heritage, or the Nature Conservancy Council, as it then was) about the impact of the introduction of sika deer to Scotland.

Sika deer represent a new part in the ecosystem of the Scottish hills, and understanding their ecology is essential to full understanding of the dynamics of that ecosystem, enabling sensible use and management of it. However sika deer have a wider importance, both economically and scientifically; as a game animal, sika are a also possible source of commercial income and as a large herbivore they are a potential threat to both plantation forestry and natural regeneration; by interbreeding with red deer, they are altering the genetic make-up of the Scottish red deer population. The behaviour of a new genome introduced to a resident population has many implications for conservation of endemic species which may be threatened by hybridisation.

The arrival of sika in Scotland represented an opportunity to investigate the genetic structure of a forming hybrid zone, to look at the way selection was acting within it and perhaps to model future behaviour of this population in a way applicable to other introductions. It was also of practical significance to understand the interaction of red and sika deer in order to first define management goals for mixed populations in various circumstances, and to then propose ways of implementing them

The project was designed initially to document the extent of hybridisation with red deer and to investigate the feeding ecology of hybridising populations as a first step to understanding both competitive interactions between genotypes and also the potential impact of hybrid populations on their habitat. It is certain that sika deer will remain in Scotland for the forseeable future and that they must be included in policies for management of the habitat.

The subsequent work has gone some way to improving our knowledge of the way in which genes may be selected after an introduction and has made a start on unravelling the complex way in which sika are affecting the red deer population. However many more questions have been raised during the course of the work, the most pertinent of which relate to how strong a barrier to gene flow would be required to stop the advance of sika genes. The answer to this would be the basis of management policy to prevent hybridisation on the mainland, but is as yet elusive. The work presented here, along with the few other studies of British sika, represent only the beginning of a full understanding of the effects of the introduction.

Chapter 1.

DEER IN SCOTLAND

In section 1.1 I review the biology of *Cervus* deer, highlighting differences, similarities and potential conflicts between red (*Cervus elaphus*) and sika (*Cervus nippon nippon*) in Scotland. The ecology of an animal is closely related to its evolutionary history (and fate) and to the limits to variation between individuals, which is ultimately the material of selection. The review intends to introduce the study animals, to set the ecological scene for deer populations in Scotland and to highlight some of the selective pressures individuals may experience. The dynamics of mixed red and sika deer populations will ultimately be determined by the relative fitness of individual genotypes. Red deer have been studied intensely for many years in Scotland (Darling, 1937; Clutton-Brock, Guinness & Albon, 1982; Clutton-Brock & Albon, 1989) and their biology is well-known compared to that of sika. Initially the two species have been treated as separate units in this thesis, although hybridisation subsequent to the sika introduction has blurred boundaries between them and may require us to think about individual traits, such as body size, varying and being subject to selection across the entire *Cervus* population, rather than within each taxon.

In section 1.2 the history of red deer populations in Scotland is briefly reviewed, and the chronology of the sika introductions follows (1.3) with short notes on the documented interaction of the two species (1.4). The chapter concludes with a summary of the aims of this study (1.5).

1.1. Biology of *Cervus*

A general outline of *Cervus* deer in Britain is given, with possible red-sika differences highlighted where appropriate, based on the behaviour of comparable pure populations The section concludes with a summary of why these differences may have arisen and asks how they may affect interactions.

1.1.1. Descriptions

Figures 1.1.1a&b show the males and females of red and sika (*Cervus elaphus scoticus* and *Cervus nippon nippon*) respectively. Red deer in Scotland are bigger than sika, though body weights vary considerably between areas for both species (Red Deer Commission, 1960 - 1993; Whitehead, 1960; Chadwick, Ratcliffe & Abernethy, in press). Fig. 1.1.1c shows the body weights of phenotypically red and sika deer from a mixed Scottish population. In both species males are larger than females.

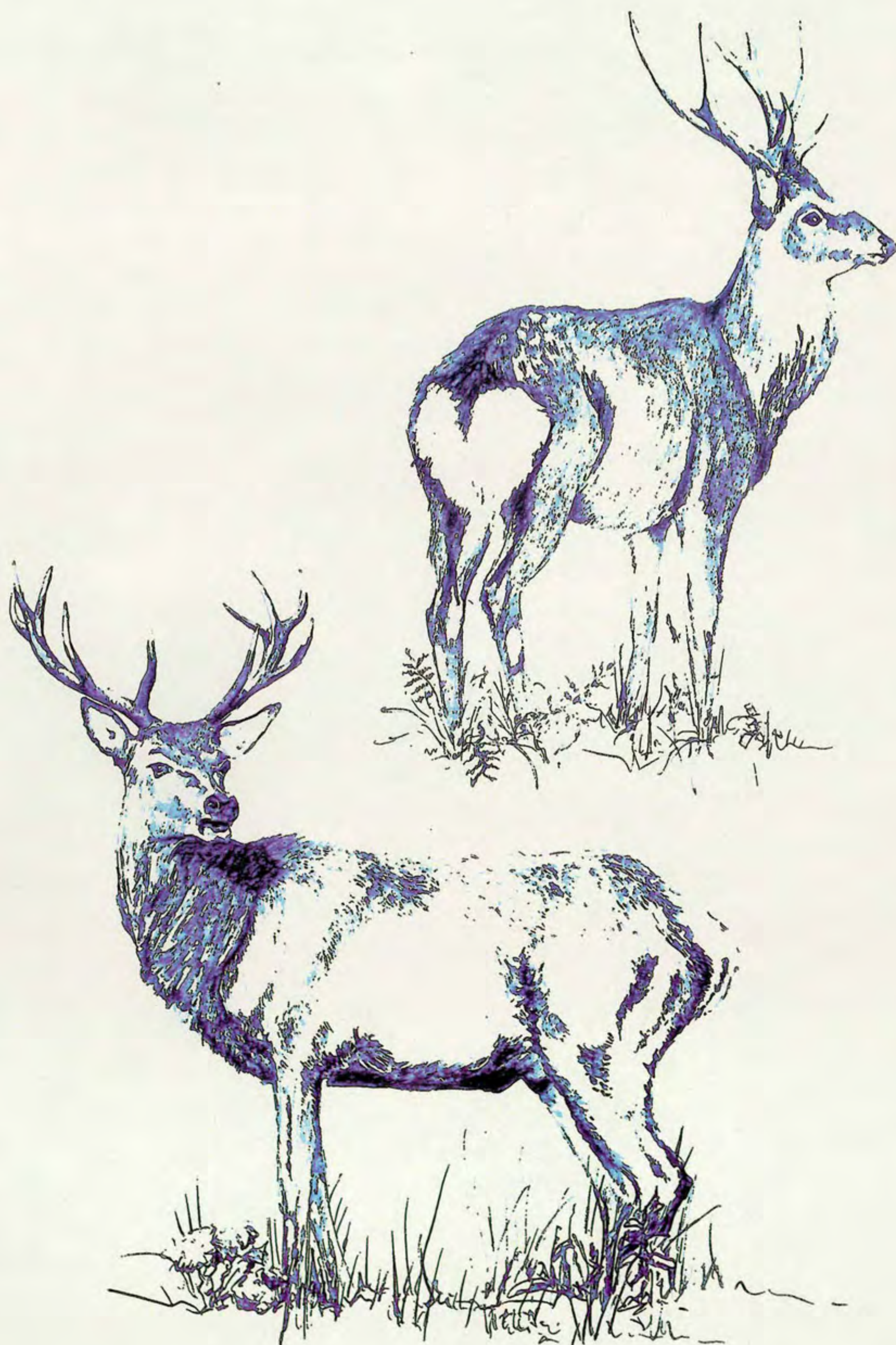
Red deer have a chestnut red summer coat, moulting to brown-gray in autumn and winter. The rump patch is cream to buff, merging to the body colour above the tail. Sika have a fawn-gray summer coat with lighter spots and a gray to black winter pelage, with the spotting becoming inconspicuous. In all seasons the caudal disc is a stark white, outlined with black above and occasionally around the legs. Sika have a variable black mark along the tail In Manchurian sika this is prominent, similar to fallow deer (see Whitehead, 1964), but in most sika seen in Scotland, which are probably of Japanese origin (Ratcliffe, 1987a), the stripe is not pronounced. Sika will flare the rump patch if alarmed. Sika also have a patch of long, white hairs over the metatarsal gland on the hock. Though this is present in red deer also, it is much less distinguishable from the coat colour (Harrington, 1979). Facial appearances of the two deer differ in the shape of the ears, which are long and pointed in red deer and broad and rounded in sika, and in the pale crescent brow marking of the sika, especially stags, which is absent in red.

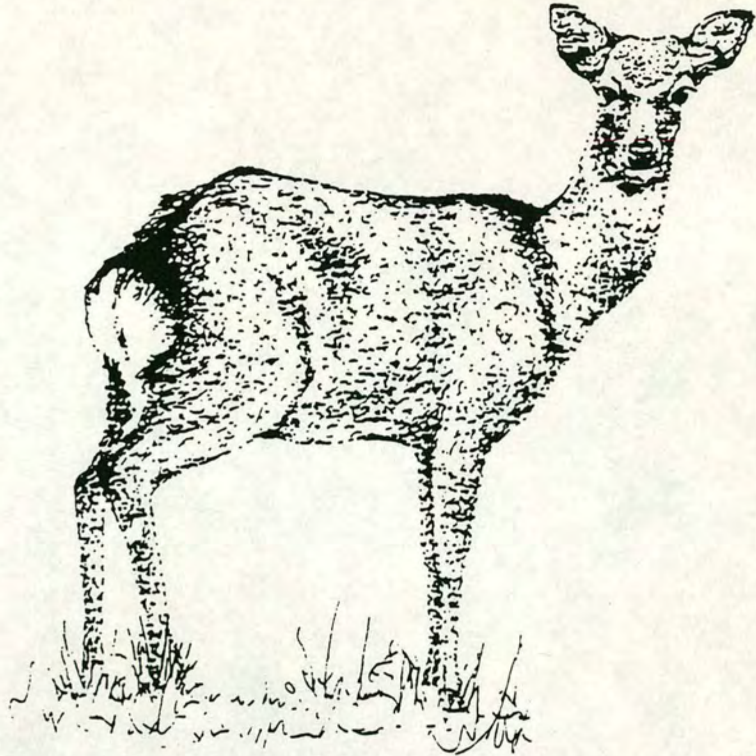
In both species only the males bear deciduous antlers. In sika these rarely grow beyond eight points (four on each antler), whilst red males commonly attain heads of 12-14 points and have been recorded with over 20 (Whitehead, 1960).

1.1.2. Habitat

In Britain red deer are assumed to have been originally a woodland animal (Lister, 1984), though severe deforestation has reduced much of the red deer's historical range to open moorland (Steven & Carlisle, 1959) and populations survive over much of Scotland without tree cover. Over the rest of its Eurasian range the red deer is generally still associated with woodlands (Dzieciolowski, 1979) and in Britain the remaining woodland populations perform better than those on open hill range

Figure 1.1.1a. Sika (upper) and red (lower) deer stags. In the smaller sika, the white rump patch is black-edged and the coat often spotted, whereas in red deer the rump is cream, the tail inconspicuous, and the coat unspotted. The pale brow crescent is very noticeable in sika stags. Antlers tend to be wider and more complex in red stags than sika.





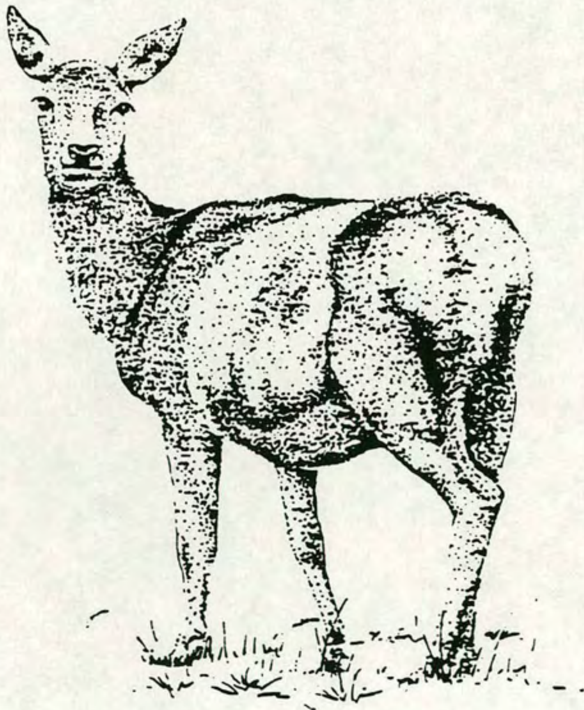


Figure 1.1.1b. Sika (*top*) and red (*lower*) females, or hinds. Note the conspicuous black edged rump patch and the rounded ears in sika. The pale brow mark of the sika is less prominent in hinds than in stags.

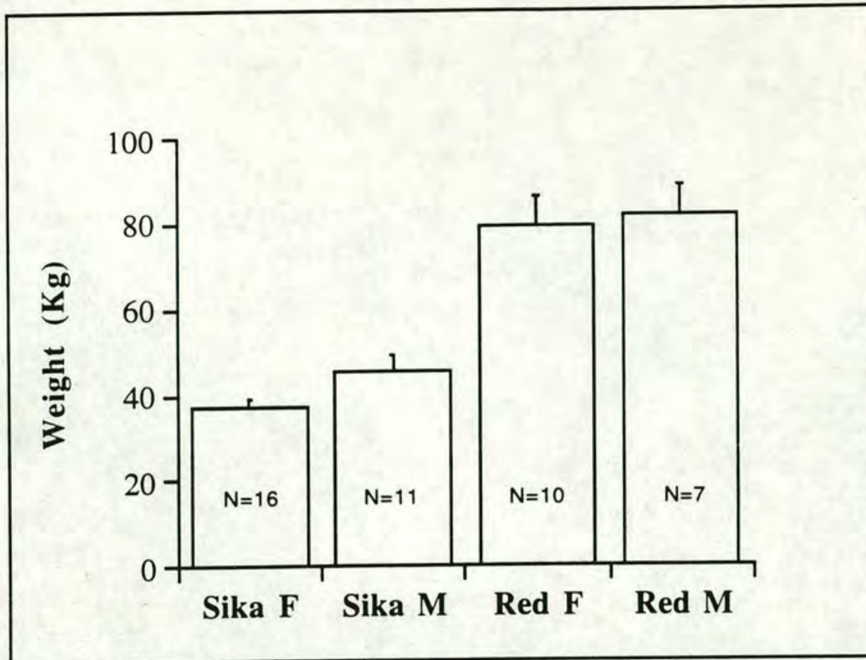


Figure 1.1.1c. Winter carcase weights of phenotypically red and sika adult deer from Inchnacardoch forest, Invernesshire. Bars show standard errors of the mean. Weights are equivalent to live weight minus blood, viscera, head and legs. Data from Forestry Commission (this study).

(Mitchell, 1973; Mitchell, Staines & Welch, 1977). Reforestation of Scotland with conifer plantations has recreated shelter habitat for deer. In the early stages of growth a plantation is particularly favoured habitat (Ratcliffe, 1987b), providing shelter and forage, however these forests become devoid of forage once they reach closed-canopy stage (Fig.1.1.2a&b). Deer may be adversely affected by lack of shelter (Verme, 1968) and Grace & Easterbee (1979) have argued convincingly that heat loss through exposure could affect the performance of open hill red deer in Scotland. They show that an equivalent animal on open ground may lose almost twice the energy of one in woodland at a given windspeed. Shelter availability has been invoked as a factor limiting deer distribution in Scotland (Staines, 1976). Native woodland, mainly oak-birch (*Quercus-Betula*) mixtures or Scots pine (*Pinus sylvestris*), do provide forage in the interior (Fig 1.1.2c&d), but this habitat is now scarce. The effect of the loss of native woodland may have been to alter both feeding and ranging behaviour, and also to reduce population performance.

Sika are also a woodland deer in their native Japan (Horwood & Masters, 1981) and in Britain have remained associated with woodlands, both deciduous and coniferous (Mann, 1982), though populations have recently been reported on open hill range (Red Deer Commission, 1993). In Japanese populations feeding is

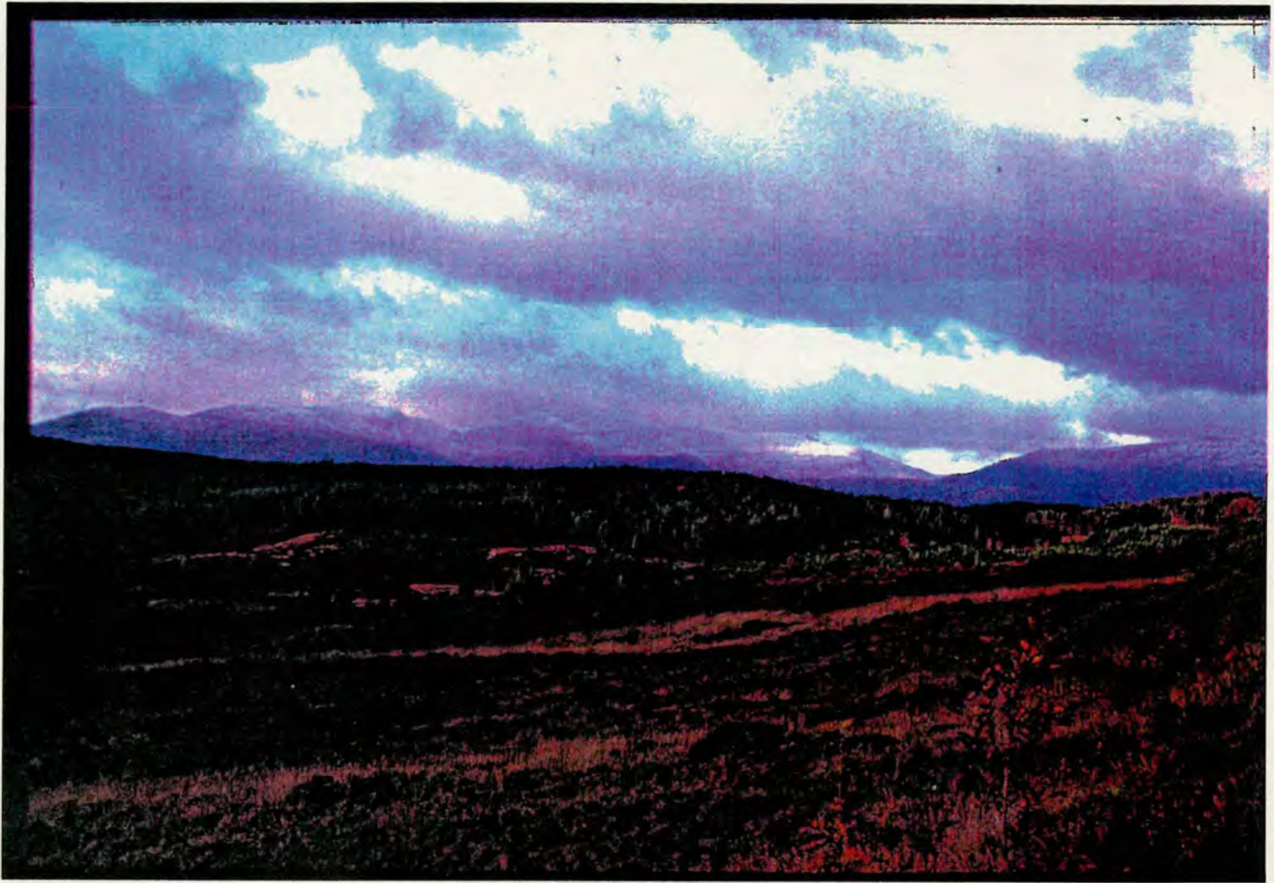


Figure 1.1.2a&b. Coniferous plantation forestry. **a**) above, shows a typical plantation area in Scotland where trees have reached the closed canopy stage. **b**) below, shows the interior of the same forest, devoid of forage material.

Figure 1.1.2c&d. Native Scottish woodlands. a) above shows a sika hind in a deciduous woodland with abundant forage. b) one of the few remaining native pine woods, Abernethy Forest, which provides excellent habitat for deer.

often nocturnal, with deer using open areas in woodland mosaics and sheltering in the tree cover during the day (Koga & Ono, 1994).

Snow cover may be range limiting in both species (Watson & Staines, 1978). In Japan a snow depth of greater than 50cm in winter defines the limit of sika range (Takatsuki, 1987).

Heat loss is greater from animals of large surface area to volume ratio. If lack of shelter results in a severe energy drain it should exert a selective pressure for increased body size (Geist, 1987), perhaps ameliorating the effect of heat loss from stags (Staines, 1974). However large size and poor body condition after the extreme demands of the rut, mean that stags have a larger absolute energy requirement, decreasing their ability to meet demands over winter and resulting in longer grazing bouts during which they are exposed to chilling. There is some evidence that male sheep have lower fat reserves and higher metabolic rates then females, increasing their heat loss (Slee, 1970). and Clutton-Brock, Guinness & Albon (1982) argue that this balance, if true in deer, may result in stags being *more* susceptible to exposure and account in part for increased winter mortality in stags. As sika are smaller than red deer (Fig.1.1.1c) they may be expected to suffer more intensely from exposure and to be selected against in populations on open range. Culled sika from woodland show greater winter kidney fat deposition (Table 1.1.2) than red deer on similar range, suggesting that, if their physiologies are otherwise similar to red deer, they may either enter the winter with higher reserves than red deer, or that their energetic demands on those reserves are lower. It is unclear if this advantage will persist in open areas.

	Sika		Red	
	Female	Male	Female	Male
Kidney Fat	0.66	0.26	0.25	0.1
SE	*0.05*	*0.06*	*0.04*	*0.001*

Table 1.1.2. Kidney fat as a measure of body condition of phenotypically identified deer in Inchnacardoch forest, Invernesshire. Rangers assess the amount of fat covering the kidney of each carcase on a scale 0-1 where 1 is totally covered, 0 is completely lacking fat. Similar, though more complex, measures have found to be representative of condition (Albon *et al.*, 1986). Data from Forestry Commission (this study). Kidney fat is assumed to be an equal measure of condition in each species.

Habitat fragmentation may be important to deer (Ratcliffe, 1985; Ratcliffe, 1987b) . A hectare that is 50% woodland distributed in a mosaic pattern with open glades will provide cover and feeding over its total area, whereas if that woodland is in one dense block, deer will need to leave cover to feed. With plantation forestry the

latter situation is the more common, although pre-thicket and growth-checked stages do increase habitat heterogeneity. The use of habitat by sika is still not well understood in Britain.

1.1.3. Feeding

Hofmann (1985) describes sika as intermediate feeders tending towards grazers and red deer as intermediate tending towards concentrate selectors, in their native habitats. In Britain both deer feed on a variety of swards and are often seen feeding in the same areas, though rarely as mixed groups (pers. obs). Stags and hinds occupy separate feeding ranges for most of the year (Watson & Staines, 1978; Clutton-Brock, Guinness & Albon, 1982; Mann, 1982; Koga & Ono, 1994). Mann (1982) found sika to be grazers in areas of coniferous plantation where they fed nocturnally on surrounding fields, but to browse more in a deciduous woodland area where shelter and feeding areas were combined. These results are largely supported by Dzieciolowski (1979) looking at sympatric red and sika in Poland.

There is a large body of literature relating to diet selection in ungulates. Much of the theoretical writing centres around the 'body size concept', which suggests that, as nutritional requirements are a function of the body size (W), scaling to $W^{0.75}$, yet absolute requirements vary with W, then larger animals require more bulk of a lower quality than small animals (Bell, 1971; Jarman, 1974) This has been applied both in interspecific and intersexual comparisons in sexually dimorphic species. Illius & Gordon (1987) modelled the allometric relationship between body size, jaw shape and bite size and suggest that only a 20% difference in body size could account for effective exclusion of the larger animal, once sward height reaches a critical threshold. This gave a good prediction of the distribution of male and female red deer foraging on short-grass preferred swards or *Calluna* heath in Scotland (Staines & Crisp, 1978; Staines, Crisp & Parish, 1982; Clutton-Brock, Iason & Guinness, 1987). There are now several studies which have found differences in male and female dispersion and diet quality in ungulates which may be explained by body size differences (Watson & Staines, 1978; Staines Crisp & Parish, 1982; Putman, Culpin & Thirgood, 1993; Weckerly, 1993), but others where the data are equivocal and other factors such as shelter use or sociality are invoked to explain habitat use (Beier, 1987; Weckerly & Nelson, 1990). In temperate habitats herbivores face most depleted resources in winter and this should be when selection is strongest. Within a guild of herbivores this may result in resource partitioning, where, if the body size concept is correct, grazing competition with smaller species forces larger species to utilise inferior resources in

times of severe scarcity (Schoener, 1986). Although the hypothesis was formulated to explain differential selection in a closely related group of species, the predictions have been upheld when applied to unrelated groups of sympatric ungulates (Gordon & Illius, 1989).

As there is more than a 20% difference in body size between red and sika it may be expected that sika could oust red from short grazed swards and this argument is used to explain the exclusion of red deer from forest colonised by sika in New Zealand (Kiddie, 1962; Davidson, 1979). Competitive exclusion of a group due to body size may not correspond to exclusion of a particular species in a hybridising and dimorphic population where body size varies both with sex and genotype.

1.1.4. Social groups, dispersal and ranging behaviour

During most of the year both species are found in single sex feeding groups, with males and females using different ranges (Clutton-Brock, Guinness & Albon, 1982; Mann, 1982; Koga & Ono 1994). The rut season is the exception, when the sexes are necessarily associating together. The behaviour of males and female during the rut is discussed separately in section 1.1.5. Like many polygynous mammals, post-natal dispersal is sex-biased in *Cervus* deer (Greenwood, 1980) and range sizes are greater in males than females.

Females

Red deer calves remain with the mother at least until the next calf is born (1-2 years) and female offspring continue to associate with their mothers throughout their lives (Guinness, Hall & Cockerill, 1979), though male calves spend little time associating with hinds after three years old (Mitchell, Staines & Welch, 1977). The mother with her calf and (perhaps yearling offspring) is the basic social unit, with associations between related hinds forming larger groups (Darling, 1937; Clutton-Brock, Guinness & Albon, 1982). Red deer female group sizes can be associations of over 100 animals (Fig 1.1.4a) and are often several dozen on moorland deer range (Red Deer Commission, 1960-1993; Whitehead, 1964).

In sika there are rarely more than ten animals together and usually 2-3 females with their calves is a maximum group size (Dzieciolowski, 1979; Horwood & Masters, 1981; Mann, 1982) Group sizes during Mann's study in the early 1980's varied from only 1.7 - 2.7 in the New Forest and 2.6 - 5.4 in a coniferous woodland in Dorset, even though both populations were at high density (approx. 20/km^2). The basic unit is still the mother-offspring pair, but sika seem less willing to aggregate than

Figure 1.1.4a. A large red hind group in open habitat in winter. Aggregations of this size are not seen in British sika populations.

red. Habitat may account for some of this variation in that most observations of red deer are from open habitat and the few of sika are for woodland populations (Harris & Duff, 1970). Dzieciolowski (1979) found that red deer sympatric with sika were found in smaller group sizes than in four other forests studied where red deer were alone, despite the area actually having the *least* forested area of his five sites. Unfortunately he does not describe the fragmentation of the habitat which may have a profound effect. It will be interesting to note the behaviour of the growing sika populations in open habitat in Britain (McLean, 1993). In extremely high density populations (600-800/km^2) on Nozaki and Kinkanzan islands in Japan, mixed-sex winter groups of up to 100 animals have been observed on preferred grassland feeding grounds (Takatsuki, 1980; Takatsuki, 1983; Takatsuki, 1984; Koga & Ono, 1994).

Lifetime ranging patterns of females are more restricted than males, a trend which is perhaps more pronounced in sika, though the ratios of daily range sizes are variable between populations (compare Darling, 1937; Clutton-Brock, Guinness & Albon, 1982, and Davidson, 1973). The extensive ranging of breeding males accounts for the increased overall dispersal (Fig. 1.1.4b). Adult sika females collared in a mixed open-deciduous woodland habitat in New Zealand were found up to two years later within 410m of the capture site, though the mean dispersal distance was 1.7 kms (Davidson, 1979). Mann (1982) and Horwood & Masters (1981) found similar movement in adult sika in England, of which 75% of marked hinds (N=12) remained within 1km of the mark site over four years and the maximum movement was of 1.2 kms. Rates of breeding sika population colonisation have been comparable in New Zealand; 0.6 km/yr (Davidson, 1973), 1.6 km/yr (Caughley, 1963) and Britain; 3-5 km/yr (Ratcliffe, 1987a).

Ranges of red deer hinds on Rum have been shown to be highly correlated to the area of short grass sward that they contain, and food distribution is likely to be of primary importance in range definition, although predation or culling pressures may also have profound influences (Staines, 1974).

Males

Male sika deer associate both with hinds and together in feeding groups, except during the rut when aggression is intense between males (Davidson, 1983). Likewise in red deer, aggregations are common between males outside the breeding season. Younger stags (immature) often form groups within an age cohort (Mitchell, Staines & Welch, 1977) unlike females who associate most closely with relatives. These male groups are loose and tend to deteriorate with age, old stags becoming increasingly solitary (Clutton-Brock & Albon, 1989). Red deer populations have been

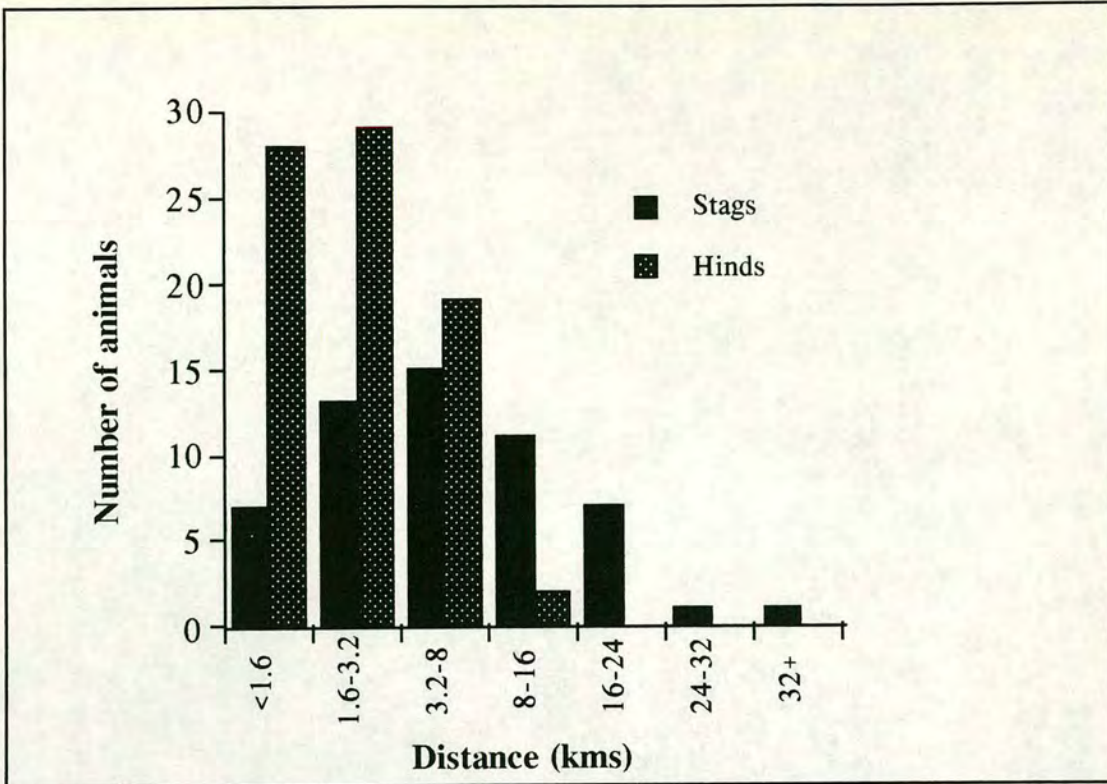


Figure 1.1.4b. Dispersal distances of adult red deer in North Ross, marked at birth and shot >2yrs old. Stags disperse long distance during the rut and may remain resident in a new area after first dispersal. There is no evidence that dispersal distance is related to age after 2 yrs. Hinds do not disperse more than 16 kms, with the majority remaining within 5 kms of the natal site. Data from Red Deer Commission, 1974 - 1988.

found to vary in the amount males and females share range. Differential use of vegetation probably largely accounts for the degree of geographical separation of the sexes (Clutton-Brock, Guinness & Albon, 1982; Clutton-Brock & Albon, 1989).

Large dispersal movements of males of both species occur annually at the rut, and in early life males may make a significant move from their natal area which is not repeated. Thus it is difficult to disentangle seasonal from lifetime dispersal without detailed and longterm observations (Davidson, 1979).

1.1.5. Breeding

Both red and sika reach sexual maturity in their first year under ideal conditions, but often in wild populations this is delayed (Mitchell & Lincoln, 1973; Blaxter *et al.*, 1974). The mating system is polygamous, with males competing for mates in an autumn rut during which the females come into oestrus. Single offspring

Figure 1.1.5. Red deer rut on open range. A stag (centre) controls a large group of hinds, attacking rival stags if challenged.

are born each year in spring (mid May to late June in Britain) and females lactate for several months, sometimes continuing to suckle the calf for over a year until the next offspring is born (Guinness, Hall & Cockerill, 1979). Fecundity in females is related to body size, condition and reproductive status (Mitchell, 1973; Albon *et al.*, 1986; Suzuki & Ohtaishi, 1994).

On open hill red deer males hold females in a harem group (Fig. 1.1.5) , with which they mate as the females come into oestrus (Clutton-Brock, Guinness & Albon, 1982). Males challenge and fight each other for harem possession and likelihood of paternity is increased with the length of time the female is held in the harem (Clutton-Brock, 1985). Thus body size and antler size and configuration, which increase the fighting ability, are related to male reproductive success (Clutton-Brock & Albon, 1985).

In woodland red deer the harem structure is less obvious, perhaps because of the physical difficulty of a stag watching a large group of hinds in woodland, perhaps because feeding patches for hinds are smaller and more dispersed in woodland, making females less willing to associate as a large group. In Britain sika have not been observed to have a structured harem system during the rut (Mann, 1982). Females tend to be found in small groups and are often not associated with the stags who remain solitary in woodlands, using a distinctive whistling call to advertise their presence to others. Males are sometimes said to behave territorially, marking territories along a woodland edge and intercepting hinds as they move from shelter to feeding areas (Horwood & Masters, 1970; Mann, 1982), though in Scotland sika stags do occasionally challenge red stags for harem ownership (E. Fraser, pers. comm). Japanese and New Zealand populations of sika are documented as exhibiting both the harem system during the rut (Kiddie, 1962; Koga & Ono, 1994) and also more territorial behaviour (Davidson, 1973; Miura, 1984).

1.1.6. Discussion

As sika and red deer are thought to be descended from a common ancestor (Lister, 1984; Geist, 1987) we could ask how and why these phenotypic, behavioural and possibly physiological differences between them have arisen? Differentiation, though not necessarily speciation; (Ayala, 1975) is thought to be the result of genetic drift and subsequent selection (natural or sexual) in isolated populations (Wright, 1931), and/or the result of accumulated genetic mutations (which may be selectively neutral) in populations isolated for long periods (Kimura, 1983). It is reasonable to assume that during the Pleistocene colonisation of Eurasia *Cervus* populations may

Summary Comparison

Red Deer

1. *Bodysize (approx.)*
   Males 110 - 180 kg
   Females 70 - 110 kg

2. *Antlers*
   Complex, including brow tine
   Up to 20 points

3. *Group sizes*
   Basic mother-offspring unit
   Large associations > 100 animals
   Sexes segregated except at rut

4. *Dispersal*
   Females remain close to the natal area, often sharing maternal range
   Males may disperse over 20km from the natal range, perhaps much more. They also disperse seasonally to rut.

5. *Feeding Strategy*
   Intermediate feeder, tending toward browser.
   Males and females choose different swards; female range is highly correlated with distribution of food patches. Males less so

6. *Mating System*
   Polygyny
   Harem sizes large on open range, smaller in woodland
   Males compete for harem control
   Challenges involve roaring and threat and can result direct combat

7. *Reproductive success*
   Female fertility is dictated by body condition, size and reproductive status, of which body condition is most important, making seasonal competition for food important in females.
   Male success depends on competitive ability in the rut.
   Fighting success is related to body size and early growth is thus important in male calves

Sika Deer

1. *Bodysize (approx.)*
   Males 30 - 60 kg
   Females 20 - 40 kg

2. *Antlers*
   Simple configuration
   Up to 8 points

3. *Group sizes*
   Basic mother-offspring unit
   Common associations of upto 10
   Sexes partially segregated

4. *Dispersal*
   Females remain close to natal area, often sharing maternal range
   Males disperse much farther though it is unknown how far, at least 20kms. Seasonal dispersal is unknown as males are secretive

5. *Feeding Strategy*
   Intermediate feeder, tending toward grazers.
   Sexes may feed in mixed groups
   Males and females may select different plant communities

6. *Mating System*
   Polygyny
   Both territorial and harem structures documented. Most populations tend toward male territoriality and harems are generally small, though systems are flexible.
   Males have a whistling cry which may warn other males of their presence in woodland

7. *Reproductive success*
   Female fertility is dictated by body condition, size and reproductive status, of which body condition is most important, making seasonal competition for food important in females.
   Male success depends on competitive ability in the rut.
   Bodysize, is likely to be important, but there is no indication that male calves are born larger than females

have been temporarily isolated by climatic change and have certainly now become effectively isolated by distance as two ends of a 'ring species'. Although the presence of differentiated characters does not imply selection over and above genetic drift, consistent directional trends in characters do, and many such trends, for instance in antler dimensions, group size, mating strategy or body size are found in the Cervidae and in particular through the *Cervus* ring (Geist, 1987). The differences between red and sika are not only phenotypic (and genotypic) but behavioural also and should be considered in combination. Differences relating to reproduction and reproductive success (fitness) are those of prime importance in the context of hybridising populations, the theme of this thesis.

If red and sika in Scotland are filling different niches (Hutchinson, 1968) and are not competing for resources, then they may exist in effective sympatry. However evidence suggests that they may overlap in diet (Kiddie, 1962; Hofmann, 1982; Mann, 1982; Hofmann, 1985) and that through hybridisation they are also competing for mates. Hybridisation involves gene flow between the two populations, allowing universally selected traits to increase throughout.

Both species have a polygynous mate system, which has been shown to correlate in mammals with increased male competition, sexual dimorphism and weaponry in males as female group sizes, and therefore the benefits of victory, increase (Emlen & Oring, 1977). In deer, the antlers' primary function is in combat (Clutton-Brock, 1982) where they have both an offensive and defensive role, the importance of good defence perhaps increasing with size as the danger (cost) of a fight increases (Geist, 1966). Antlers represent a huge investment by males which would be expected to correlate with a high potential gain to their owners. As male reproductive success is entirely determined by ability in competition for females, there being no male parental care, there is large variance in male success (Clutton-Brock, 1985) and selection would be expected to favour traits that enhance competitive ability (Trivers, 1972). Competitive weapons like antlers should therefore be under intense selective pressure. (Clutton-Brock, Albon & Harvey, 1980) compared the antler length of size-matched species with different levels of polygyny and found that in those where males were able to monopolise most females, antlers were largest. The effect was more pronounced in sika than red and sika also have higher sexual dimorphism (Whitehead, 1964 and see above) thus we might expect to find that sika are more highly polygynous, yet in introduced populations (without red deer present) this seems not to be the case (Dzieciolowski, 1979; Horwood & Masters, 1981; Mann, 1982), implying that there may be other factors influencing mating system (Bubenik, 1987).

Clutton-Brock & Albon (Clutton-Brock & Albon, 1985) also show that within a species body weight and age are the best predictors of fighting success and harem holding, which are highly correlated to true reproductive success (Clutton-Brock & Albon, 1989) and when matched for these factors, differences in antler dimensions are only very weakly correlated to success. If sika and red males are competing for mates within a polygynous system it seems that sika males must be disadvantaged due to their absolutely smaller body size, though variation in fighting technique may afford them some success (Kiddie, 1962; Geist, 1966; Clutton-Brock *et al.*, 1979).

The breakdown of the harem structure in the transition from woodland to open habitat has been noted in several deer species (Harrington, 1979; Bartos & Zirovnicky, 1982; Mann, 1982; Takatsuki, 1983; Hanley, 1984; Miura, 1984; Fraser & Sweetapple, 1992) and in mammals the decline in group size with increasing vegetation density occurs in many species (e.g. White, 1993). As sika display predominantly the characteristics of a woodland deer in small group sizes, high-pitched calls and some territory marking (see above), whereas scottish red deer seem to be more typical of an open-range species there may be a habitat component to the social structures and behaviour found in mixed red-sika populations.

In polygynous deer, competition for food is likely to be most intense in females, who rely on short term annual improvements in body condition for reproductive success (Albon, *et al.*, 1986; Suzuki & Ohtaishi, 1994) . Thus, in the absence of severe predation, female ranges should be dictated by food availability. Clutton-Brock, Guinness & Albon (1982) have shown that in red deer on Rum this prediction is upheld. Red and sika females would be expected to be in direct feeding competition in sympatric populations. This competition is reduced by selection of different plant communities (Gordon & Illius, 1989) or geographical separation, but competition may be intense especially in times of low abundance (winter). When variation in reproductive status and body condition are accounted for fertility in red deer hinds is negatively correlated with body size (Albon *et al.*, 1986). This may describe an advantage for the sika trait of small size.

A large part of the nutritional requirement of ungulates is explained by body size, even between species (Bell, 1971), though mate competition is between males, food competition will include all animals. In mixed populations will smaller sika displace red hinds on similar sward? By the same measure, will sika hinds be the best overall competitors? Will competitive interaction result in winter resource partitioning? Winter diet in mixed populations and evidence for genotypic effects on resource use are examined in Part 3.

.c.1.1.7. Summary

- In polygynous deer male fitness is determined by combative success, favouring large body size, antler size and dimorphism.
- Although sika have relatively large antlers and are more dimorphic than red they appear to be less polygynous in British populations
- Differences in mating systems may have a species-specific component, but are also likely to be influenced greatly by habitat.
- In Britain red deer populations tend toward a harem structure, sika tend toward a male territorial system, but there is obviously considerable overlap and in mixed populations the behaviour of hybrids is not known. The outcome of hybridisation between red and sika, and the resulting modification of quantitative traits may shed light on the factors that truly bestow fitness advantages.
- Fitness is determined differently in the sexes and, as such, traits universally favoured in males may not be so in females and *vice versa*
- Females may be in interspecific feeding competition
- As feeding competition has a body size component red females may also be in competition with sika males.
- As, in an introduction, the migration of individuals is critical to the spread of the new population (see also Chapter 4) data on the ranging behaviour of sika, and the competitive interactions with resident red deer will be expedient to understanding the factors potentially limiting species ranges.

1.2. Red Deer in Scotland

1.2.1. History

Scottish red deer populations have been closely associated with man throughout history as a staple food source (Jarman, 1972; Lister, 1984), but in the last three centuries this relationship has been changed dramatically as a result of social revolution in the human population (Red Deer Commission, 1981). During the 15th and 16th centuries Scotland was gradually deforested as timber demands increased, a situation common to much of Europe (Ritchie, 1920). This lead to the creation of

tracts of heath land and rough sward below 600m, the natural tree line, though these were largely unused for pasture. After 1745, after the breakup of the clan system of land stewarding, sheep farmers rapidly spread north to claim this resource and the deer population seems to have declined as a result of grazing competition from high sheep density (Ritchie, 1920) However the industrial revolution in England, providing a wealthy Victorian gentry who took up Highland deer stalking, soon reversed this trend. In the 1800's landowners were buying up enormous tracts of land as 'deer forest' or stalking estates and clearing sheep off (Mitchell, Staines & Welch, 1977; Red Deer Commission, 1981; Scottish Natural Heritage, 1994) Red deer numbers seem to have steadily increasedfrom then (Fig. 1.2.1a) (Watson, 1983).

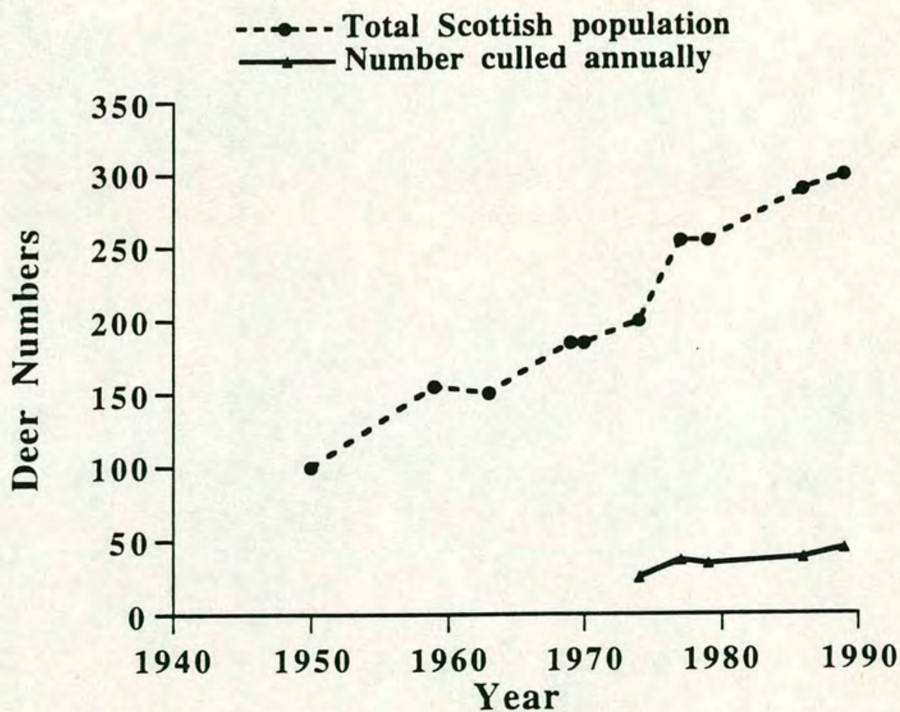


Figure 1.2.1a. The increase in red deer numbers in Scotland. Data are from the Red Deer Commisson Annual Reports (1960-1993). Numbers are calculated for each of 48 counting blocks, assuming equal density across the area of the block. Some authors (Clutton-Brock & Albon, 1989; Callander & MacKenzie, 1991) believe these figures are too low and should take into account local differences in density due to variation in habitat quality. Current estimates lie below 300,000, due to heavy culling and high winter mortality in the last three years.

The detrimental effect of dense deer populations and particularly marauding stags, had already become a source of great strife in the Highlands between crofting populations and estate owners (Clutton-Brock & Albon, 1989), and early this century the impact of a rising deer population was noted on native woodlands and other habitats (Ritchie, 1920; Darling, 1937). In 1948 the Deer (Scotland) Act was put through Parliament to address this conflict. It allowed farmers to kill marauding deer on enclosed land. In the decade that followed deer suffered continued and occasionally inhumane poaching pressure and in response to this in 1959 the Act was amended and the Deer (Amended) Scotland Act instigated close seasons for the killing of red deer. These were set in 1962 and remain as October 21st to June 30th for stags and February 16th to October 20th for hinds, though deer damaging crops may be shot at any time. The Act also provided for the inception of the Red Deer Commission to control and conserve red deer populations in the country. The Red Deer Commission's authority was later extended by the Deer (Amendment) Scotland Act, 1982, to control of sika, roe and fallow deer. The new Commission began regular deer counts in Scotland and estimated the population to be at 150,000 in the early 1960's. The estimate was around 300,000 in 1990 (Red Deer Commission, 1960-1993), but severe culling pressure and winter mortality in the last three years are thought to have reduced that figure (Gordon-Duff-Pennington, 1994). Continued pressure from commercial deer estates, forestry groups, conservationists and land access groups have shaped the role of the Red Deer Commission and thinking on the management of red deer in Scotland. In recent years conflict of interest between these groups (Callander & MacKenzie, 1991; Grant, 1994) together with rising deer numbers has forced a major rethinking of the way deer and land in Scotland are managed (Scottish Natural Heritage, 1994) and legislation regarding deer is currently under review .

1.2.2. Current interests and concerns.

Red deer cause concern in three major areas of land use; agriculture (including commercial forestry), stalking and leisure use of the hills. The major points on which people in these areas are in conflict are reviewed in both the Scottish Natural Heritage policy paper on red deer (1994), and the Rural Forum report on wild red deer management (Callander & MacKenzie, 1991). One central problem exists from which others have stemmed: the goal of managing deer populations - more deer or less deer? Traditionally farmers and foresters have aimed for few deer, culling heavily if possible, whilst stalkers have wanted many deer, culling lightly in favour of stags

which also represent capital value. As Highland property is valued on game returns, one stag shot is worth around £20,000 on a property price. The economic value of highland red deer is primarily in the experience of open ground stalking, rather than the worth of the venison, making hind culls uneconomic. Attempts at commercial venison farming have been largely unsuccessful (Blaxter *et al.*, 1974) and the cost of bringing carcases off the hill makes sale of wild shot venison unattractive, although increased marketing is changing this. Those interested in the deer and their habitat as resources of aesthetic value fall between these two, not wishing to eradicate the deer, but trying to reduce their impact on the habitat, particularly native forest, which in Scotland is severely pressurised from overgrazing and lack of natural regeneration.

Much of the conflict between people managing deer range has arisen from the juxtaposition of estates managed for different purposes, but dealing with essentially the same deer population. To try to reconcile these differences the Red Deer Commission, with the support of the Forestry Commission, Scottish Natural Heritage and the Scottish Landowners Federation have established local deer management groups (DMG's) which bring together local land managers and interested parties to decide common policy and culling targets. The wide publication of data documenting changes in sex ratio, antler size, body condition and fertility associated with increasing hind density on Rum (Clutton-Brock, Guinness & Albon, 1982; Clutton-Brock & Albon, 1989) and research into the effect of target culls on population growth (Ratcliffe, 1987b) have done much to alter traditional estate management practices, where the emphasis was on increasing hind numbers and culling trophy stags.

There is unanimous agreement that the deer population in Scotland is too high to be compatible with *any* of the current management goals: commercial forestry damage is high; deer wintering grounds are degraded by overgrazing or have been lost to fenced forestry, resulting in winter mortality and poor carcase quality; native woodlands are unable to regenerate. The Red Deer Commission has a stated goal to reduce the population by 100,000 (33%) as a first step to future management that will aim to conserve the red deer as an integral part of thriving forest and heathland ecosystems. There is increasing evidence that deer do have a positive role to play in forest ecosystems, creating gaps and structural diversity that may even increase regeneration (Sykes, 1992) and restoration of a balanced forest ecosystem is an objective of the Red Deer Commission and Scottish Natural Heritage. The Commission has a difficult role to play in balancing the demands of the forestry, leisure and stalking lobbies, but population reduction seems an essential first step.

Sika deer have shown higher reproductive rates than red in scottish forests (Chadwick & Ratcliffe, unpub.), they are secretive and are proving hard to control

(McLean, 1993), they hybridise with red deer and they have smaller body sizes and smaller antlers. These characteristics seem to satisify no-one. The foresters and conservationists are worried by increasing population growth and therefore damage, the stalkers by reduced trophy sizes and carcase weights, and the aesthetes by the challenge to the "Monarch of the Glen", a symbol of Scottish heritage. In the next section I review the history of sika deer in Scotland.

1.3. The Sika Introductions

1.3.1.The arrival of sika in Britain

Sika deer were first brought to Britain 1860 when the Zoological Society of London imported both Japanese sika (*Cervus nippon nippon*) and Dybowski's sika (*C. n. hortulorum*) to the zoo at Regent's Park (Whitehead, 1950; Ratcliffe, 1987a). At the same time Viscount Powerscourt brought four animals, a male and three females, to his park at Powerscourt in Wicklow, Eire (Powerscourt, 1884). The Powerscourt sika were kept in a closed 1000 acre park alongside several other cervid species; red deer, wapiti, axis deer and sambur. The axis deer (chital, *Axis axis*) are said to have expired fairly rapidly in the damp climate of Wicklow, as are the sambur (*Cervus unicolor*), which lived only a few years and though several hybrids were produced with red deer, these also died. The presumed effect of the climate on these deer motivated the Viscount to bring sika to Powerscourt, thinking that they would cope better with the harsh weather. This they proved to do and within 25 years of their introduction the four founders had increased to over 100 deer (Powerscourt, 1884). They also hybridised with the red deer, and in the recognised hybrids (probably the F_1 generation only) the mother was always a red deer.

1.3.2. Translocations, escapes and introductions

Viscount Powerscourt made gifts of sika to many landowners during the late nineteenth century, and though he does not specify the numbers involved, he states in his 1884 paper that he believes all the new herds to be thriving. These herds were then in Ireland at Muckross in Killarney, Glenstal in Limerick, Castlewellan in Co. Down,

Colebrook in Co. Fermanagh, and in England at Melbury in Dorset and Aylesbury in Berkshire.

There are still park populations surviving in Aylesbury and Melbury and in three other places from these very early documented movements of sika, but Ratcliffe (1987) documents many feral populations in the British Isles which have unknown origins. The present feral populations can be traced back to 13 founder populations, only one of which is certainly Japanese sika and which gave rise to the present herds at Dawyck in Peebleshire and Pixton in Devon. These herds are a melanistic strain without the white rump patch and with an entirely black coat, perhaps *Cervus nippon keramae* (Whitehead, 1964). Of the other feral populations, not only is the subspecific status of the founders unclear, but many parks that held deer at the turn of the century had both red deer and Manchurian and Japanese sika, potentially producing both inter- and intra-specific hybrids before other parks were stocked (Whitehead, 1950).

The two populations studied in detail here are of unknown origin, but believed to be Japanese sika, as are most of the wild British populations (Whitehead, 1964; Lowe & Gardiner, 1975). The Bowland and Poole basin populations in England were thought to be Manchurian sika (Delap, 1967), but subsequent study of cranial morphometrics has classed them as Japanese sika (Lowe & Gardiner, 1975; Ratcliffe *et al.*, 1992).

1.3.3. Establishment of sika in Scotland

Several authors have reviewed the status and distributions of sika populations in Britain (Whitehead, 1964; Whitehead, 1972; Horwood & Masters, 1981; Carne, 1982; Ratcliffe, 1987a; Rose, 1994) the most extensive of which is Ratcliffe's (1987a) paper, which describes in detail the colonisations of Argyll and Invernesshire. The distributions assessed by those authors, along with records held by the Institute of Terrestrial Ecology Biological Records Centre, are shown in Fig. 1.3.3. Several populations in Scotland have become well established and are increasing their numbers and extending their range; the Dawyck herd in Peebleshire, and the populations in Kintyre, the Great Glen and Sutherland cover most of the scottish sika, but there are also populations around Tulliallen in Fife, Ledgowan in Rossshire, Glenmazeran in Invernesshire, and Berriedale in Caithness (Whitehead, 1964; Ratcliffe, 1987a). Introductions were also made in the early 1900's at

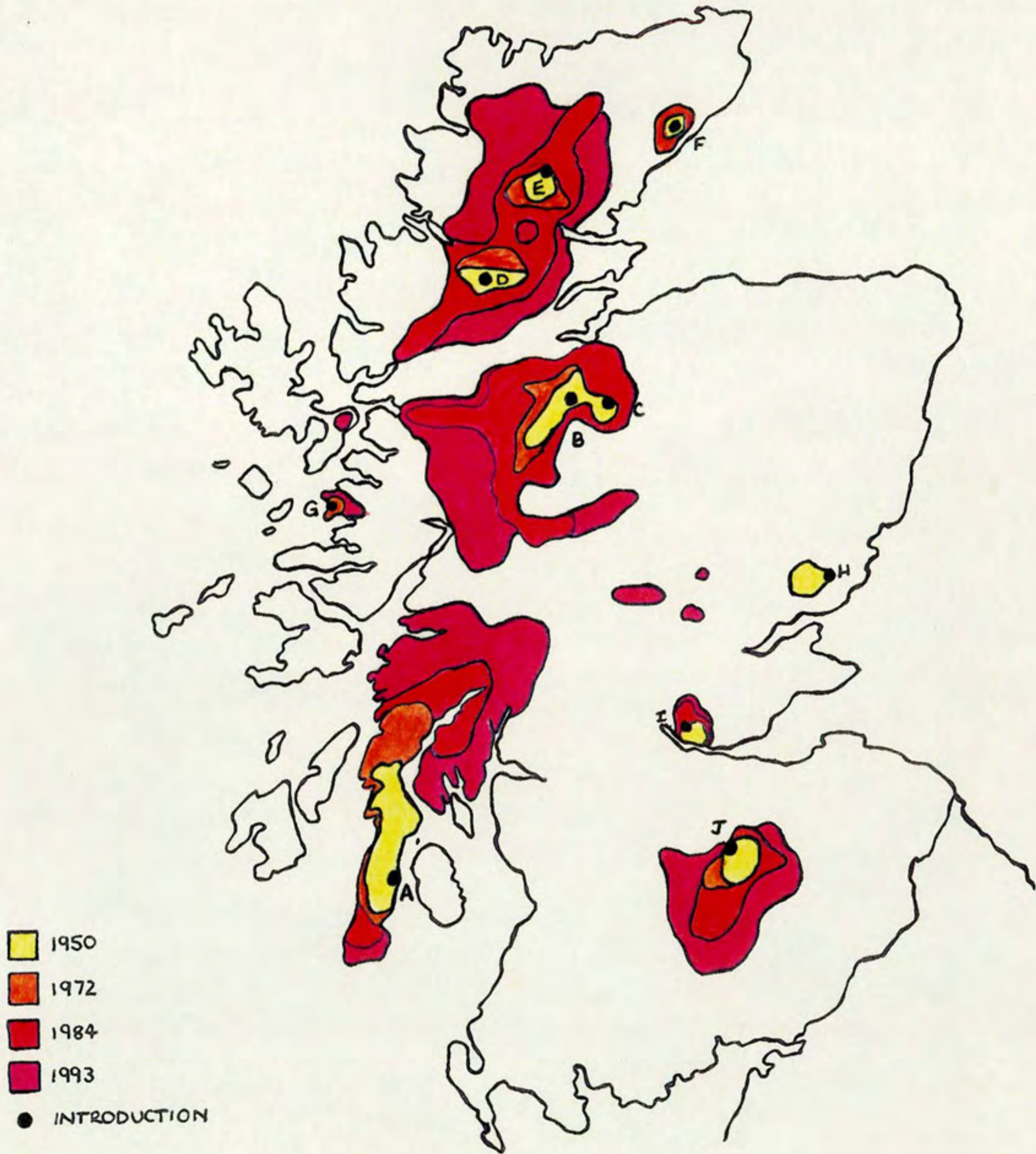


Figure 1.3.3. Sika range expansion in Scotland. Ranges are mapped from Biological Records Centre data of sightings and so give maximum ranges, rather than breeding population ranges. Introduction points are marked **A**) Carradale, **B**) Aldourie, **C**) Glen Mazeran, **D**) Rosehaugh, **E**) Ledgowan, **F**) Berriedale, **G**) Loch Morar, **H**) Kinnaird, **I**) Tulliallen, **J**) Dawyck. In the most recent records (1993) sika have been sighted in several new areas, most notably on the Isle of Skye.

Rosehaugh on the Black Isle and at Kinnaird in Angus, but sika are no longer reported in these areas (Rose, 1994).

Sika have tended to colonise through woodlands, making much less use of open hill areas than red deer (Ratcliffe, 1987). Their local distribution in much of upland Scotland is restricted to the glen bottoms where remnants of deciduous oak-birch woods remain, or to areas of dense conifer plantation, such as those along the banks of Loch Ness (Horwood & Masters, 1981). The exception to this is the Sutherland population which is rapidly expanding onto open range (Red Deer Commission, 1993) and now forms around 5% of the North Ross area population (C. McLean, pers. comm.). Prior to the 1980's this was exclusively red deer range, monitored regularly by Red Deer Commission counts (Commission, 1960-1993).

1.3.4. Range expansion in the Great Glen

There were two introductions of sika to Invernesshire (Fig.1.3.3), at Glenmazeran in 1900 and at Aldourie in 1898 (Whitehead, 1964). The herd at Glenmazeran apparently did not perform well and remained within the glen for several years which Whitehead (1964) attributes to the harsh winters and lack of shelter. By 1962, however, the population had reached 30 individuals from around 6 original founders (from the Fawley Court herd, Ratcliffe, 1987) and is now established and expanding (C. Lavin pers. comm.). In contrast, the Aldourie introduction of 8 animals, from the Sutherland herd, established a resident population very quickly and sika were seen up to 32 miles south of Aldourie by 1952 (Whitehead, 1964). This population now ranges throughout the Great Glen and sika, presumably originating here, are seen in to the south in Glen Roy and Glen Spean, and at Kingussie and Newtonmore at the east end of Glen Spean; to the west of Loch Ness in Glen Urquhart and Glen Affric, Glen Moriston and Glen Garry (Rose, 1994; pers. obs.). The range is possibly even larger than this.

1.3.5. Range expansion in Argyll

Eleven sika deer were brought to Carradale in Argyll (Fig. 1.3.3) in 1893 and were kept enclosed on Carradale headland until the First World War, when presumably less attention was paid to the fencing and escapes were made. Whitehead (1964) states that "a few" deer swam around the fence where it reached the shore and

established a feral population in the woods around Carradale in the early 1900's. This population has expanded throughout the last century, colonising throughout the Kintyre peninsula, around the north of Loch Fyne and into the Cowal peninsula. The Crinan canal at the head of Kintyre provided a temporary barrier to spread but this had been crossed by the 1960's and subsequent spread through the area to the north was rapid. By the early 1980's sika were found on the Cowal peninsula, over 350kms by land from Carradale (Ratcliffe, 1987a). In Argyll there are detailed records of first sightings of sika in particular districts from which maximum speeds of colonisation can be estimated. Ratcliffe estimates the mean rate of colonisation at 3-5km/year, but notes that occasional stag sightings may precede resident hinds by 10 years. Interpretation of these data is somewhat confounded by the likelihood of hybridisation. If hybrids are unlikely to be recognised as sika, then these figures will underestimate the impact of any successful matings of a few sika stags. However, once sika densities become high enough that hybrids are crossing back to sika, a sudden rapid increase in sika-like animals may occur, despite the introgression of sika genes having been at a fairly consistent rate. This is in fact what has occurred in Argyll, and (anecdotally) in other areas of Scotland.

1.4. Hybridisation

1.4.1. Overview

Gray's (1972) 'Checklist of Mammalian Hybrids' records interbreeding between many species of *Cervus* and between members of *Cervus* and other genera within the Cervini (see Chapter 2). Between red and sika Bartos, Hyanek & Zirovnicky (1981) report a hybrid zone between parapatric Chinese wapiti and Manchurian sika at the Ussuri river in east Manchuria, but this is the only example I know of hybridisation between red and sika in their natural ranges. Post-introduction hybridisation is reported in New Zealand (Davidson, 1973), Czechoslovakia (Bartos, Hyanek & Zirovnicky, 1981; Bartos & Zirovnicky, 1981; 1982), England (Lowe & Gardiner, 1975), Scotland (Ratcliffe, 1987a; Abernethy, in press) and Eire (Harrington, 1973, 1979, 1982). Figure 1.4.1 shows an F1 hybrid from a red female sika male cross (Harrington, 1979).

1.4.2. Hybridisation of red and sika in Scotland

The chance of hybridisation between wild red deer and Japanese sika was originally thought to be low as body sizes were considerably different, even though animals in Powerscourt park had hybridised easily (Powerscourt, 1884). This view persisted until the latter half of this century when hybrids were reported in many areas of Britain (Whitehead, 1950, 1964; Delap, 1967; McNally, 1969; Lowe and Gardiner, 1975) and in other populations (Davidson, 1973; Bartos, Hyanek & Zirovnicky, 1981). Concern over the possible hybridisation with red deer was still slow to arise, despite reports of rapid and complete introgression in populations of red and sika in Ireland (Harrington, 1973,1982) and England (Lowe and Gardiner, 1975) as these researchers believed that either the larger, mainland form of sika (*C. n. hortulorum*) was involved (Lowe & Gardiner, 1975) or that the original hybrids were of park origin and that admixture continued through backcrossing and breeding of the F_2 generation through reduced size difference in the hybrids. Ratcliffe (1987) reviewing the status of sika deer in Britain reported many cases of putative hybrids in Scotland where the feral sika were thought to be Japanese races. He then voiced serious concern over the genetic impact of sika on scottish red deer.

1.5. The aims of this study

The major gaps in the knowledge of sika in Britain were highlighted by Ratcliffe in his 1987 review of their status and distribution. He saw the lack of data on population dynamics, dispersal and density of populations as a major obstacle to the formulation of management plans, and the lack of information on the extent and incidence of hybridisation as a problem for conservation strategies for scottish red deer (see also (Greig, 1973). The need for a thorough assessment of extent and distribution of hybrids in Scotland has since also been declared a priority, alongside a survey of mixed population demographics and ecology, in the civil service policy paper on red deer (Heritage, 1994).

The advent of molecular markers suitable for populations surveys has paved the way for a new branch of biology - the synthesis of population genetic and ecological data (Burke, Seidler & Smith, 1992; Avise, 1994). Sika in Scotland present both an ecological problem and a rare situation in population genetics, especially in mammalian systems. This study aims to apply the new approach, combining molecular and ecological methods, to these deer populations.

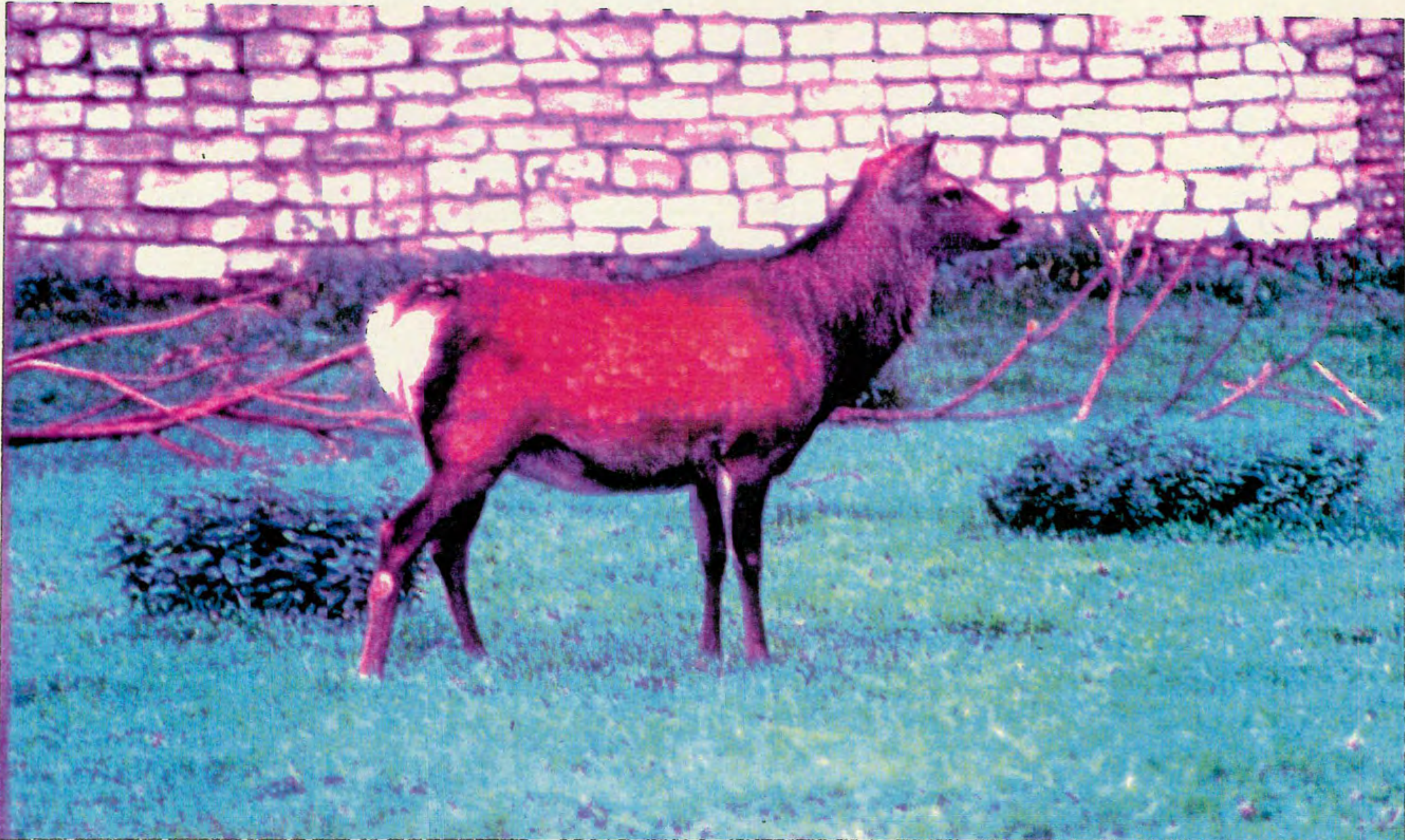


Figure 1.4.1. An F1 red-sika hybrid. The mother in this case was a sika hind. Note the white, sika-like, metatarsal gland and rump, but red like body shape. Hybrids in the field are very difficult to recognise after the F1 generation.

Specific aims are to:

- Ascertain the extent of hybridisation in two large mixed populations in Scotland.
- Describe the genetic structure of the hybrid zones.
- Model the future genetic structure of the hybridising populations.
- Examine functional effects of hybridisation on diet selection.
- Provide information on which to base management decisions, if appropriate, for the genetic conservation of both scottish red deer and introduced sika.

Chapter 2

THE CERVIDAE

This section contains a brief description of the distribution and taxonomy of the red and sika deer in Britain. Much more detail is available on both species in the literature (e.g. Whitehead, 1964; Harris & Duff, 1970; Mitchell, Staines & Welch, 1977; Clutton-Brock, Guinness & Albon, 1982; Mann, 1982; Putman, 1988; Clutton-Brock & Albon, 1989), but most is beyond the requirements of this study. Here the characteristics of *Cervus* are outlined to allow readers to put the following work in context and to enable them to appreciate its implications.

2.1. Distribution

2.1.1 Origins of Cervus

Two major theories of the mechanism of *Cervus* evolution have developed and are reviewed by Lister (1984), Groves & Grubb (1987) and Geist (1987). The first, and more widely accepted theory, supported by all three of these reviews, is that a common, probably sika-like, ancestor arose in eastern Asia, and its descendants colonised west across Asia and Europe and east across the Bering Strait into North America. From this main colonising lineage, branches established to the south and north in Eurasia (Fig. 2.1.1a). Geist (1987) explains these as the result of minor colonisations from glacial refugia as the Pleistocene ice ages periodically forced populations south, in some cases leaving relict populations in southern districts. Selection and genetic drift during this consistent migration west have produced the races of red deer and wapiti found in Eurasia. A cline in antler form, body size and other quantitative traits can be recognised both across this geographical cline and in the fossil record, supporting the hypothesis (Lydekker, 1898; Lister, 1984; Geist, 1987) .

The second theory of *Cervus* origins designates a wapiti-like deer as the common ancestral form living in east Europe (Harrington, 1985). Colonisation

Figure 2.1.1a. Origins of *Cervus* in Eurasia. The ancestral form is thought to have been sika like, perhaps *Cervus perrieri*, arising in Asia in the Middle Pliocene. Antler forms become more complex across the western migration route, and in general body sizes increase, though European red deer are smaller than the continental maral or asiatic wapiti now found in Siberia. Cervids colonised the Americas across the Bering Straits, giving rise to the present North American wapiti. The Odocoileini also arose in Eurasia, but divergence probably occurred before the colonisation of America.

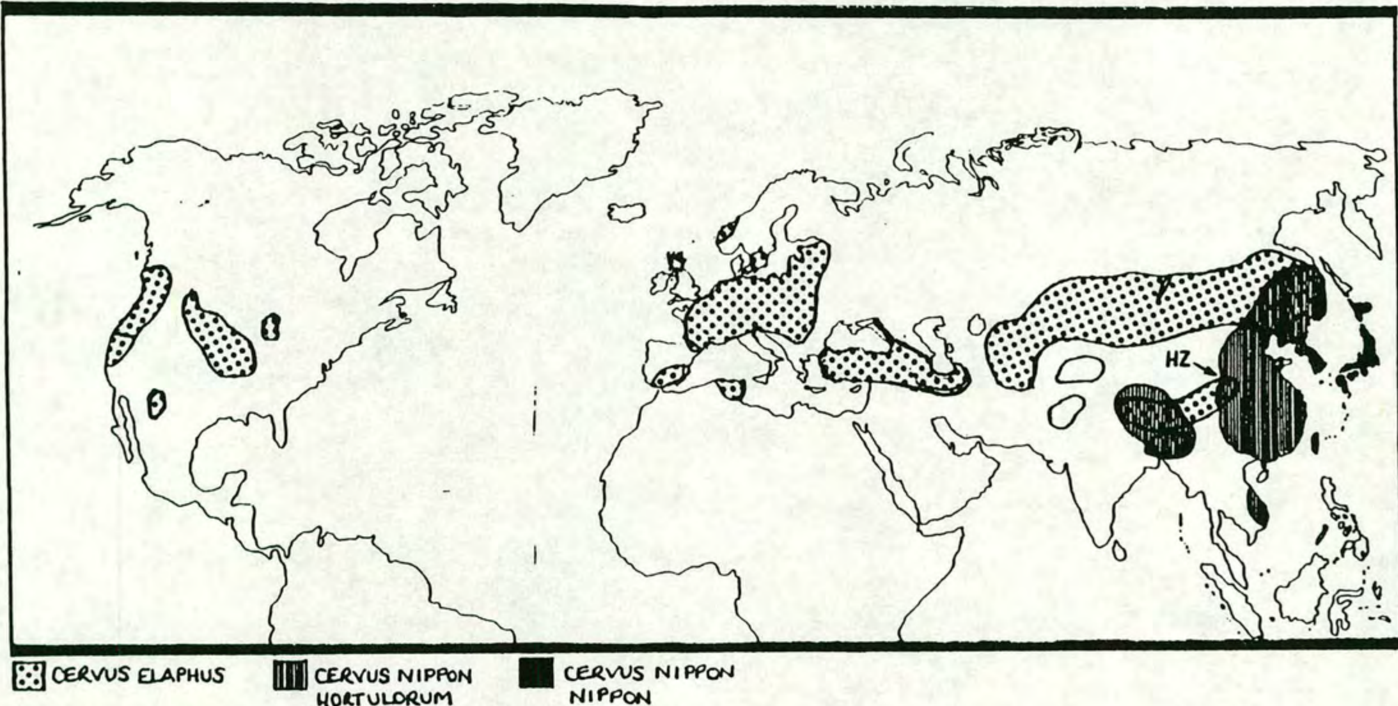


Figure 2.1.2 , The natural ranges of *Cervus elaphus* and *Cervus nippon*.. The various subspecies ranges are not differentiated, but are indicated in Table 2.2.1b. Asiatic and North American *C. elaphus* are sometimes classified as *Cervus canadensis* subspecies (see section 2.2). *C. elaphus* subspecies are known as red deer in Europe, wapiti in Asia and as wapiti or elk in North America. Hybridisation is reported between *C.e. xanthopygus* and *C.n.manchuricus* at the Ussuri river (marked **HZ**). Other cases of reported hybridisation are subsequent to introductions of one or both species in other countries.

of Japan across a series of land bridges in the late Pleistocene then gave rise to the present-day sika, whose reduced size is an adaptation to island life. Harrington supports this hypothesis with immunoelectrophoretic data showing reduced variation in sika from Ireland, which he explains as evidence for multiple founder effects during these colonisations. Unfortunately the reduced variation in his sika sample can be adequately explained by the single founding event when sika were introduced to Ireland (Linnell & Cross, 1991) and does not therefore necessarily support this hypothesis.

2.1.2. Current distribution

Currently cervids are found across the whole of the Palearctic region. The distributions of the subfamilies Odocoileinae and Cervinae are broadly parapatric, though they are in sympatry over regions of North America. The distributions mapped in Figure 2.1.2 follow Whitehead (1972). Hybridisation is found between introduced populations and in a few natural populations, though designation of hybrid zones between races is difficult when the degree of differentiation between them is unclear. Known regions of hybridisation are indicated.

2.2. Taxonomy

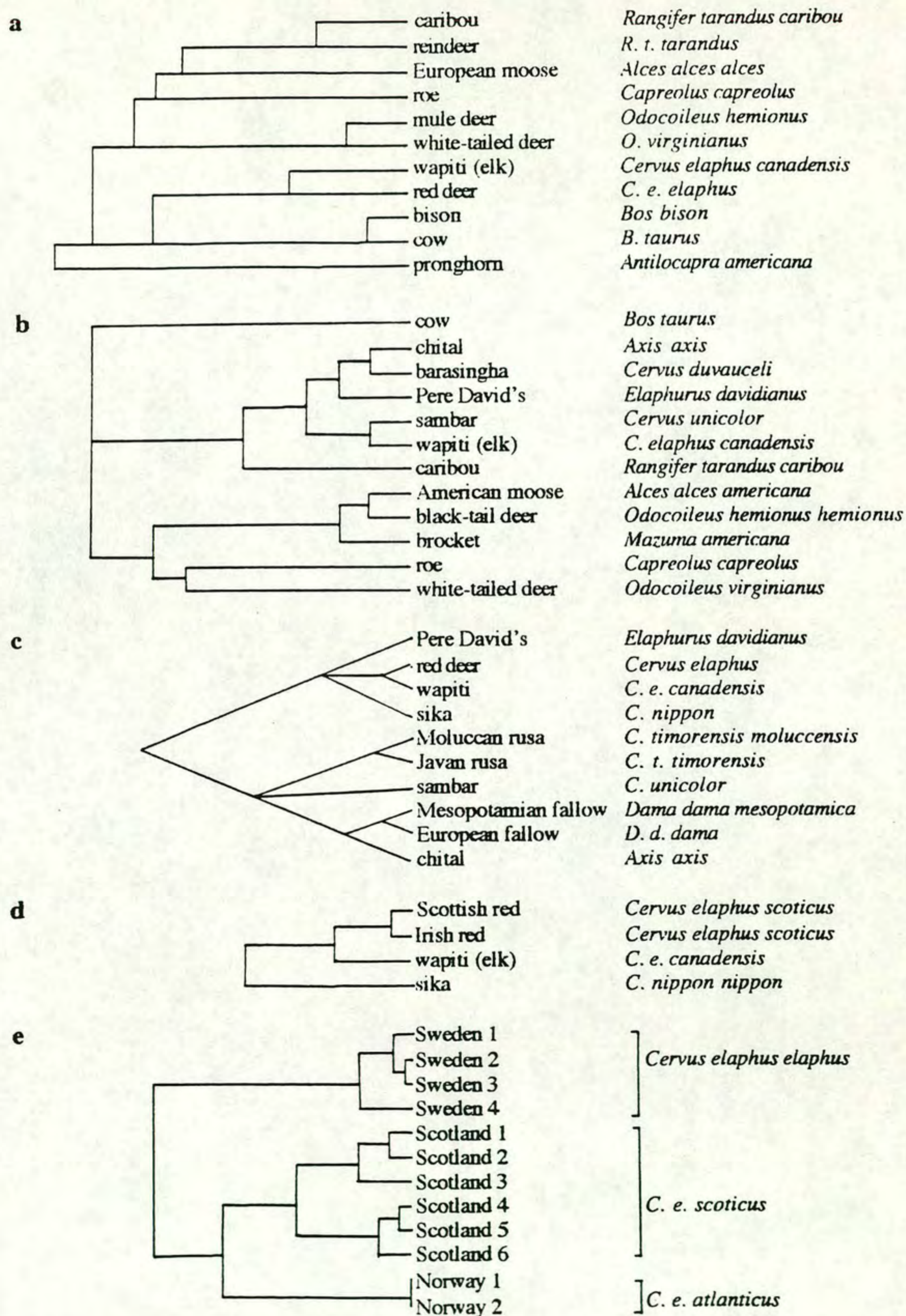
In order to discuss the implications of hybridisation between red and sika deer, or indeed between any two taxa, it is essential also to understand their prior relationship on an evolutionary scale. Unfortunately the taxonomy and evolutionary history of the family Cervidae, which includes red and sika, is still the subject of considerable debate, the roots of which are important to any conclusions about the significance of their hybridisation (Caughley, 1971). In this section, I review current knowledge and opinion, consensus and disagreement, on the classification and origin of the two groups. In the discussion in Part 4, I refer to the points raised by this debate and their implication for the status of sika and hybrids in Scotland.

Figure 2.2.1a. Classification of Red and Sika Deer.

The following account is derived from the work of Ellerman & Morrison-Scott (1951) and Simpson (1945) themselves working largely from Lydekker's (1898) account. Classification up to the family level has been widely accepted, but substantial debate persists on taxonomic classification within the subfamily and especially within the genus *Cervus*, to which both red and sika deer belong (see text).

Rank	Taxon	
ORDER:	**ARTIODACTYLA**	
Suborder:	SUIFORMES	
Infraorder:	SUINA	
Family:	Suidae	
Suborder:	TYLOPODA	
Family:	Camelidae	
Suborder:	**RUMINANTIA**	
Infraorder:	TRAGULINA	
Family:	Tragulidae	
Infraorder:	**PECORA**	
Superfamily:	**Cervoidea**	
Family:	Giraffidae	
Family:	**Cervidae**	
Subfamily:	**Cervinae**	(Pleisiometacarpalia)
Tribe:	Hydropotini	
Tribe:	Muntiacini	
Tribe:	Cervini	
Genus:	Dama	
Genus:	Axis	
Genus:	**Cervus**	
Subgenera:	Rusa	(H. Smith, 1827)
	Rucervus	(Hodgson, 1838)
	Thaocervus	(Pocock, 1943)
	Panolia	(Gray, 1843)
	Sika	(Schlater, 1870)
	Przewalskium	(Flerov, 1930)
	Cervus	(Linnaeus, 1758)
Genus:	Elaphurus	
Subfamily:	Odocoileinae	(Telemetacarpalia)
Tribe:	Capreolini	
Tribe:	Alcini	
Tribe:	Odocoileini	
Tribe:	Rangiferini	
Superfamily:	Bovoidae	
Family:	Bovidae	
Family:	Antilocapridae	

Figure 2.2.1b. Dendrograms showing relationships within a) Artiodactyla (Baccus *et al.*, 1983). b) Cervidae (Cronin, 1991). c) Cervinae (Emerson & Tate, 1993). d) Cervus (Linnell & Cross, 1991). e) *Cervus elaphus* (Gyllensten *et al.*, 1983).



2.2.1. Debate over classification of the Cervidae

The classification of the Cervidae has been the subject of academic endeavour since Linnaeus first gave systematic names to deer in 1758. Intense debate has arisen and still continues over the origins and relatedness of members of the genus *Cervus*, which comprises both Old and New world wapitis and moose, pan-European red and fallow deer, Asian axis and rusa deer and the oriental sika. Several recent papers have tackled the systematics of this group (Gustavsson & Sundt, 1968; Baccus *et al.*, 1983; 1987; Miyamoto, Kraus & Ryder, 1990; Emerson & Tate, 1993) or portions of it (Lowe & Gardiner, 1974, 1975, 1989; Lister, 1984; Herzog, 1988; Linnell & Cross, 1991; Cronin, 1991b) and whilst some themes arise, many differences are still outstanding in the final descriptions of the group. Figure 2.2.1a shows a classification of the cervids to subgenus level, based on the work of Simpson (1945) and supported by Ellerman & Morrison-Scott (1951). This classification up to the subfamily division of the Cervinae and Odocoileinae, based on the presence or absence of the lateral metacarpals (Brooke, 1848) is now largely accepted, although relationships between the tribes and membership of the genera, subgenera, species and subspecies remain contested (1990). (Scott & Janis, 1987) review the superfamily Cervoidea and include the Moschidae and Antilocapridae as sister groups to the Cervinae, whilst the Giraffidae are considered tentatively as a sister group to the Bovidae in the Bovoidea. This is the main diagreement in classification below the subfamily level.

Early studies used morphological traits for systematic classification and described evolutionary links based on clines in characters like body size or antler configuration (Brooke, 1878; Lydekker, 1898; Simpson, 1945; Ellerman & Morrison-Scott, 1951; Lowe & Gardiner, 1974, 1989; Lister, 1984; Geist, 1987), though generally more recent works have examined the genetical basis for classification, both at species level (Gustavsson & Sundt, 1968; McDougall & Lowe, 1968; Harrington, 1979; Baccus *et al.*, 1983; Herzog, 1988; Dratch & Gyllensten, 1991; Fennessey, Tate & Johnstone, 1991; Linnell & Cross, 1991; Emerson & Tate, 1993) and subspecific level (Feldhammer *et al.*, 1982; Gyllensten *et al.*, 1983).

Within the genus *Cervus*, depending on the author, up to 13 subspecies of sika (Whitehead, 1972) and 18 subspecies of red deer (Ellerman & Morrison-Scott, 1951) have been classified, though in most documents less are described. In conjunction with this

Japanese Sika Deer		
1838	Cervus nippon	Temminck
1845	Cervus sika	Temminck
1846	Cervus japonicus	Sundevall
1878	Cervus manchuricus minor	Brooke
1884	Sika schlegeli	Heude
	S. s. fuscus	Southern islands, Japan
	S. s. hollandianus	Southern islands
	S. s. infelix	Goto islands
	S. s. brachypus	Goto islands
	S. s. orthopus	Kobe
	S. s. blakisoninus	Nippon & Yezo
	S. s. dolichorinus	Nippon & Yezo
	S. s. legrandianus	Nippon & Yezo
	S. s. yesoensis	Yezo (Hokkaido)
	S. s. sylvanus	Nippon & Yezo
	S. s. aplodontus	north Tokyo
	S. s. mitratus	Tokyo
	S. s. xendaiensis	Sendai, Nippon
1888	Sika paschalis	Heude
	S. p. aceros	Goto islands
	S. p. rex	Goto islands
	S. p. dejardinus	Goto islands
	S. p. marmandianus	Goto islands
1893	Cervus sica	Lydekker
1897	Cervus sica typicus	Lydekker
1897	Sika sendaiensis	Heude
	S. s. schizodonticus	Tokyo
	S. s. ellipticus	Sendai, Nippon
	S. s. elegans	Sendai, Nippon
	S. s. minoensis	Mino
	S. s. rutilus	west Tokyo
1898	Sikallis daimius	Heude
	S. d. regulus	Goto islands
	S. d. sicarius	Goto islands
	S. d. consobrinus	Goto islands
	S. d. latidens	Goto islands

European Red Deer			
1758	*Cervus elaphus elaphus*	Linnaeus	South Sweden
1898	*Cervus elaphus typicus*	Lydekker	Sweden
1777	*Cervus elaphus hippelaphus*	Erxleben	Ardennes, France
1822	*C. e. germanicus*	Desmarest	Ardennes, France
1822	*C. e. albus*	Desmarest	(albino form)
1845	*C. e. albifrons*	Reichenbach	Germany
1874	*C. e. varius*	Fitzinger	Germany
1903	*C. vulgaris*	Botezat	France
1903	*C. v. campestris*	Botezat	Rumania
1903	*C. v. montanus*	Botezat	Carpathian Mts
1907	*C. balticus*	Matschie	E. Prussia
1907	*C. albicus*	Matschie	Silesia
1907	*C. rhenanus*	Matschie	Hessen, Germany
1912	*C. bajovaricus*	Matschie	Bavaria
1912	*C. elaphus neglectus*	Matschie	Posen, Germany
1912	*C. e. visurgensis*	Matschie	Rhineland
1912	*C. e. debilis*	Matschie	Rhineland
1912	*C. e. saxonicus*	Matschie	Saxony
1906	*Cervus elaphus atlanticus*	Lönneberg	West Norway
1906	*Cervus elaphus scoticus*	Lönneberg	Glenquoich, Scotland

Table 2.2.1a. Historical classifications within the present subspecies *Cervus nippon nippon* (Japanese sika) and the four European subspecies of red deer; *Cervus elaphus elaphus, C.e. hippelaphus, C.e.atlanticus* and *C.e. scoticus*. Gyllensten *et al.*. (1983) also recognise *C.e. germanicus* as a potential present European red deer subspecies which could have contributed to present scottish populations, as may have other more distantly related Cervinae. Various classifications of subspecies recognised today in *Cervus* are given in Table 1.1.2b and Fig. 1.1.2b.

uncertainty over specific or subspecific status of many populations of deer, many subspecies have also been renamed several times over the last two centuries. Table 2.2.1a shows the various namings and subdivisions of the subspecies involved in this study; the Japanese sika, now *Cervus nippon nippon*, and the Scottish red deer, designated *Cervus elaphus scoticus*. Due to the inability of recent genetic studies to find a firm basis for the subspecific status of the Scottish red deer (Lowe & Gardiner, 1974; Gyllensten *et al.*, 1983; Bruford *et al.*, in prep.); the taxonomic classification of the other Eurasian red deer are also reviewed. It is obvious that some of these early systematists often recognised several subspecies from the same location, particularly island groups in *C.nippon*. Groves & Grubb (1987) cite 18 cases of holotype specimens of subspecies known to or likely to have come from translocated animals of uncertain origin. Most of these subspecies are not now recognised.

Table 2.2.1b shows the various historical classifications *within* each present-day species of all Eurasian red deer (*Cervus elaphus*) and sika (*Cervus nippon*). The nineteenth century tendency to classify subspecies on a very fine scale is clear in Heude's catalogue of thirteen japanese sika subspecies in his *Sika schlegeli* species, now known as *Cervus nippon nippon*.

Figure 2.2.1b shows dendrograms generated from five studies of cervid populations at the various taxonomic levels. Though these broadly agree and can be followed as an evolutionary path from order to species as in the classification in Fig. 2.2.1a, there are discrepancies between studies. Note the close relationship between *Cervus elaphus* (subspp. *C. e. canadensis*; Canadian wapiti) and sambar in Cronin's phylogeny (b) generated from mitochondrial DNA types, which differs markedly from the classification of Emerson & Tate (c) where divergence of sambar precedes divisions within the red-sika-wapiti clade. Table 2.2.1c gives Nei's (1972) genetic distances (D) and genetic identities (*I*) calculated from the same works and again there is much variation in the degree of relatedness calculated between and within the three subspecies groups.

2.2.2. Defining the relationship between taxa: *Cervus elaphus scoticus* and *C. nippon nippon*

This confusing array of classifications, with disagreement at most levels, demonstrates the problem of defining the relationship between Scottish red deer and Japanese sika prior to hybridisation. It is accepted by all contributors to the debate that red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) are separate species, yet

Range	Ellerman & Morrison-Scott, 1951.	Whitehead, 1972	Groves & Grubb, 1987	Ratcliffe, 1987 (sika only)
Sweden	*Cervus elaphus elaphus*	*Cervus elaphus elaphus*		
N. Europe	*C. e. hippelaphus*	*C. e. hippelaphus*		
West Norway	*C. e. atlanticus*			
Britain	*C. e. scoticus*	*C. e. scoticus*	*Cervus elaphus elaphus*	
Corsica & Sardinia	*C. e. corsicanus*	*C. e. corsicanus*		
Spain & Portugal	*C. e. hispanicus*	*C. e. hispanicus*		
Algeria - Tunisian border	*C. e. barbarus*	*C. e. barbarus*		
Carpathian Mts			*C. e. montanus*	
S. Russia & Asia Minor	*C. e. moral*	*C. e. maral*	*C. e. maral*	
Russian Turkestan	*C. e. wachei*	*C. e. bactrianus*	*C. e. bactrianus*	
West Mongolia		*C. canadensis wachei*		
Chinese Turkestan	*C. e. yarkandensis*	*C. el. yarkandensis*	*C. e. yarkandensis*	
Kashmir	*C. e. hanglu*	*C. e. hanglu*	*C. e. hanglu*	
Siberia & Tibet	*C. e. wallichi*	*C. e. wallichi*	*C. e wallichi*	
N. Tibet	*C. e. macneilli*	*C. canadensis. macneilli*	*C. e. macneilli*	
Altai - Baikal	*C. e. asiaticus*	*C. c. asiaticus*	*C. e. sibricus/canadensis*	
Mongolia	*C. e. alashanicus*	*C. c. alashanicus*	*C. e. alashanicus*	
Manchuria, E. Siberia	*C. e. xanthopygus*	*C. c. xanthopygus*	*C. e. xanthopygus*	
Tian-Shan Mts, China	*C. e. songaricus*	*C. c. songaricus*	*C. e. songaricus*	
Kansu, China	*C. e. kansuensis*	*C. c. kansuensis*	*C. e. kansuensis*	
N. China	*C. n. mandarinus*	*C. n. mandarinus*	*C. n. hortulorum*	
Manchuria		*C. n. hortulorum*		
Mancuria & Korea	*C. n. hortulorum*	*C. n. manchuricus*	*C. n. manchuricus*	
Shansi, China	*C. n. grassinanus*	*C. n. grassinanus*	*C. n. grassianus*	
S.E. China	*C. n. kopschi*	*C. n. kopschi*		
Vietnam		*C. n. pseudaxis*	*C. n. pseudaxis*	*C. n. hortulorum*
Formosa (Taiwan)	*C. n. taiouanus*	*C. n. taiouanus*	*C. n. taiouanus*	
Sichuan, China			*C. n. sichuansis*	
Hokkaido (Yezo) Japan		*C. n. yesoensis*	*C. n. yesoensis*	
Honshu, Japan			*C.n. aplodontus*	
Tsushima, Japan			*C. n. pulchellus*	
Hondo, Japan		*C. n. centralis*		
Kyushu, Japan	*C. n. nippon*	*C. n. nippon*	*C. n. nippon*	*C. n. nippon*
Mageshima, Japan		*C. n. mageshimae*		
Yakushima, Japan		*C. n. yakushimae*		
Kyuku, Japan	*C. n. keramae*	*C. n. keramae*		

Table 2.2.1b. The various classifications of Eurasian red (top) and sika deer subspecies. North American *Cervus elaphus* subspecies are not included.

Comparison		D	I	Study
Red	*Cervus elaphus scoticus* (between populations)	0.001		Linnell & Cross, 1991
		0.0027		Gyllensten et al., 1983
	Cervus elaphus elaphus (between populations)	0.0014		Gyllensten et al., 1983
	Cervus elaphus atlanticus (between populations)	0.000		Gyllensten et al., 1983
	Cervus elaphus sub spp. mean distance	0.016		Gyllensten et al., 1983
Wapiti	*Cervus elaphus canadensis* (between populations)	≈0.005		Dratch & Gyllensten, 1985
Red Sika	*Cervus elaphus scoticus* *Cervus nippon nippon*	0.13		Linnell & Cross, 1991
	Cervus elaphus *Cervus nippon manchuricus*	0.5	0.6	Emerson & Tate, 1993
Red Wapiti	*Cervus elaphus scoticus* *C. e. canadensis* (wapiti)	0.05		Linnell & Cross, 1991
	Cervus elaphus scoticus *C. e. canadensis*	0.0248		Dratch & Gyllensten, 1985
	Cervus elaphus *C. e. canadensis*	0.32	0.72	Emerson & Tate, 1993
	Cervus elaphus elaphus *C. e. canadensis*	0.29	0.749	Baccus et al., 1983
Sika Wapiti	*Cervus nippon nippon* *C. e. canadensis*	0.06		Linnell & Cross, 1991
	Cervus nippon manchuricus *C. e. canadensis*	0.39	0.67	Emerson & Tate, 1993

Table 2.2.1c. Genetic relationships between red, sika and New world wapiti populations from different studies. Clearly there is a lack of agreement as to absolute genetic distances between taxa though variation within subspecies is lower than that between them. All distances are calculated after Nei (1972). Distances between red and sika are higher than those found between other hybridising mammalian taxa (D = 0.0-0.073, D = 0.037), but close to mean D between taxa in 20 well-studied hybrid zones (D = 0.147; Barton & Hewitt, 1985). Maximum D between hybridising taxa in Barton & Hewitt's review was 0.49 between *Bombina bombina* and *B. variegata*.

estimates of the genetic distance between them vary from 0.13 - 0.5 depending on the populations sampled, and all estimates are lower than the average distance between large grazing mammals (0.849, Baccus *et al.*, 1983) or between most mammalian species (Avise, 1974) . Obviously red and sika cannot be considered to be true biological species under the definitions of Mayr (1966) or Dobzhansky (1970), who used reproductive isolation as the criterion for species status. Not only is there

continuous deer range between the present day sika and European red deer populations, across which deer may interbreed freely, but when two of the subspecies are brought into secondary contact they generally seem capable of producing fertile hybrids (Caughley, 1971; Bartos & Zirovnicky, 1981; McClymont, Fenton & Thompson, 1982; Abernethy, 1994). Indeed Lowe & Gardiner (1975) classify all mainland forms of sika as hybrids of Chinese wapiti (*Cervus elaphus* subspecies) and Japanese sika, and are of the opinion that mainland sika cannot be classified as belonging to *any* species single species.

Generally it appears that requirements for reproductive isolation of species have been ignored in the Cervidae (Baccus *et al.*, 1983). There are several possible factors in this, the first is perhaps more a socioeconomic reason than a biological one in that as the hunting of deer has been important to European peoples for millenia (Jarman, 1972) and has had a reputation in recent centuries as the sport of the nobility. People have therefore wished to define membership, and thus ownership, of populations on a fine scale. Likewise the predilection for taxonomy in the latter nineteenth century gave people 'ownership' of species or subspecies by naming them. The intense amount of historical interest in deer morphology, especially body size and antler form, is suggested as a contributory factor in the proliferation of cervid species, and especially subspecies, beyond the common expectations. Geist (1983, 1987) holds the view that rapid morphological change in ornate characters (perhaps coded by relatively few genes) is driven by social competition in colonisers of new and plentiful environments - the dispersal phenotype. When individuals have energetic resources beyond their metabolic requirements they can invest in 'luxury' tissues or ornaments involved in mate competition, i.e. antlers. He explains the increasing complexity in antler form as the result of colonising the seasonal temperate zone where a long summer provided an excess of nutrients to animals whose overall size was determined by maintenance requirements in the winter; long cold winters selecting for larger body sizes with lower metabolic rates and lower heat loss. However the growth of large sexual ornaments is common to (male) animals with a polygynous mating system, and may need no more explanation in the cervidae than elsewhere (Kavanagh, Clutton-Brock & Harvey, 1981). Large morphological variation would provide a biological basis for intricate classifications of populations, without necessarily requiring genetic divergence or reproductive isolation.

Even if genetic divergence associated with these phenotypic traits was relatively large, an alternative explanation for the perceptible lack of genetic differentiation in *Cervus* subspecies is in the high mobility and therefore continued, though low level, gene flow between geographically distant populations of large

grazing mammals (Baccus *et al.*, 1983; Georgiadis, in press). As above, there is evidence that deer populations across Eurasia have been widely influenced by man through hunting and trade which recently, and perhaps historically, has certainly involved translocation of animals, enhancing geneflow between populations (Jarman, 1972; Lister, 1984). Much of the influence of this trade would have been felt between the designation of species during the 1800's and the assessment of genetic differences in the last decade or two. This may be a factor in the large genetic variability (high heterozygosities and numbers of polymorphic loci) in populations known to have a high immigrant contribution e.g. Scotland (Dratch & Gyllensten, 1985). The counter argument to the claims of overzealous designation of subspecies in the last century is that the restocking and moving of deer populations in the interim period have blurred the boundaries of what were more clearly differentiated populations. In their study of pocket gophers in North America, Patton & Smith (1989) found a higher than expected level of differentiation between geographically distinct races of a *Thomomys bottae* population. They explained this as a result of the low level of migration between demes and highly stable territoriality amongst adults, contrary to the situation in the deer populations.

It seems reasonable to say that the *Cervus* populations of Eurasia cannot be regarded as panmictic, although it is far from clear how they should be subdivided and where the boundaries between taxa lie. There has been a considerable amount of work in recent years on the biology of hybridising taxa and the importance of understanding hybrid zones in unravelling the complexities of evolution and speciation (and hence improving both taxonomic classification and rendering taxonomy a more useful tool in describing the world's biodiversity. The following section reviews the literature on hybrid zones with a view to the concepts relevant to the introduction of Japanese sika to Scotland and their subsequent hybridisation with native red deer.

Throughout the remaining sections of the text I shall refer to all Japanese sika in the broad definition of Ratcliffe (1987a) as *Cervus nippon nippon* . I will refer to all the Eurasian subspecies of *Cervus elaphus* as red deer (after Groves & Grubb, 1987) and to the variety of red deer inhabiting Scotland, the putative *Cervus elaphus scoticus* as Scottish red deer. Where other subspecies or races are mentioned I will define the nomenclature as appropriate.

Chapter 3.

HYBRID ZONES

3.1. The definition of species

The previous chapter serves to highlight the practical difficulty of defining species both in terms of their evolutionary origins and status and in terms of their membership by individuals. The problem recurs at all taxonomic levels in the Cervidae, and is also common in other systematic groups (Barton & Gale, 1993). In spite of the fact that every biologist can recognise particular levels of organisation and groupings of animals and plants, species have not been defined by any single concept in biology.

In 1937 Dobzhansky proposed a working definition of species;

> "Species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism, or perhaps by a combination of several such mechanisms."

and this was followed in 1942 by Mayr's definition of the biological species, which has remained the most widely used of various subsequent species concepts. Mayr defined species as

> "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups."

The usefulness of the *biological species concept*, as Mayr's paradigm became known, was thought to be that it gave unambiguous boundaries, reducing confusion for taxonomists. In recent years, however, it has become apparent that this is also its major shortcoming. In the plant kingdom botanists have long recognised hybrid varieties and their parents as distinct 'species' and these remain phenotypically (and probably genetically) distinct groups (Arnold, 1992). Recently animal biologists have likewise realised how widespread the phenomenon of persistent hybridisation is. Barton & Hewitt (1985) give 150 examples of interspecific hybrid zones in a wide variety of organisms and Hewitt (1989) states that in certain groups, for example African birds, one third to one half of all species are subdivided by hybrid zones. The

biological species concept has no way to accommodate interspecific hybrid zones and does not recognise the subtle distinctions of within species differentiation.

During the last decade several alternative species definitions have arisen in answer to this failing of the biological species concept and these are reviewed by Templeton (1989) and Avise (1994). The *evolutionary species* (Simpson, 1951) defines species as

> "populations or groups of populations that share a common evolutionary fate".

This definition was instigated to cope with extinct as well as present species, but has not been widely accepted due firstly to the problems of discerning a "common evolutionary fate" in existing populations and secondly because it gives no clue to a functional mechanism for species existence. The *recognition species* (Paterson, 1985) developed from the isolationist principles of the biological species, but saw the same behavioural 'isolating mechanisms' as holding members of a species together through recognition, rather than precluding individuals through pre-mating barriers. Similar to the biological (isolation) species concept the recognition species idea is flawed in that it does not deal with asexual organisms, nor does it address the problem of classifying hybridising taxa.

Templeton concludes his 1989 review by proposing the *cohesion species* which he defines as

> "the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms."

He goes on to define cohesion mechanisms in terms of genetic and demographic exchangeability, such that any member of a species may exchange genes with any other and each member occupies the same Hutchinsonian ecological niche. Although this allows most individuals to be allocated to a species, it does not allow for heterogeneity between individuals within a species and thus should subdivide many existing species, possibly to an unworkable extent. After all each individual is in fact different and cannot be fully exchangeable with another. The problem remains to define the point at which individuals differ so greatly that they are no longer regarded as conspecific. Templeton has simply moved the goal posts.

Cracraft (1983) moved away from the ideas of reproductive isolation or cohesion and defined the phylogenetic species concept, which states that species are

> "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent"

Implicit in this definition is that the cluster should be monophyletic. Although this does get round the obstacle of reproductive isolation, it suffers from the difficulty of defining a pattern of ancestry in a group and perhaps more seriously from the problem that, if the phylogeny is sensitive enough, even a family pedigree can be recognised as a unique pattern of parental descent. Again the dilemma is who to include and who to exclude.

Within the plant world Grant (1981) defines the 'syngameon' as

> "the most inclusive unit of interbreeding in a hybridising species group."

This creates a new level of organisation of biodiversity and discards many of the problems of more limiting species definitions, but it cannot deal with directional gene flow through member populations, resulting, in extreme cases, in the ends of a 'ring species', or syngameon, that are infertile together. In avoiding the overdivision of species it has left the definition perhaps too limitless.

Returning to the original problem of dealing with species in the genus *Cervus*, we find that none of the species definitions have adequately solved it. Though Grant's 'syngameon' is perhaps the best description of *Cervus*, which would then place all members in a single taxon, it does not help us define membership of species within the hybridising group. If we return to Mayr's biological species concept of a reproductively isolated unit, we can in fact find it by looking at the syngameon, and discarding the search for a robust definition of 'species' at the higher level of differentiation. It is perhaps more useful to use such unambiguous definitions as the biological species concept when the biological world is truly divisible into discrete units, rather than forcing populations into arbitrary pigeonholes. A single definition of 'species' at the level we currently wish for it is in fact extremely difficult, if not impossible, but the debate has lead us toward a greater appreciation and discussion of the factors contributing to the organisation of life. Because 'species' as single units *can* be adequately described in terms of their mean characters it may be more applicable to talk about variation in each character found in *Cervus*, highlighting those that are shared or differentiating between groups of individuals, than to consider suites of characters collectively as those defining species.

It cannot be denied that the current 'species' unit does play a valuable role in the description of biodiversity. Simpson starts his 1945 monograph on classification with the sentence

> "It is impossible to speak of the objects of any study, or to think lucidly about them, unless they are named."

which is the essence of taxonomic endeavour. Species provide a method of organising the groups of creatures we undoubtedly recognise as discrete and different, but there are interesting questions in why we are able to recognise such groupings, and in how they remain separate, which can be asked without demanding a definition applicable to all currently recognised species (Barton, 1993). It should not be forgotten that nomenclature is simply a descriptive tool and does not *necessarily* imply a biological event.

3.2. The systematic problem of hybrid zones.

In all the species concepts a major hurdle has been the presence of interspecific hybrid zones. Evolutionary biologists are now looking to understand these areas of species boundaries, and the factors that maintain their stability, as a key to understanding the speciation process (and thus perhaps gaining the ability to define species) (Barton & Hewitt, 1989).

Since Darwin & Wallace's presentation of the theory of natural selection to the Linnean Society in 1858 and Darwin's subsequent publication of "The Origin of Species" in 1859, the understanding of natural selection and speciation have been the life's work of many. The literature on the subject is now immense (e.g. Dobzhansky, 1940; Mayr, 1963; Lewontin, 1967; Otte & Endler, 1989).

The principle of evolution occurring through various selection patterns on ancestral forms, forming an 'evolutionary tree' of divergent sibling species is largely uncontested. Not so the circumstances under which selection may be able to initiate divergence. A point of keen debate is whether populations may diverge and speciate in sympatry or parapatry, or whether geographic isolation (allopatry) is essential for genetic divergence (Mayr, 1942,1963 and others).

Hybrid zones have been explored as potentially shedding light on these issues because, as we have seen, they violate the species concepts of most biologists (Hewitt, 1988 ; Harrison, 1990). In hybrid zones, taxa that are genetically (and usually morphologically) distinct interbreed, yet remain distinct despite geneflow between them. As the isolation element of species concepts was instigated in the belief that gene flow between populations *prevented* divergence, this phenomenon requires explanation. Do hybrid zones separate species in the process of divergence? Could hybrid zones have arisen in sympatry or are they the result of contact after allopatric divergence? What is the fate of hybridising taxa: speciation or integration? Do hybrid zones play a role in speciation? If they do it is likely to be through reinforcement of

characters that influence reproductive isolation as, when hybrids are selected against, a certain amount of reproductive effort is wasted on hybrid matings, reinforcing the selective advantage of recognising and mating with a like-type - a conspecific (Butlin, 1987, 1989)

In order to assess the possible role of hybrid zones and to test these theories, the strength of selection on hybrids, the effects of heterozygosity at different loci or combinations of loci and the recombination of homozygous loci of different descent, the interaction of genotype and sex, and perhaps most importantly the interaction between genotype and a varying environment must be understood.

3.3. Describing Hybrid Zones

3.3.1. Overview

The study of hybrid zones has flourished in the last decade. Barton & Hewitt (1989) review over 170 hybrid zone studies and the literature has continued to expand in the intervening five years (e.g. Harrison, 1990; Mallet *et al*, 1990; Arnold, Bennett & Zimmer, 1990; Fennessey, Tate & Johnstone, 1991; Sperling & Spence, 1991; Arntzen & Wallis, 1991; Butlin, Ritchie & Hewitt, 1991a,b; Wayne & Jenks, 1991; Kohlmann & Shaw, 1991; Paige, Capman & Jennetten, 1991; Arnold, 1992; Sanderson, Szymura & Barton, 1992; Jackson, 1992; Barton & Gale, 1993; Georgiadis, 1993; Berry *et al,* 1993; Rieseberg & Wendel, 1993; Sites, Reed & Barton, in press; Abernethy, in press). They have been explained in two main ways. One is that hybridisation occurs at the boundary between two allopatric populations and hybrid numbers are maintained by constant meeting and mating of the parental types. Selection against hybrid genotypes within the parental populations prevents hybrids spreading, restricting them to a band between the parental ranges (Barton & Hewitt, 1985). Selection may either be acting to favour particular parental genotypes in particular habitats: exogenous selection (Haldane, 1948), or may act against recombinant individuals, regardless of habitat: endogenous selection (Key, 1968; Bazykin, 1969). The latter type of model is termed a 'tension zone' (Key, 1968) as the zone is maintained by the tension between opposing forces of dispersal and selection.

The second explanation is that in a narrow ecotone, hybrid individuals have a selective advantage due to some environmental parameter (Moore, 1977). Outside this ecotone the advantage is lost and individuals with mixed genetic traits are selected against. In this case the width of the zone is independent of dispersal distance, but is

dictated by the habitat. The steepness of the transition in gene or phenotype frequencies may be related to the sharpness of the habitat boundary, the strength of association between the parent type and its habitat and the strength of selection favouring the hybrids.

Under both these scenarios, the hybrid zone is only an impermeable barrier to genes that are under direct selection (Barton, 1979; Barton & Bengtsson, 1986). Those that are selectively neutral will not influence the survival of the hybrid and may thus diffuse across the taxon boundary thus although differentiated taxa may remain so at loci under selection, they will merge at neutral loci. Hybridising taxa are differentiated at loci that affect the quantitative traits that define them and for traits that reproductively separate, though do not isolate them. In fact, a barrier to neutral alleles does exist in most hybrid zones as associations between them and selected loci prevent introgression until they are broken down by recombination (Thomson, 1977; Barton, 1986 & Bengtsson). The isolation of populations may occur with very little genetic divergence if the genes directly influence reproductive isolation, as in European races of shrews, which differ through multiple chromosomal rearrangements rendering crosses infertile (Searle, 1984) or if selection is intense on a few loci i.e. *Heliconius* butterflies, which differ at only a few genes coding for coloration, yet are strongly selected for wing patterns involved in mimicry, preventing introgression between types with different wing patterning (Mallet, 1986).

In many ways these examples are leading us to consider the unit of selection in the hybrid zone to be individual traits of the organisms and the genes that control them rather than the whole organism. This can be extended to the consideration of genes, rather than individuals, in species contexts and alters many of the problems exposed above with attempting to fit species definitions. In the investigation of the *Cervus* hybrid zone it seems more interesting to look at the fate of individual traits and genetic loci than to try to define either the original (or resultant) sika and red deer.

In the next sections I shall review hybrid zone theory in terms of the behaviour of individual loci. The interactions between them, of course, eventually define the elusive "red" or "sika" deer. The developing hybrid zone between red and sika in Scotland contains many complex elements which are illustrated in other hybrid zones. These include a dispersal-dependent wave of advance of some selected loci, genotypic (endogenous) selection and probably habitat-related (exogenous) selection. The details of genetic patterns in the *Cervus* hybrid zone are discussed in Part 2, this section is intended to lay a framework for interpretation of those patterns.

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3.3.2. Dispersal-independent clines

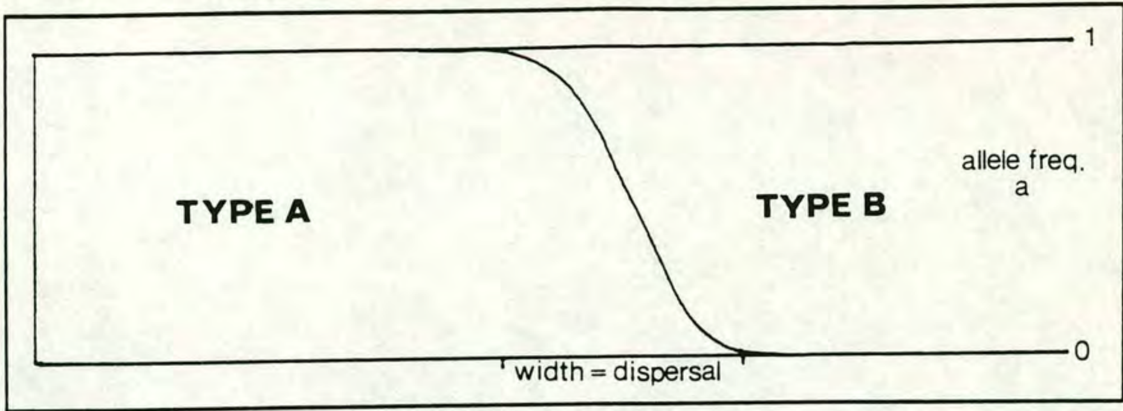
Moore's (1977) theory of *bounded hybrid superiority* is the classic example of a dispersal-independent hybrid zone. In this case the hybrids are fitter than either parent in the ecotone between two parental ranges, along which the hybrid zone is found. Beyond this ecotone the advantage is lost and hybrids or the 'wrong' parental types are selected against. Examples of hybrid advantage do exist, for instance the hybrids of red and black oak (*Quercus* spp.) do not germinate well under forest canopy, but do better than either parent in cleared areas (Templeton, 1989) Thus hybrid superiority exists in a continually cleared forest patch, or at a forest edge, but will not persist if the forest goes to maturity. Evidence in the literature of a hybrid zone maintained in this way is rare but not absent (Littlejohn & Watson, 1973). Hybrid swarms, the production of a hybrid population in a particular locality which then form a distinct and fertile population, could be seen as examples of hybrid superiority, but do not behave in the dynamic fashion of hybrid zones which continue to interact with parental taxa. In the plant world this is accepted as a mode of speciation (Caisse & Antonovics, 1978; Grant, 1981), but examples are rarer in animals (Wayne & Jenks, 1991).

Another example of a dispersal-independent system is that of the *balanced polymorphism*. Here a trait is favoured in local environments and is found at equilibrium frequencies across the population range. This may be viewed as a cline if local environments change transitionally, but the dispersal of individuals is unlikely to affect its shape. The polymorphism can exist at a single locus and Barton & Gale (1993) give the example of the Hb^s allele involved in the sickle cell anaemia trait, which varies with the incidence of malaria across Africa.

3.3.3. Tension zones

Tension zones are generally narrow bands at species boundaries created by a balance between dispersal of parental forms into the zone, and selection against hybrids. Zone widths are related to the dispersal distances of the parental types whereas the steepness of the transition or 'cline' in gene frequencies is related to the strength of selection against the hybrids, i.e. the reduction in hybrid fitness compared to the parental types (Barton, 1979b) The extreme case of an inviable hybrid would lead to the absence of heterozygote individuals and a cline in allele frequencies

Fig. 3.3.3. A typical cline in allele frequencies maintained by balancing strong



selection against heterozygote individuals and immigration of parental types.

plotted against geographic distance whose width equals the dispersal distance of the organism (Fig. 3.3.3)

Barton & Gale (1993) show that the mode of selection acting in a hybrid zone, either environmental or genetically determined, does not markedly affect the shape of resultant clines at individual loci, and that certain inferences about selection strength and mean fitness of populations can be drawn from data on cline shapes, widths and concordances between loci regardless of the mehanism of selection acting. If dispersal (geneflow) is approximated as the standard deviation of the distance between parent and offspring (σ), selection = s, homozygotes have a fitness of 1 and heterozygotes a fitness of 1-s, then the ratio between dispersal and selection is proportional to the cline width at each locus in terms of a characteristic scale of selection, l, such that $l = \sqrt{(\sigma^2/2s)}$ (Bazykin, 1969). Cline width is then $4l$.

Clines at different loci can exist separately, dependent on the selection experienced at each locus, however associations between loci tend to pull clines together (linkage disequilibria, see below, section 3.3.5). Loci that are selectively neutral will not themselves affect the fitness of a hybrid and may flow across the cline once they are dissociated from selected loci (Thomson, 1977; Barton, 1979).

As tension zones exist through genetical and demographic forces, rather than environmental ones, they can be perturbed by changes in dispersal, population density or fitness of the parental types. Barton (1979), has investigated tension zone dynamics to show that tension zones can effectively be trapped in low density troughs which limit dispersal or be arrested by physical barriers to gene flow (see also Barton, 1986). A good example is the hybrid zone between chromosomal races of the alpine grasshopper *Podisma pedestris* which lies along a density trough created by inhospitable habitat (Jackson, 1992).

In theory a tension zone will only remain stable in a homogeneous environment if the absolute fitnesses of the two parental taxa are equal. If not the fitter type will eventually swamp the less fit one. In nature absolute equality of fitness seems unlikely to be the case and most tension zones are stabilised by an underlying environmental gradient or change that alters the fitness of the parental types. The zone will move toward the less fit parent, coming to equilibrium at a point where fitness is balanced in the parental taxa (Kohlmann & Shaw, 1991). Examples of tension zones that lie along environmental gradients are common, for instance the well-documented zone between fire-bellied and yellow-bellied toads, *Bombina bombina* and *B. variegata*, runs along the border between the Carpathian foothills and the Danube plain (Szymura & Barton, 1986). Likewise the zone between the mice *Mus musculus* and *M. domesticus* which follows a climatic isocline in northern Europe (Hunt & Selander, 1973). As habitat boundaries are often themselves zones of intergradation (Anderson, 1948) it follows that the habitat-fitness associations that determine the overall range of the taxa may also be effective on a finer scale within the hybrid zone. In the next section I will review case studies of hybrid zones where a strong relationship between genotype and habitat is found within the zone, structuring its genetic architecture. I then go on to consider the effect of habitat within possible 'tension zones'.

3.3.4. Mosaic hybrid zones

Within a zone of hybridisation the population is rarely a random mixture of the possible genotypes from parental gametes. This may be due to selection against recombinant types, as in the tension zone, but may also have an environmental component as particular recombinants, or individual alleles are favoured in particular habitats. (Rand & Harrison, 1989) investigated the relationship between soil type and genotype in a hybrid zone between the crickets *Gryllus pennsylvanicus* and *G. firmus* in Connecticut. They found a striking difference in frequencies of isozyme, morphological and mtDNA traits between populations at geographically close sites, but on sand or loam soils. There was also experimental evidence for a similar level of (directional) reproductive isolation between populations on adjacent patches of different soil type as there was between populations on opposite sides of the zone. *G. pennsylvanicus* is found on loamy, inland soils, whilst *G. firmus* is typically of sandy, even beach habitats. In Connecticut these habitats are interleaved by ridges of loam running to the coast, whilst river corridors bring sandy soils inland. They

suggest that the current structure of the zone may be the result of habitat-specific colonisation, but state that the crickets must have been genetically divergent before such a colonisation in order for the *pennsylvanicus* mtDNA haplotype to have reached high frequency in both habitats consistently across the zone. They invoke selection as maintaining the steep transitions at soil type boundaries. Rand and Harrison propose a model for the mosaic hybrid zone (redrawn in Fig. 3.3.4a) which differs from that of the conventional cline. They suggest that, although on a broad scale the hybrid zone may have the characteristics of a tension zone, if genotypes are restrained by habitat on a fine scale, the hybrid zone will no longer have the freedom to move. However just as the tension zone is able to move toward a population of lower density or fitness (Barton, 1979a; Barton & Hewitt, 1985), increased ecological tolerance of one genotype, or a certain type of recombinant, allows it to colonise into the opposite parental habitat, also shifting the zone. If habitat patches of one type become very small, populations within them may be swamped by the more frequent type, also shifting the zone.

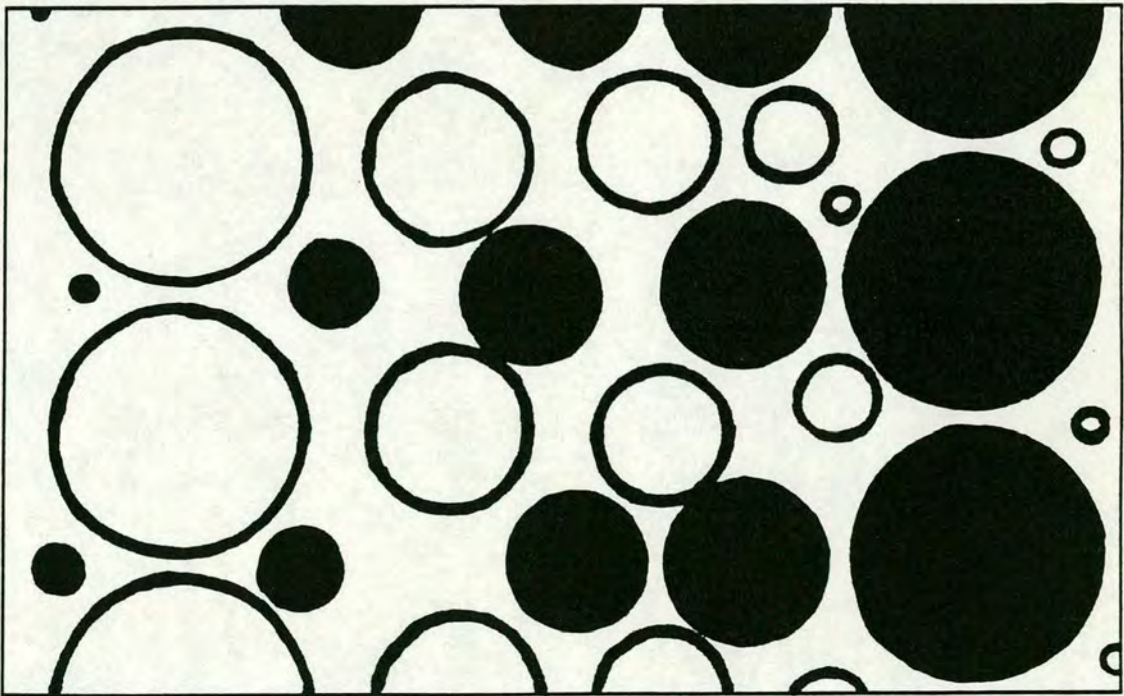


Fig. 3.3.4. Distribution of genotypes in mosaic hybrid zones.

Recently other 'tension zones' thought to be maintained purely by genetic forces have been found to have a finer scale ecological dependence. For example the *Bombina* hybrid zone, where within the zone *B. variegata* genes tend to be found in temporary puddles and genes from *B. bombina* in permanent ponds (Gollmann, in press; MacCallum, in prep.). In this case the habitat dependence is not as marked as in

the *Gryllus* example, but gene frequencies across the broad cline are significantly affected by 'micro'habitat. The Mexican lizards, *Sceloporus grammicus*, show a similar affiliation to small scale habitat changes in the hybrid zone between chromosomal races as Bombina do (Sites, Reed & Barton, in press). These latter two examples may differ slightly from the *Gryllus* zone in that adults show active preference for habitat patches which exist on a smaller scale than their potential dispersal distance. The crickets experience local selection in habitat patches that are relatively large. In the *Cervus* zone the large potential dispersal of the organism makes it likely, in Scotland, that habitat patches will exist on a smaller scale.

3.3.5. Associations between loci: linkage disequilibrium

Although clines may exist at each locus, loci are bound together within individuals who disperse packages of genes jointly in gametes. Although many genes may not affect the fitness of an individual, their flow across a hybrid zone is affected by the selected loci within that individual's genome (Slatkin, 1975; Barton, 1983) Different loci will respond to differing selection pressures. The formation of gametes by meiosis and subsequent sexual reproduction recombines parental genomes presumably at random, and will break down associations within the genome. However, if selection acts more strongly on some loci, or some gene combinations (epistatic selection), than others the pattern of associations seen in future genomes will not be random (Lewontin & Kojima, 1960). Loci on the same chromosome stand a much higher chance of remaining together during gameteogenesis than loci on different chromosomes; a relationship which intensifies as loci are found closer together on the chromosome. This structural bond is termed linkage. However unlinked loci (on different chromosomes) may also be found in non-random association in a hybrid zone i.e. in *linkage disequilibrium* (Lewontin & Kojima, 1960). The term linkage disequilibrium is rather a misnomer (Hedrick, 1987; Barton & Gale, 1993) as it does not refer to loci 'linked' in the conventional sense above, nor does it necessarily refer to instability, as disequilibrium might imply. It has nonetheless come into common usage in population genetics for describing non-random patterns of pairwise associations between loci, and it will be used in this context in this thesis.

Consider two segregating loci which have alleles P and Q, R and S respectively at frequencies p, q, r and s. In a finite, sexual population, recombination would be expected to break down chromosomal associations within a given genome

by a half ($r = 0.5$) at each recombination event if random mating were occurring. Associations between linked loci will be slower to break down and will, in addition, depend on the rate of chiasma formation. Gametes in the next generation will be formed as PR, PS, QR, QS at frequencies *pr*, *ps*, *qr*, *qs*. Disequilibrium is defined as the difference between the observed frequency of the PR gamete and its expected frequency *pr* (Weir, 1979).

Random mating and equal selection on each genotype is an unlikely scenario in nature. Non-random associations between loci on separate chromosomes can be created by selection against certain gene combinations or assortative mating between the genotypes. In a hybrid zone, the constant influx of 'parental' types which are sampled before recombination also increases the effective lack of recombinants (Barton, 1979b; Barton & Hewitt, 1985).

Unfortunately gametic disequilibrium, as above, is difficult to measure in natural populations for which only genotypic frequency data are available as the gametes contributing double heterozygotes cannot be deduced; the union of PR/QS, or of PS/QR ?. In these cases linkage disequilibrium measures are a composite of two components; cross-gamete and within-gamete disequilibria. (Weir & Cockerham, 1978; Weir, 1990). Disequilibirum measures can be standardised by the combined gene frequencies $\sqrt{pqrs}$ to reduce the frequency-dependence of the measure, although this can never be entirely accounted for (Hedrick, 1987). Barton (1983) shows that in clines maintained by selection against heterozygotes, a proportion of the alleles at each locus (those in the heterozygotes) are effectively not available for recombination in the next generation. Estimates of genotypic linkage disequilibria must compensate for this, reducing the alleles by a factor equivalent to the heterozygote deficit observed. Heterozygote deficit is calculated as Wright's F_{is} (Wright, 1965), the deviation from Hardy-Weinberg expectations at each locus (see Chs. 4 & 5 for analysis of Fis and linkage disequilibrium coefficients).

Linkage disequilibria are a common feature of many hybrid zones e.g. *Bombina* (Szymura & Barton, 1986), *Gryllus* (Rand & Harrison, 1989), *Sceloporus* (Sites, Reed & Barton, in press) among others. Barton & Bengtsson (1986) show that they can significantly impede the gene flow across the hybrid zone, increasing the concordance of clines, effectively pulling them together, which generates more disequilibrium (Slatkin, 1975). Often inferences about disequilibria in wild populations must be made from genotypes after recombination and even after dispersal and selection. In these cases it is difficult to disentangle the cause of the deviation from random expectations, and linkage disequilibrium statistics may need to be interpreted alongside other measures of population process. Cytonuclear disequilibrium;

disequilibrium between the cytoplasmic genomes and the nuclear genotype, is created in the same way as gametic disequilibrium. Assuming random mating, in zygotes, cytonuclear genotypes are in Hardy-Weinberg proportions with frequencies of cytonuclear types predictable from the frequencies of gametes. Selection in later life, and also dispersal, can then create both nuclear and cytonuclear disequilibrium in the population.

3.4. Creating hybrid zones

3.4.1. Waves of advance

In 1937 Fisher produced his theory on the wave of advance of an advantageous allele. In it he described the behaviour of a single favourable mutation (allele A) arising uniquely in a population. The mutation bestowed an advantage such that relative fitnesses of the genotypes are $aa = 1$, $Aa = 1+s$, $AA = 1+2s$, where $s << 1$. For a population in which the standard deviation of the parent offspring dispersal distance is σ, the change through time in p allele frequencies for a locus with allele p, q then describes a travelling wave

$$dp/d\tau = (\sigma^2 /2)\, d^2p/dx^2 + spq \qquad [3.1]$$

The x term is a spatial scale. Fisher uses terms of absolute distance, but it may also describe movement through demes or portions of demes. The speed of the wave front will depend on the starting conditions, population density, dispersal distance and the strength of selection. If selection becomes very strong Fisher's wave formula cannot be used as the wave no longer approximates to diffusion. At any instant the population at the wave front has allele frequency $spq / 1+sp$ where $1+sp$ is equivalent to the mean fitness (ω). For very small values of s, even at high values of p ($0<p<1$, $0<s<1$)), this term is close to 1 and thus allele frequencies equal spq. However for large values of s, the term becomes significant and the mean fitness of the population changes. This prevents an analytical solution to the wave without complex recursions of this term.

If dispersal is bounded, Fisher's wave moves at speed $\sqrt{2\sigma^2 s}$, but if long range migrants occur, new populations can be established beyond the front of the wave (Fig. 3.4.1), resulting in a patchy advance.

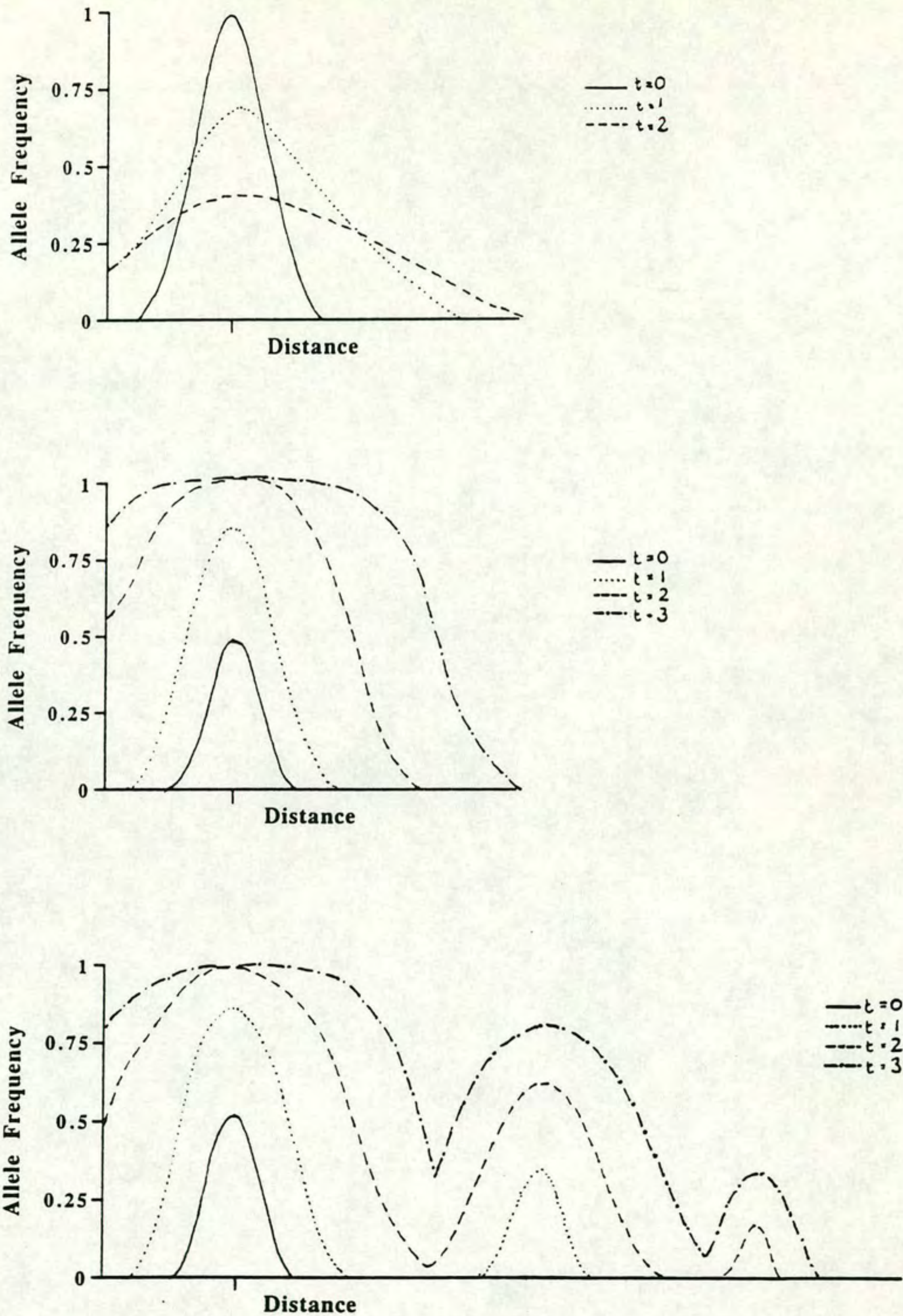


Figure 3.4.2a-c. The waves of advance of alleles under various scenarios of selection and dispersal. a) Decay of neutral allele frequencies. Dispersal reduces frequencies at the introduction point. In this case total number of copies of the allele remains constant, though this could be altered through random drift. b) Fisher's wave of advance of a selected allele. Dispersal and selection strength influence the speed of propagation of the wave. Populations behind the wave are fixed for the allele. c) Long distance dispersal of a selected allele can result in a ragged advance as, effectively, a series of introductions and expanding waves merge.

3.4.2. Introductions

A unique situation occurs when populations that have accumulated mutations whilst in allopatry are brought into secondary contact. If, following an introduction (secondary contact), a small founder population is able to hybridise with an extant population, this is analogous to the instantaneous arisal of a huge number of mutations. In most populations selection is rarely strong as the incidence of favourable mutations is low, affecting single loci in single individuals, but the conditions in an introduction alter this. Not all the introduced alleles will be advantageous, but it is likely that a proportion will be. If we assume additive selection, which may be inaccurate but is likely to be true of at least a portion of these new alleles, then $S = \sum s$, and may be large.

A neutral allele introduced at high frequency may be expected to decline at the introduction site as individuals disperse away (Fig. 3.4.2a). If a newly introduced population has a selective advantage at many loci and is effectively at a new 'adaptive peak' relative to the extant population, it may send out immigrants propagating a simultaneous wave of advance across many loci (Key, 1968; Barton & Hewitt, 1985). The conditions and success of the founders is crucial to the establishment of a population as in the initial generations numbers are low and without a substantial increase in relative fitness at at least some loci, the new alleles will be swamped. If the selective advantage is sufficiently strong or the neighbourhood size low, advantageous alleles will spread throughout the population pulling it to the new adaptive peak (Rouhani & Barton, 1987a). Initially introduction will create strong linkage disequilibria in the colonised populations as alleles derived from the founders will cluster in few descendants. Subsequent recombination will reduce this effect, separating loci with differing selection coefficients (Baird & Abernethy, submitted). Migration of individuals from the founding population will decrease local density and increase the risk of swamping (assuming it is countered by inward migration of resident genotypes).

This is similar to the scenario envisaged in phase III of Wright's (1931) "shifting balance". Wright saw species as existing at peaks on an 'adaptive landscape'. The adaptive landscape is a multi-dimensional surface defined by interactions between each locus in the genome and each environmental parameter influencing the organism. Populations could move between peaks as a result of genetic drift. He did not include mutation as a method of a population moving to a new peak as introducing a new allele effectively introduces a new dimension to the fitness surface, however once new

alleles are in the genepool of a hybridising population, this dimension is added to the surface occupied by the extant population. Again, introduction of a new genome is effectively the simultaneous arisal of many mutations.

As in the wave of advance of a single locus (Fisher, 1937) the performance of alleles at the leading edge of the wave is crucial to the propagation of the wave as here the colonising alleles are always rare. If the selective advantage is lowered or lost in a newly colonised neighbourhood, for instance because of a density trough or a habitat change, the wave will be stopped. Increase in allele frequency behind the wave may then allow it to move on by sheer weight of numbers, however if this does not occur the result may be the evolution of a tension zone (Hewitt, 1988).

The advance of a multi-locus wave could also be stopped if the advantage the founders had was due entirely to epistatic or dominance effects. Fitness will then be reduced by recombination. Under these conditions migration becomes a critical factor in the initial stages as it will tend, by decreasing local density, to increase recombination. If the migration is sufficiently limited, even a selective advantage caused by epistasis may enable gene frequencies to increase. To date there is very little literature on the possible effects of epistatic selection in introductions.

3.4.3. Moving Zones

The behaviour of a group of clines following introduction of a new genome raises interesting questions. It is rare that the advance of universally favoured alleles is documented, as they are likely to have swept through the entire population relatively quickly after secondary contact (Hewitt, 1988). How strong does selection have to be to propagate a wave in various population densities? Of the few genetical studies that are in the literature of post-introduction hybridisation, most document complete admixture of the population, without the formation of a stable zone (Woodruff & Gould, 1987; Echelle & Connor, 1989).although theoretically one could arise at a barrier to gene flow, or through ecological change. How strong need a barrier be to halt the wave?

Initial linkage disequilibrium will hold the genome together, but in the absence of selection recombination will reduce associations by half each generation. If selection is spread over many loci the chance of association between a neutral and a selected locus increases. How well does selection over many loci hold clines together? How does this relationship vary for the extremes of a single locus or very many loci? Do linkage disequilibria between loci alter the speed or shape of the wave?

A group of clines may move together, remaining concordant and coincident immediately after the introduction, simply because selection coefficients are insufficiently different to separate them in a small time frame. In this case clines produced by different forms of selection acting independently but with similar force on each locus would be indistinguishable from those produced by a single selective pressure acting on a multi-locus trait. However, linkage disequilibria would be expected to be strong and persistent in the latter case, but to decay in the former (Abernethy, in press). Understanding how large the ratio of selection coefficients need be to separate clines in 10, 100 or 1000 generations and how many genes are involved in a selected trait may enable us to predict how a genome will break down, and which elements may persist coherently in a hybridising population.

3.5. Directional introgression

3.5.1 Overview

When taxa meet in a hybrid zone there is the potential not only for the genotypes to behave differently, but for the sexes to do so, and for sex and genotype to interact. In 1922 Haldane observed that "when in the F_1 offspring of two different animals one sex is absent or sterile, that sex is the heterozygous sex" (Haldane, 1922). and this rule is true in many case studies (Arnold, 1993; Avise, 1994). This sort of asymmetry however does not affect the direction of the original crosses. The advent of uniparentally inherited cytoplasmic markers (mitochondrial DNA in animals; chloroplast DNA in plants) has enabled population genetics to establish cytonuclear genotypes for individuals in hybrid zones and to test hypotheses concerning the direction of hybridisation and the fitness effects in each sex of the parental types and the hybrid offspring (Asmussen, Arnold & Avise, 1987, 1989; Arntzen & Wallis, 1991; Arnold, 1993).

Intersexual fitness differences, mating preferences or dispersal differences can create asymmetrical clines or, in the extreme, unidirectional introgression into one taxon. Examples of unisex F_1 sterility in hybrid zones include cases caused by parasite inheritance (Hoffman & Turrelli, 1988) and by the phenomenon of cytoplasmic male sterility (Mackenzie, 1991; Rieseberg & Wendel, 1993). Surprisingly this does not dramatically affect the cytonuclear genotype frequencies in the hybrid zone (Arnold, 1994). Other zones show that although both sexes are fertile in the F_1, matings between parents are always in one direction with respect to sex. For instance in the

Hyla tree frogs, F_1 offspring are always the result of a cross between *H. gratiosa* females and *H. cinerea* males (Lamb & Avise, 1986) and in North American cottonwood trees where all F_1 progeny are crosses between Fremont females and narrowleaf males (Paige, Capman & Jennetten, 1991).

Asmussen, Arnold and Avise produced a recent suite of papers analysing associations between nuclear and cytoplasmic genotypes and inferring mating systems in a hybrid swarm from the observed cytonuclear architecture of the population [Asmussen, Arnold & Avise, 1987, 1989; Arnold, Asmussen & Avise, 1988; Arnold, 1993). This work is reviewed in the next section.

3.5.2. Cytonuclear disequilibria

In two populations that have diverged such that they are respectively fixed for alleles A and B at a nuclear locus and for types M and m in the mitochondrial genome, the parental cytonuclear genotypes are then AAM and BBm. If these taxa hybridise they will produce cytonuclear genotypes at frequencies shown in Table 3.5.2a. Expected frequencies of a) gametic associations between the nuclear allele and mtDNA type and b) genotypic associations between the nuclear genotype and mtDNA type. Deviations from the expectations of random union can be described as disequilibria in the same way as nuclear linkage disequilibrium (section 3.4.5).

	Nuclear genotypes			
Cytoplasm (mtDNA)	AA	AB	BB	Total
M	u_1	v_1	w_1	x
m	u_2	v_2	w_2	y
	u	v	w	1

Table 3.5.2a. Cytonuclear genotype frequencies in a hybridising population. (After Asmussen, Arnold & Avise, 1987).

For a population at which A and B are at frequencies p and q respectively and M, gametic cytonuclear disequilibrium can be defined as the frequency of AM gametes - (freq. A)*(freq. M), or

$$D = u_1 + {}^1/_2 v_1 - px \qquad [3.2]$$

Genotypic disequilibrium has three components, corresponding to the three possible nuclear genotypes:

$$D_1 = \text{freq. AAM} - (\text{freq.AA})*(\text{freq.M}) = u_1 - u\text{x} \quad [3.3]$$

$$D_2 = \text{freq. AaM} - (\text{freq.Aa}) *(\text{freqM}) = v_1 - v\text{x} \quad [3.4]$$

$$D_3 = \text{freq. aaM} - (\text{freq.aa}) *(\text{freqM}) = w_1 - w\text{x} \quad [3.5]$$

therefore genotypic and gametic disequilibria are linked in the relationship

$$D = D_1 + {}^1\!/_2 D_2 = -D_3 - {}^1\!/_2 D_2 = {}^1\!/_2 D_1 - {}^1\!/_2 D_3 \quad [3.6]$$

The most interesting component in a hybrid zone is D_2, which describes genotypes in the heterozygote class. In a unidirectional system like the cottonwood trees above, where the frequency of one cytonuclear genotype in the heterozygote class is absent, interpretation of mating system is simplified, but in less obviously directional hybridisation the relationships between the components of disequilibrium have been used to infer the strengths of reciprocal crosses (Cronin, Vyse & Cameron, 1988).

Asmussen, Arnold & Avise (1987) suggest an alternative disequilibrium measure, d, or *residual disequilibrium*, which has statistical significance as an interactive term.

$$d = (q - p)D - {}^1\!/_2 D_2 \quad [3.7]$$

They then use relationships between gametic (D), genotypic (D_1,D_2,D_3) and residual (d) disequilibria to classify patterns of cytonuclear disequilibria and test hypotheses about mating pattern within a hybridising population.

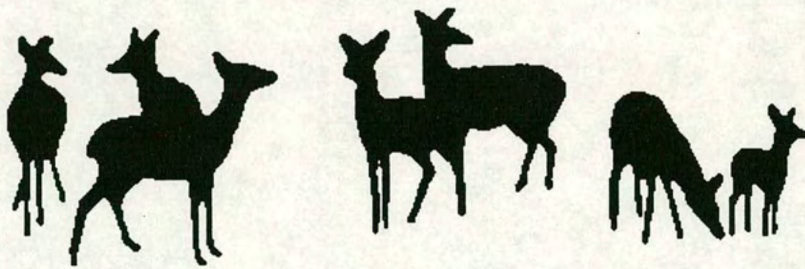
Asmussen, Arnold and Avise have developed their model of cytonuclear disequilibria on a case study of a population of 305 *Hyla* tree frogs, sampled in a single event in 1986 (Lamb & Avise, 1986; Asmussen, Arnold & Avise, 1987). These frogs inhabit a pool and the surrounding area, but are effectively a closed population. They are random mating with respect to nuclear genotype, but the matings are directional in the F_1 cross (*gratiosa* females with *cinerea* males, see above), creating strong cytonuclear disequilibria. The initial model assumed random mating, no migration and no selection, discrete non-overlapping generations, exclusively maternal inheritance of mtDNA and a closed population of infinite size. Clearly most of these assumptions are violated in hybrid zones that are maintained by a balance of selection and migration and display non-random mating (Barton & Hewitt, 1985,1989). The models have since developed to include epistatic interactions that influence mate choice (Arnold, Asmussen & Avise, 1988), non-random mating and migration of parental

types into the zone (Asmussen, Arnold & Avise, 1989) and genetic drift in finite populations (Fu & Arnold, 1992; Arnold, 1993). Despite these modifications the models remain inapplicable to a moving hybrid zone where colonists are likely to have heterozygous nuclear genotypes, creating disequilibria simply by dispersal, or to situations where selection acts against hybrids, reducing the effective size of the heterozygote class available for computation of D_2. Calculation of cytonuclear disequilibria under these circumstances are extremely analytically complex. To date the models do not combine analysis of nuclear and cytonuclear disequilibria, although the two are equivalent under random mating in a closed system. A compound analysis would be a much more powerful approach (Abernethy, 1994).

3.6. Summary

- Conventional species definitions do not adequately describe the relationship or taxonomic status of hybridising taxa that remain distinct despite gene flow.
- In hybridising taxa it is most useful to consider the behaviour of individual genes or traits and their interactions than to try to define the status of individuals within the population.
- Hybrid zones are maintained by a balance of dispersal and selection.
- Zones maintained by purely endogenous selection against recombinant genotypes are tension zones and in theory exist independently of habitat variation
- Exogenous selection may act to favour hybrids in an ecotone (bounded-hybrid superiority) or to disadvantage hybrids and favour particular parental types in different habitats.
- More than one type of selection may be acting in a given zone.
- Within a hybrid zone, habitat preferences may act on a fine scale to form a mosaic of genotype-habitat associations.
- Associations between loci, caused by non-random mating, genotype-specific selection or dispersal, can alter the genetic architecture of a hybrid population and form a barrier to gene flow.
- Differential selection on, or dispersal of, the sexes may cause directional introgression of genes across a zone.
- Introducted genomes must have a strong selctive advantage to survive at initial low frequencies.
- A wave of advance of an advantageous allele is unlikely to be halted by a density trough but may be stopped by a fitness change at a habitat boundary.

- If the initial advantage of the introduced population is epistatic this will be lost through recombination unless the fitness advantage also has an additive component.



Chapter 4.

METHODS IN CLINE ANALYSIS

4.1. Study Areas and Sites.

4.1.1. Overview

Two study areas were chosen in Scotland. These covered the largest continuous sika phenotype ranges; the main site in Argyll and the second in the Great Glen (see Fig 4.1.1), but had differing topography, climate and vegetation. Each area has a resident population of red deer and sika deer. The history of the introduction of sika to these areas is reasonably well documented (Ratcliffe, 1987a). The introductions were of similar numbers of animals, both occurring early this century. Although at the onset of the study the extent of genotypic introgression of sika into the red deer population was unknown, it was presumed that these areas would be likely source of hybrids. Samples were also collected in forests beyond the resident sika range, as reports of far-ranging sika stags were not uncommon (Ratcliffe, 1987a; Red Deer Commission, 1960-1993).

Within the large study areas, sampling sites were chosen in forest blocks managed by the Forestry Commission; 10 forests in Argyll, 6 forests in the Great Glen. These were chosen on the basis of availability. Access to tissue samples was made available by the Forestry Commission, from normal culling operations aimed at controlling population size (Ratcliffe, 1987b). Access to the land for censusing and observations was also allowed. At each forest, tissue samples and phenotypic data were collected and in a selected central site rumens were also collected.

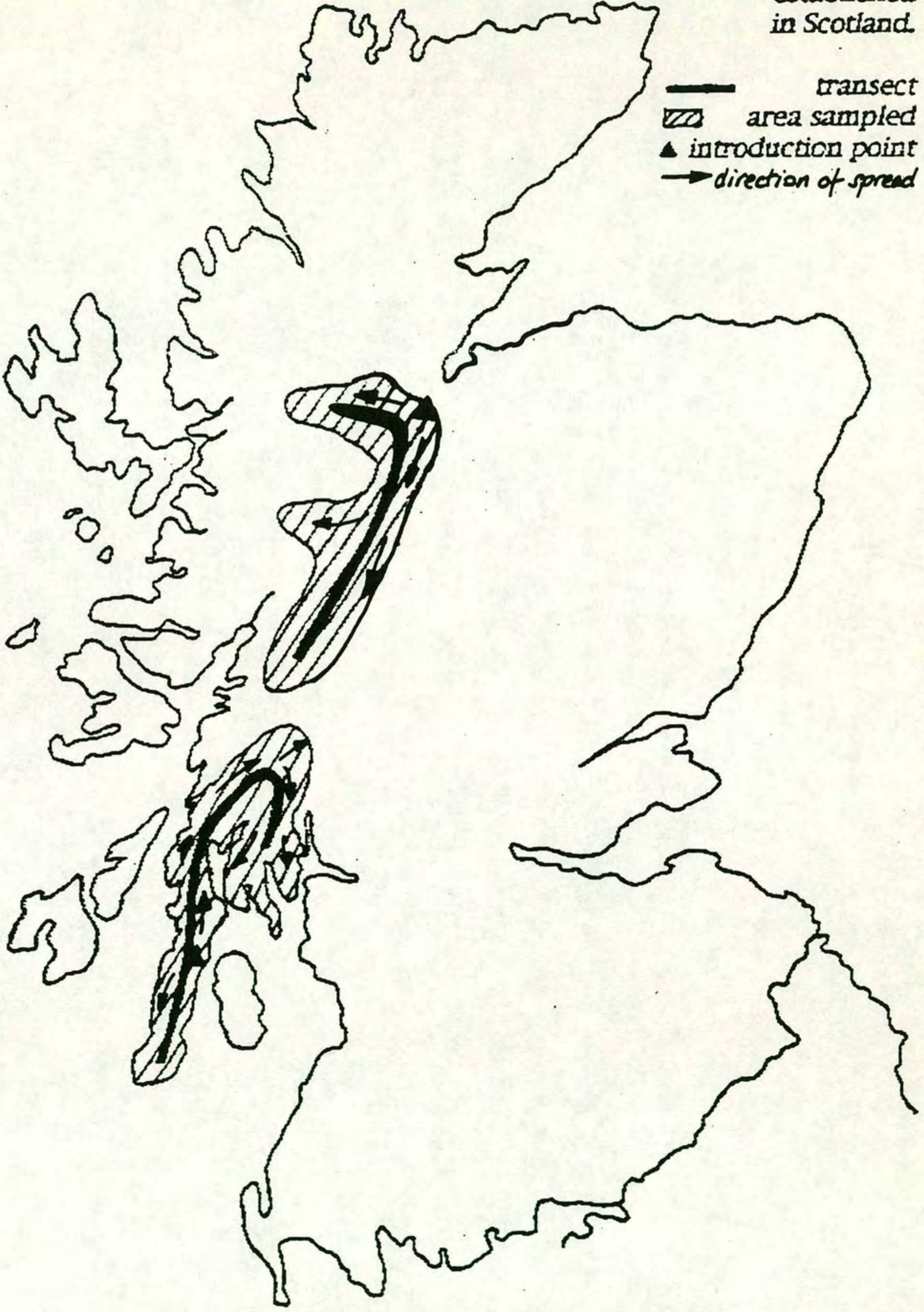


Figure 4.1.1. The transects established through mixed red and sika range in Scotland.

4.1.2. Argyll

Topography

The study area in Argyll, on Scotland's west coast, spans the Kintyre peninsula, the mainland area from the Crinan canal north to Loch Awe and east to the head of Loch Fyne, and the Cowal peninsula (Fig. 4.1.2a). The highest land is north of Loch Fyne, reaching 1130m (3708 feet) at Ben Lui, but most of the area, including all ground on the Kintyre and Cowal peninsulas, is below 600 m (2000 feet). This land is a series of rough, small hills and glens mainly running north-south and characterised by thin, acidic, rocky soils. The lochs create physical barriers to dispersal from the introduction site at Carradale in Kintyre (55°31'N, 5°30'W), making the system essentially linear. Although deer will swim considerable distances and have been sighted crossing Loch Fyne (Stuart & Stuart, 1848 , H. Gibb pers. comm.), this is likely to be an infrequent occurrence and will contribute little to overall dispersal.

Sampling forests are marked on the overlay of Fig. 4.1.2a. Distances from the introduction site and numbers of deer sampled are given in Table 4.1.2a.

Forest	Minimum Distance overland	Number sampled
South Kintyre	-70	2
Carradale	0	40
Achaglachgach	60	34
Knapdale	123	35
Kilmichael	138	9
Birdfield	188	46
North Cowal	283	48
East Cowal	313	13
Glendaruel	358	12
South Cowal	386	22

Table 4.1.2a. The details of sampling forests in the Argyll study area. Each forest was hunted by a ranger, employed by the Forestry Commission, who took samples from all red and sika deer culled between October 1991 & March 1992.

Climate

Argyll has a warm temperate maritime climate, influenced by the Gulf Stream. Mean monthly temperatures at Carradale range from 3°C in January to 11°C in July and mean annual rainfall from 1305mm in South Cowal to 2358mm at the head of

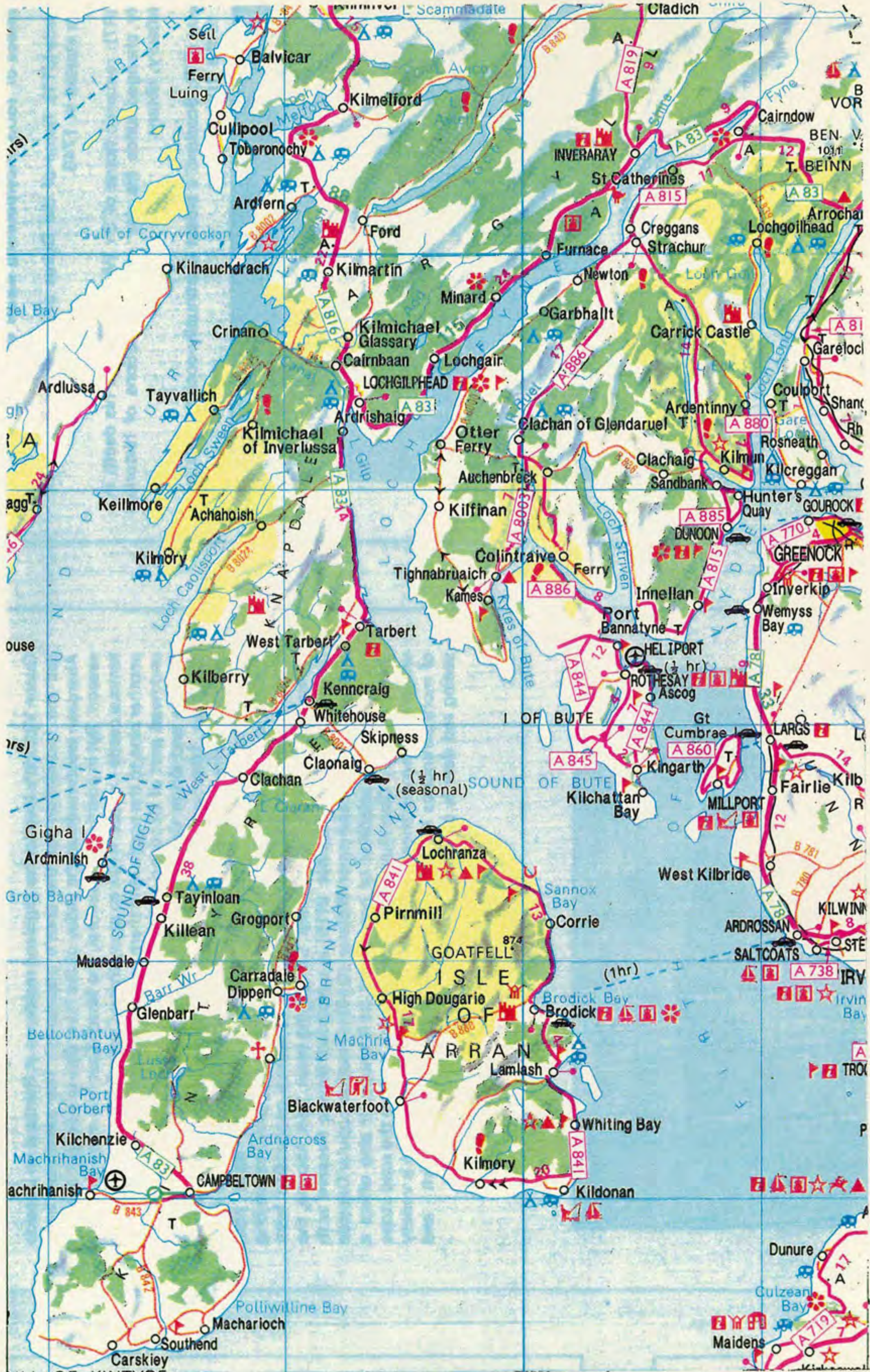


Figure 4.1.2a. The study area in Argyll. The overlay shows the forests sampled, which are referred to by name in the text.

Figure 4.1.2b. Typical habitat mosaic in Argyll. Deciduous and coniferous woodland, heath, mires and grasslands are found in small, mixed patches.

Loch Fyne. Prolonged snow cover is rare except on the high ground to the north of Loch Fyne where average cover is 4.2 - 6.5 days over the period November - April (Bickmore & Shaw, 1963). The area does experience strong south-westerly winds, mainly in the spring and autumn.

Vegetation and Land Use

The habitat can be broadly defined as a mixture of commercial conifer plantations, pockets of native deciduous woodland and *Calluna-Molinia* moorlands. Very little agricultural land exists in either area. The low valley bottoms have rough grazing for cattle, and there are sheep on the uplands. The plantation forestry is predominantly Sitka spruce (*Picea sitchensis*), but contains stands of Norway spruce (*Picea abies*), Lodgepole pine (*Pinus contorta*), larches (*Larix* spp.) and small numbers of other conifer varieties. Generally trees are planted and clear-felled in blocks of several hectares over an approximately 40 year rotation, dependent on species and conditions. Most plantation blocks are fenced against deer in the planting and establishment stages, but fences are often holed and deer have fairly free access after trees reach pre-thicket size, i.e. above 1m tall.

Deciduous woodlands are found along glen bottoms, loch sides, or in other sheltered areas, and are dominated by native species of birch (*Betula* spp.) and oak (*Quercus petrea*). Ash (*Fraxinus excelsior*), rowan (*Sorbus aucuparia*), willows (*Salix spp.*) and holly (*Ilex europaea*) are found frequently in these woods with other species as rarer occurrences.

Wet *Calluna* moors and Callunetum associations (McVean & Ratcliffe, 1968) are generally devoid of trees, though occasional Scots pines (*Pinus sylvestris*) birches and rowans exist. Although dominated by heather and grasses, other herbs, sedges and heaths are found in association with them (Rodwell, 1991b). Other communities cover only small areas (<5% total study area) and are more fully described below.

Vegetation communities often occur in small patches of a few hectares and boundaries between them are diffuse (i.e. Fig. 4.1.2b). This applies even to commercial plantations which are unevenly planted due to poor soils or steep slopes and which suffer greatly from uneven growth and windblow which break them up.

Deer population

In Argyll, "a few" sika (probably around a dozen) escaped from Carradale estate in the late 1890's (Whitehead, 1950). Their descendants were not reported further than the Carradale headland until 1911, but then spread quickly through the

surrounding area (see Chapter 1). Contemporary red deer densities were low for the immediate 30-40 km radius (Whitehead, 1964). Though actual red deer densities are not available, comparison with other populations designated of "low density" in the same text gives an estimate of 1-5 per km^2. Cervid densities throughout the Kintyre peninsula are now high, though exact counts are again unavailable. Estimates lie between 15-30 deer per km^2 in preferred habitat (Forestry Commission, unpub.). Censuses made during this study found local densities of 20-32 deer per km^2 in pre-thicket plantation (see below), but were insufficient to calculate absolute numbers on the peninsula. As thicket plantation has been previously found to be preferred habitat for cervid deer (Ratcliffe, 1987b), counts in this study were expected to give lower than maximum densities. Increases in deer density have been documented throughout Scotland during the last century and are probably due to decreases hunting pressure and improved habitat ,with the advent of commercial forestry.

The sika phenotype (as described above, Chapter 1) remains recognisable as distinct from red deer and resident sika-like individuals persist to the head of Loch Fyne, some 200kms from the introduction site, their frequency declining with increasing distance. Occasional sika males are sighted in south Cowal up to 400kms away from Carradale, but these sightings are rare and unpredictable. Approximate sika phenotype frequencies were assessed in each population, scoring animals as either 'sika' or 'red' dependent on the majority of the characters that were seen well enough to be assessed. Proportions of animals of mixed red and sika phenotype characters were recorded during the pilot study for this work (Knapdale forest, 1990) where animals were observed closely in a small study area (Abernethy & Thorne, unpub.). They were found to comprise around 17.2% of that population. However, field assessment of individual phenotypic characters was not thought to be reliable during censuses at a longer range of observation as often either a) all characters were not seen in an individual or b) the species origin of a character observed was uncertain. Most characters varied continuously throughout the population rather than exhibiting two discrete states.

4.1.3. Great Glen

Topography

The Great Glen (also called Glen Albyn or Glen Mor) cuts through the highlands of Scotland between the Monadhliath mountain range and the West Highlands. Loch Ness, Loch Oich and Loch Lochy lie along its length, joined by the

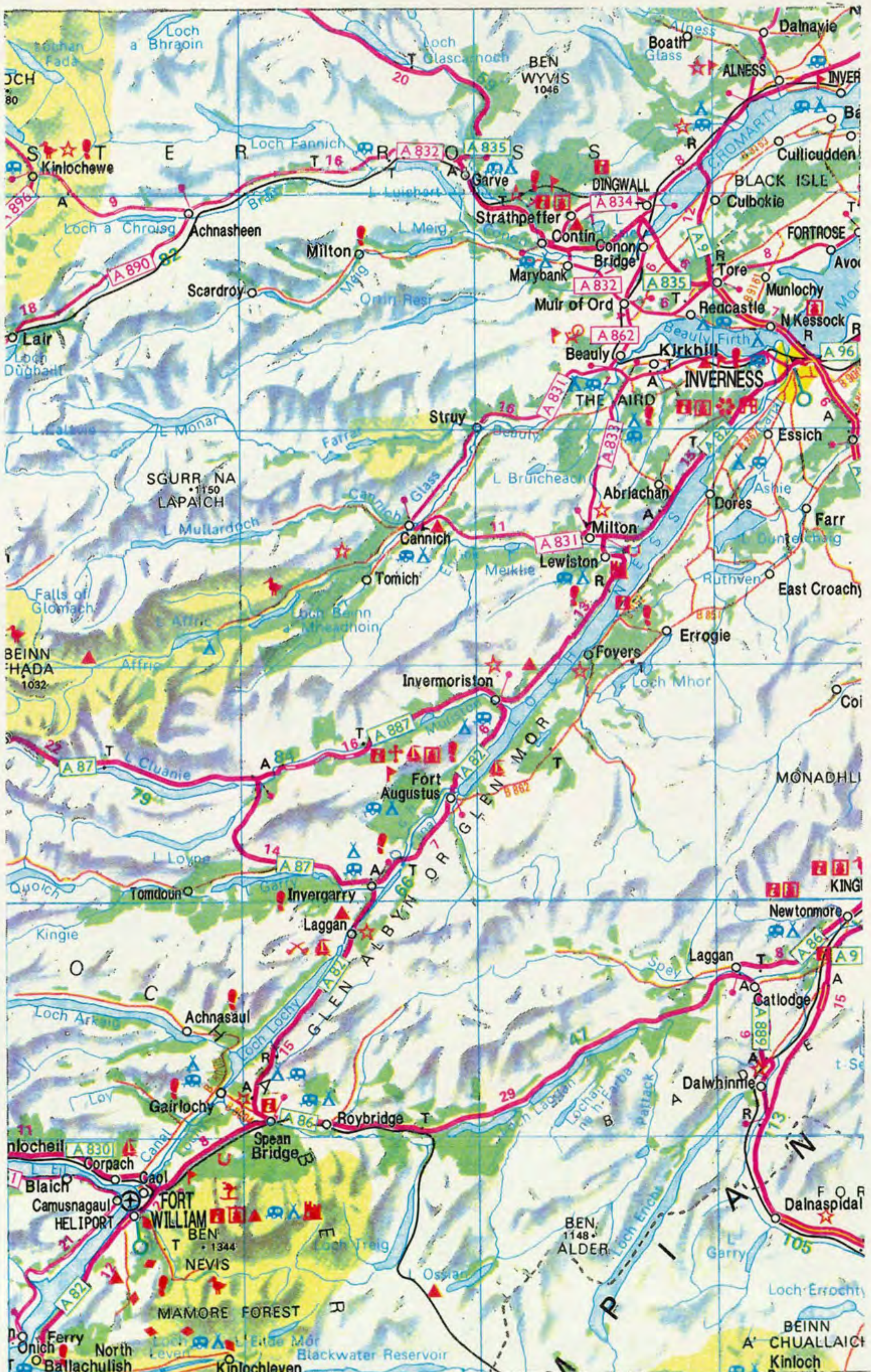


Figure 4.1.3a. The study area in the Great Glen. The overlay shows the forests sampled, referred to by name in the text.

Caledonian canal. To the north-east and south-west land rises fairly steeply to summits of over 1000m. The valley bottom is at sea level (Fig. 4.1.3a). Forests were sampled n the Great Glen (4 sites) and in Glen Urquhart, Glen Affric and Glen Morrison to the west. The sika release was at Aldourie (grid ref) at the north east tip of Loch Ness. As in Argyll there is a possibility of dispersal across the loch, though this is considered to make a relatively tiny contribution to overall dispersal patterns. Details of Great Glen sampling forests are shown in Table 4.1.3a.

Forest	Minimum Distance overland	Number sampled
Farigaig	13.75	23
Knockie	27.5	11
Inchnacardoch	36.5	32
Laggan Wood	48.5	7
Laddie Wood	53	34
Glen Urquhart	41.5	7
Glen Affric	42.5	17

Table 4.1.3a. The sampling forests in the Great Glen study area. Each forest was hunted by a single ranger, employed by the Forestry Commission, who took samples from all red and sika deer culled between October 1992 & February 1993.

Climate

The Great Glen is away from the warming influence of the gulf stream and is considerably colder in winter than Argyll. Temperatures vary from a January mean 4°C to July mean 14°C, with considerable variation due to altitude. Rainfall in the Glen is slightly lower than Argyll, with an annual mean of 1045mm, though there is also considerable variation in the hills from east to west. Due to the rain shadow effect of the western mountains, the eastern side is drier. Annual average snow lie for the period 1916-1950 was 10.2 - 17.0 days over the winter, November to March, but snow falls may begin earlier and continue later in the year (1963). Winds are westerly prevailing and can be strong on the high, exposed ground especially during autumn and winter.

Vegetation and Land Use

As in Argyll the major vegetation types are commercially planted conifer forest, deciduous woodland remnants, but Dry*Calluna*-moorland is more frequent than wet heath (1991b). Short, herb-rich grassland (*Agrostis-Festuca* associations) exist in large areas of the uplands in maosaic with Callunetum communities, though above 600m communities are montane (Rodwell, 1991c).

Figure 4.1.3b. Forest blocks in the Great Glen. The interior of these plantations often provides little forage, though shelter benefits deer.

Dry *Calluna* moor is almost entirely heather with few sedges and the coarse grasses. Patches of continuous habitat are large (several hundred hectares for patches of conifers or dry *Calluna*) and habitat boundaries tend to be well defined (e.g. Fig. 4.1.3b).

Except for the timber plantations and hill sheep, agriculture is confined to the valley bottoms. Even here it is scant as there is little flat land between the loch side and the hill side. There is no arable land of significant size.

Deer populations

At Aldourie in the Great Glen, the herd escaped from a park to which 4 animals had originally been brought and these may be considered as the founding population (Whitehead, 1950; Whitehead, 1964). In Invernesshire red deer densities were thought to be locally high (perhaps 10-12 per km^2), but distribution patchy. It is therefore uncertain what the density of the population immediately adjacent to the Aldourie estate may have been (Whitehead, 1964). Sika-like deer have spread through woodland habitat along the east shores of Loch Ness and along the smaller glens that run east from the Great Glen. They are also established on the western side of Loch Ness in Inchnacardoch, Glen Urquhart and Glen Affric, probably arriving by a route around north end of the loch, but possibly also swimming across. The sika phenotype in this area remains distinct and different from that of the red deer. Sika remain in close association with forestry in the Great Glen, although red deer populations are found at high density on the open ground.

4.2. Census Methods

4.2.1. Overview

There is a considerable debate concerning the merits of various methods of censusing deer populations in Scotland (Ratcliffe, 1987b; Red Deer Commission, 1960-1993). Widely used observational methods include vantage point counting (Forestry Commission) or group sweeping of an area (Red Deer Commission). Indirect methods involve pellet group counting (Neff, 1968; Ratcliffe, 1987b) or slot counting (Bergerud, Wyett & Snider, 1983). Recently the Red Deer Commission have

also experimentally counted areas using thermal imagery from a light aircraft, though this is so far unsuccessful in woodlands (Gordon-Duff-Pennington, 1994).

In this case the indirect methods were unable to distinguish red-like from sika-like deer and so were irrelevant to the question in hand.

4.2.2. Observational Methods.

Team Sweep Counts

The sweep principle employs a team of observers who walk across a known area of ground, noting the time, group composition, position and direction of movement of any deer seen. Observations are then pooled at the end of the sweep and a calculation made of the minimum number of deer required to account for them. Its strength lies in the ability to count densities on large tracts of land, but its weaknesses are in the number of observers required, their disruption of the population (which may drive deer before the counters, resulting in overestimates of density) and the inability to count areas of woodland which are sufficiently large to prevent deer from being flushed by beaters.

Vantage point counts

The vantage point count requires that a large tract of homogeneous habitat with clearly defined boundaries, be adequately viewed by a single observer. This observer then counts all deer viewed over a three hour (minimum) period on each of 2-4 consecutive days and calculates a density for the known visible area (Ratcliffe, 1987b). The three hour minimum is imposed to allow for deer ruminating. During rumination deer 'lie-up' in a sheltered place and are likely to be missed by an observer, who will see them when they resume grazing. Rumination bouts are generally less than three hours (Ratcliffe, 1987b). Accuracy of the estimate can be cross-checked if a second vantage point is available overlooking an overlapping tract of ground. This method deals well with forested areas (before canopy closure) if the vantage point is sufficiently high above the trees to see gaps between them, e.g. counting the opposite side of a steep glen. The counting period should also be extended to 4-5 hrs to ensure that deer within the area will be likely to move through a gap when feeding. The method suffers most from the assumption that the area counted is representative of a larger area and the validity of this is difficult to assess. There can also be problems in finding a suitable vantage point in relatively flat or undulating land.

The vantage point method was emloyed here primarily as it requires only a single observer, but also because it is able to deal with forested areas which predominate in these study areas.

Census routes

Where visibility is good over open country, animals can be counted by an observer walking a route from which all of a predetermined area can be seen (e.g. Clutton-Brock, Guinness & Albon, 1982). This method is likely to move animals unless the route is well-chosen and upwind, i.e. along a ridge, looking into a glen. It can be effective for assessing the proportions of animals of a particular type in a population; stag: hind ratios or red: sika, asuming all animals are equally visible. If a large amount of 'dead' ground exists out of view, or if animals can enter and leave the area unobserved then it is difficult to calculate an absolute density from this method and for this purpose a vantage point should be used. The route method has the advantage of coping with relatively flat terrain, but is not suitable for forestry where visibility is low.
Both the vantage point method and census routes were employed here primarily as they require only a single observer. They were chosen depending on the site topography and degree of forestation..

4.3 Laboratory Methods

In this section I describe laboratory techniques used in the genetical part of the study. Although the genotypes are necessary in interpreting aspects of red-sika population ecology in later sections, the methods are primarily of importance here. Several methods, such as pouring of gels and extracting DNA, or the recipes for standard buffers are well-known procedures in molecular biology and basic protocols can all be found in Sambrook, Fritsch & Maniatis (1989). I have relegated my exact methods for these and suppliers details for commercial buffers etc. to appendices, giving a brief overview in the text. Less well-known methods are presented in full in the text.

4.3.1. Tissue Collection and Storage

At each forest tissue samples were collected from all cervid deer shot by Forestry Commission rangers. Roughly equal numbers of hinds and stags were culled each week. Stags were shot under licence RWY/CT/27/1/1, issued to the Forestry Commission by the Red Deer Commission. In Argyll, samples were collected over the 1991-1992 hind season (October 21st - February 28th) and in the Great Glen collections were made over the following, 1992-1993, season (same dates). In each case sample collection was carried out from December to March. In each forest culling was carried out on a daily basis and rangers hunted in all forest types from planting to felling, but included a only small portion of unplanted land. Rangers culled deer when they were seen, regardless of phenotype. This may bias sampling if one type is less visible than the other, but this bias is impossible to assess as it will also affect the apparent composition of the population in the census. It was assumed that the sampling was not biased to one phenotype in the absence of any such evidence, though I do not discount the possiblity.

Approximately $1cm^3$ of both kidney and neck muscle tissue were taken from each animal and frozen to -20°C within 24 hrs of death. These samples were taken by the rangers at the time of culling. Samples were collected weekly from field freezers and brought to Edinburgh for storage at -70°C until processing. Time until processing varied from 4-6 months for isozymes or 1-12 months for DNA markers.

4.3.2. DNA Extraction and Storage

DNA was extracted from a subsample of kidney tissue from each animal and from the foetuses collected from Knapdale. Extractions were performed using a standard phenol : chloroform technique, modified from Sambrook, Fritsch & Maniatis (1989). The procedure involves denaturing of non-DNA proteins, by digestion with proteinase K, followed by their removal in a phenol or chloroform suspension. Isolated DNA is then precipitated in 100% alcohol. The exact protocol is given in Appendix 4.3A. DNA quality and approximate concentration were assessed by electrophoretic separation on an agarose checking gel and comparison to known standards (Appendix 4.3B). Isolated DNA was stored at -20°C until use. Working DNA concentrations for the following procedures (generally a 10x to 100x dilution of the extraction) were assessed by trial and error on a test panel of the extracted DNA.

Subsamples at the appropriate concentration were then kept at -20°C in the lab., whilst stocks were stored at -70°C.

4.3.3. Amplification And Electrophoretic Separation Of Microsatellite Marker Loci.

Overview

Marker loci in the genomic DNA were amplified by polymerase chain reaction (PCR), and visualised by polyacrylamide gel electrophoretic separation of the resulting radiolabelled fragments. A similar (non radio-active) technique was applied to mitochondrial DNA (mtDNA), with an additional step involving restriction digestion of the PCR product (see below; Protocol 4C). As PCR is essentially the same reaction, under slightly varying conditions, for any DNA fragment, it is discussed fully here and only briefly noted in the section on mtDNA markers. The protocol for mtDNA amplification is given in section 4.3.4, together with considerations specific to that reaction.

4.3.3.1. The polymerase chain reaction.

The polymerase chain reaction (PCR; (Mullis & Faloona, 1987) generates multiple copies of a DNA template by the action of a nucleotide polymerising enzyme. It is a quick and relatively simple technique and can be used on degraded or 'dirty' DNA, as it targets small, specific portions of the template strand. Even a single template molecule can be amplified to high copy number, lowering sample requirements to tiny fragments of tissue. The development of PCR has made the screening of DNA polymorphisms possible for large sample sizes and greatly increased the scope of empirical population genetics (Avise, 1994).

The basic principle behind the PCR reaction is a threefold process of temperature cycling. First the template DNA is denatured, usually at 94°C, although particularly guanine and cytosine (GC) rich regions may require higher denaturing temperatures (Hoelzel & Green, 1992). Secondly, at a lower temperature (around 55°C), 'primers' (synthesized oligonucleotides designed to be homologous to the sequence flanking the target DNA) anneal to the template at either end of the target sequence. Finally a copy of the target sequence is synthesised from free nucleotides by the polymerase enzyme. This step is necessarily at the enzyme optimum temperature, which for the most commonly used polymerase is 72°C. The three part cycle is repeated several times until yield of product DNA copies reaches a maximum (0.3-

1pM, or 25-40 cycles; (Gyllensten, 1989), later cycles replicate from initial copy strands to give an exponential yield.

PCR protocols and reaction mixtures vary according to the template DNA strand and primers used. Primer design is important in determining the specificity of the product. Less than 100% homology between the primer and the template, especially at the 3' end where annealing is initiated, can result in non-specificity, as can short primer length (Moore *et al.*, 1991). Primer annealing temperature is determined by the primer GC: AT (adenine & thymine) content, and to a lesser extent by the sequence order of the nucleotides (Suggs *et al.*, 1981). The most commonly used rule for estimating annealing temperatures of known primers is

T_{melt} (°C) = 2(A+T) content + 4(G+C) content

$T_{ann.}$ (°C) = T_{melt} - 5

Generally a high GC content in primers allows higher annealing temperatures and this gives better specificity in the product. Primers may anneal to each other if annealing temperatures are low, creating 'primer-dimer' artefact product and wasting primer copies in the reaction mix. A 40-60% GC content, in primers of 17-24 base length is a good rule of thumb (1992). The lengths of the temperature cycle stages are dependent mainly on the lengths of the primers (annealing stage) and the length of the template (polymerisation stage).

The building blocks of PCR are free nucleotides. These are commercially available in the form of (neutralised) deoxynucleotide triphosphates (dNTP's) from most molecular biology suppliers. They are added in equal concentrations per nucleotide to the reaction mix to a final total concentration of 50-200µM. Too great a concentration of nucleotides can lead to misincorporation, whilst too few will limit the yield of the reaction. Misincorporation is a serious problem if the product is to be sequenced, but can lead to problems for other PCR product applications also.

The efficiency of the reaction can be fine-tuned by additives which improve the performance of the polymerase enzyme. DNA polymerases were first discovered in thermophilic bacteria, including *Thermus aquaticus* which now produces the widely commercially available PCR polymerase, *Taq* polymerase (Innis *et al.*, 1988). The following comments and protocols are based on the use of *Taq* polymerase for PCR.

Magnesium chloride ($MgCl_2$) can profoundly affect the efficiency of the reaction and is incorporated in most PCR buffers. Low $MgCl_2$ reduces yield, but high $MgCl_2$ concentrations increase non-specificity. Often an essential step in PCR protocol design is an first trial-and-error test run, using serial concentrations of $MgCl_2$ in the reaction mixture. Non-ionic detergents can improve yield by reducing *Taq* inhibitory SDS (sodium dodecyl sulphate) contamination from the DNA extraction process.

Dimethyl sulphoxide (DMSO) or formamide are occasionally used to increase primer specificity in GC rich regions by reducing secondary structure of the template strand, but they decrease *Taq* activity and so reduce yield. Often the use of reaction additives is a matter of altering the balance between specificity and yield, rather than ensuring the success of the reaction.

PCR can potentially amplify DNA fragments from a large number of samples in a very short time. As it amplifies a specific region (assuming the use of specific, rather than universal primers) genetic divergence within that region can be interpreted in the light of prior expectations for sequence conservation. Rates of both random and non-random sequence change vary throughout the genome (Dover, 1982; Britten, 1986). Population genetic studies are now able to detect and interpret variation at the sequence level with increasing confidence and in considerably larger numbers of animals than were possible before PCR became available (e.g. Allendorf & Leary, 1988; Pemberton *et al.*, 1988; Hall & Muralidharan, 1989; Hale & Hoffman, 1990; Jones, 1990; Arntzen & Wallis, 1991; 1991; Hall, 1992; Turelli, Hoffman & McKechnie, 1992; Hall & Nawrocki, 1994; Bancroft, Pemberton & King, in press)

4.3.3.2. Microsatellites

Microsatellites are short lengths of DNA composed of a series of variable number dinucleotide (or occasionally trinucleotide) repeats, flanked by variable sequence (Litt & Luty, 1989). Typically they are hypervariable in repeat iterations as transcriptional errors inserting or deleting repeats are common. This leads to varying fragment lengths, detectable by electrophoretic separation after PCR amplification priming from the flanking sequence. Fragment sizes represent Mendelian inherited alleles present at the locus. Microsatellites are common in all higher vertebrates (Sarkar, Paynton & Sommer, 1991) and often PCR priming sequences will amplify microsatellites in taxa congeneric with, or even more widely divergent from those in which they were designed (Moore *et al.*, 1991). Primers for many microsatellite loci are now available and have been used to good effect in variability studies of wild populations (1991; Hughes & Queller, 1993; in press) The level of variation is less than that at minisatellite loci (Bruford *et al.*, 1992) but finding microsatellites and screening variation is simpler, making microsatellite techniques well suited to studying variation at the population level.

Microsatellites do have two significant draw backs in a) the presence of null alleles and b) rapid mutation rate of alleles. These are not grave problems in many studies as long as their existence is recognised and dealt with. Null alleles are alleles

which fail to amplify from the primer sequence, perhaps because they incorporate a change in the priming site. An individual homozygous for the null allele will give no band on the gel and it is likely that the sample may be discarded, leading to overestimation of other allele frequencies in compensation for the deficit. If the individual is heterozygous for the null allele and another, it will appear homozygous for the other allele, leading to overestimation of that allele frequency and underestimation of the level of heterozygosity in the sample. Inheritance tests on individuals of known pedigree and on mother-offspring pairs can be used to identify mismatches and trace null alleles (Pemberton & Slade, in press), but in most wildlife studies these samples are unavailable. The possible presence of null alleles in the population can pose a problem in these cases.

The mutation rate of microsatellites is known to be high. If the rate of mutation is of the same order of magnitude as the introduction of new alleles by immigration, then conclusions cannot be drawn about population process and demography from microsatellite data. Generally this time frame will not be a problem, but in cases where migrants are rare and populations have been separated over several hundred generations, the origin of rare alleles should be considered carefully. The problem of parrallel evolution of microsatellite alleles may be greater than in other markers as the structure of a microsatellite - a chain of two or three nucleotide repeats - makes it likely that each mutation by slippage adds or deletes a repeat, rather than altering the fragment length by a random amount. The problem is not serious for many applications, but should be taken into acount when choosing microsatellite markers to investigate population differentiation.

In this study, already available ungulate microsatellite loci were screened on a panel of known species individuals. This panel comprised 3 sika from a zoo population, 6 sika from the present Carradale herd in Argyll and 6 sika from Glen Mazeran in Invernesshire, 6 red deer each from the islands of Rum (samples from J. Pemberton) and Mull off the west coast of Scotland. The *BOVIRBP* and *OarFCB193* loci amplified species-specific alleles in a test panel of known individuals and gave a good product yield under existing conditions. They thus enabled identification of red x sika hybrids and were chosen for population screening. The species-specific status of alleles at the *BOVIRBP* locus has since been independently confirmed (M. Bruford, 1994, pers. comm.). The loci were previously isolated in other ungulates; *BOVIRBP* in cattle (1991), modified and supplied by D. McHugh, Trinity College, Dublin) and *OarFCB193* in sheep (Buchanan & Crawford, 1993).

BOVIRBP primers are:

Forward	5' TGT ATG ATC ACC TTC TAT GCT TC 3'	23mer
Reverse	5' GCT TTA GGT AAT CAT CAG ATA GC 3'	23mer

Oar FCB193 primers are:

Forward	5' TTC ATC TCA GAC TGG GAT TCA GAA AGG C 3'	28mer
Reverse	5' GCT TGG AAA TAA CCC TCC TGC ATC CC 3'	26mer

Genbank accession no. LO1533.

Protocol 4A: PCR amplification of microsatellite loci from genomic DNA

Preprepare
- Working conc. DNA
- Primers @ 10pM/µl
- 1M DMSO (dimethyl sulphoxide)
- 10x PARR-excellence™ buffer
- 0.05M $MgCl_2$
- dNTP's (deoxynucleotide triphosphates) @ 1mM dATP, dGTP, dTTP ; 0.1mM dCTP
- dH_20
- *Taq* polymerase @ 10 units/µl (in freezer)
- $dCTP^{32P}$ @ 1mM

a) Switch on PCR machine to warm up. In this case a Hybaid 'Omnigene' was used.

b) Programme in reaction cycle.

Reaction cycle

i) 2 mins at 93°C
ii) 7 cycles of 30 secs at 93°C
60 secs at 52°C
30 secs at 72°C
iii) 25 cycles of 30 secs at 89°C
60 secs at 54°C
30 secs at 72°C

c) Calculate recipe for enough 'cocktail' for a 10um reaction for each sample, plus a control.

Protocol 4A continued

Cocktail for 1 x 10µm reaction

On ice	*µl*	
DNA	1.0	
Primer 1	0.2	
Primer 2	0.2	
DMSO	1.0	
'PARR-excellence'™	1.0	
$MgCl_2$	0.1	
dNTP's	4.0	
dH_2O	6.0	
Taq polymerase	0.05	(add penultimately)
dCTP ^{32P}	0.025	(add last)

d) Make up appropriate cocktail **on ice**.

e) Add 1µl DNA to each PCR tube. NOTE ORDER AND LABEL).

f) Add cocktail up to 10µl.

g) If the PCR machine requires an oil seal on the sample to prevent evaporation, add 1 drop mineral oil to each tube.

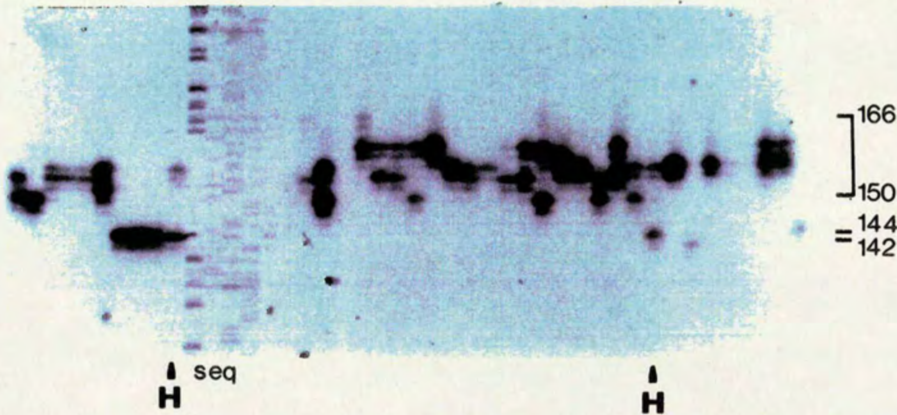
h) Place in PCR machine and start reaction cycle immediately.

i) When reaction is finished, store products at -4°C until electrophoresis.

4.3.3.3. Polyacrylamide gel electrophoretic separation

Varying length fragments, corresponding to alleles were amplified by the PCR technique above. These were then separated by electrophoresis on polyacrylamide gel. 'Sequagel®' preprepared 6% polyacrylamide gel was used (supplied by National Diagnostics, Georgia, USA), polymerised with TEMED (99% N,N,N',N'-Tetramethyl ethylenediamine; Sigma Chemical Co.), and 10% ammonium persulphate. Gels were poured in sequencing plates, giving 48 or 96 sample capacities. Running buffer was 1x TBE (Appendix 4.3c). Gels were allowed to set for 1 hour and then pre-run at 70mA, 2000V to achieve a temperature of 50-55°C before loading. For loading, 4µl of 5x TBE-Bromophenol blue loading dye (Appendix 4.3c) was added to each sample and 1-2µl per sample of this mix loaded onto the gel. If the PCR reaction was oil-sealed, samples were removed from below the oil meniscus with a fine pipette tip. A four lane sequence ladder was run on each gel to give a size standard. This is a single strand DNA clone of published sequence (supplied by USB™, USA), cut sequentially at each base by an endonuclease, to provide a 'ladder' of fragments differing in size by a single base. From the published sequence the size of

Figure. 4.3.3a
Microsatellite DNA polymorphisms
A. BOVIRBP locus
Red specific alleles gave fragment sizes of 150-166 base pairs
Sika specific alleles gave fragment sizes of 142-144 bp.
Fragments are shown against a DNA sequence ladder (SEQ).
Heterozygote red x sika individuals (H) are seen directly to the left of the sequence and in the 9th lane from the right.



B. OarFCB193 locus.
Red specific alleles gave fragment sizes 97-119 bp.
The Sika specific allele gave a fragment size of 129 bp.
The individual in the centre of the gel (X) had alleles outside of either the sika or red range. This animal subsequently typed oddly at all other loci and was believed to be a contaminated sample.

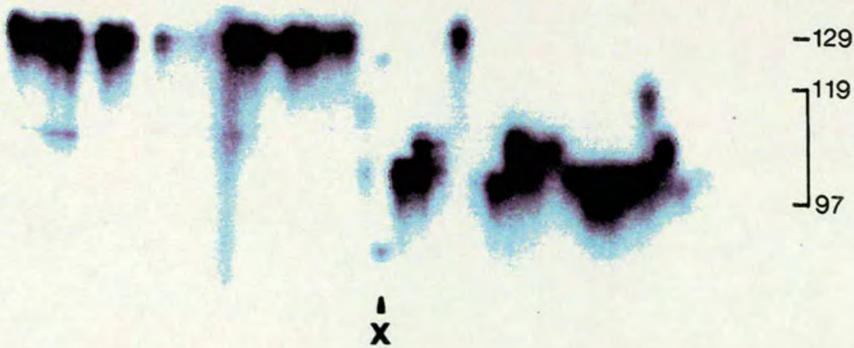
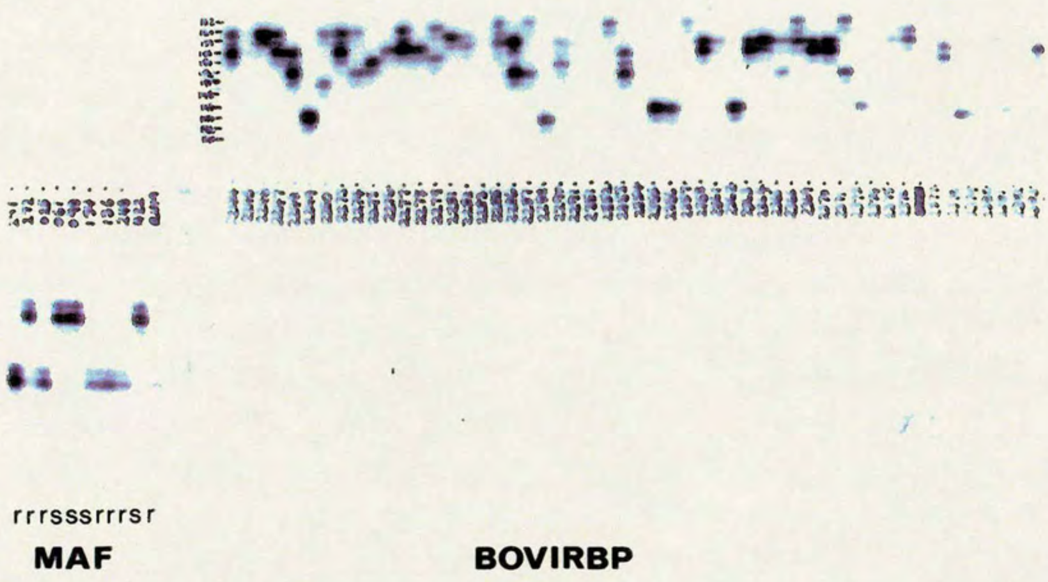


Figure.4.33b
Screening the population.
A screening gel for the microsatellite locus BOVIRBP. Red individuals are found in the 150-166 base pair region, sika at 142 or 144 bp. A DNA sequence ladder is used to score fragment sizes. To the left of the sequence is a trial of the locus MAF 23, which proved non-diagnostic. The test panel of known species individuals are marked below. Numbers on the gel lanes indicate individual sample numbers from the Great Glen populations.



corresponding unknown fragments can be calculated. Gels were run at 55°C (50mA, 2000V) for 2-3hrs using separation of the loading dye colour bands to assess the position of the DNA. At the end of the run, gels were removed from the plates by blotting onto filter paper sheets, protected with plastic wrap, and dried on a heated vacuum drier. Once dry, radioactivity levels were counted with a hand-held Geiger counter and an appropriate length autoradiographic exposure made. Generally a 5-24 hr exposure at -70°C, with intensifying screens was adequate, though time and the use of screens varied according to the activity on the gel.

4.3.3.4. Scoring the microsatellite gels

Alleles on the gels were scored according to size. Species-specific size classes are shown in Figs. 4.3.3a. Red allele sizes were designated from the test panel and from allele sizes found in a sample of 229 Rum red deer scored by J. Pemberton and J. Slate (unpublished data). Several red alleles were present at each locus (7 at *BOVIRBP*, 11 at *OarFCB193*) and animals were scored as 'red' regardless of the particular alleles they carried, though these were recorded. Sika alleles were designated from the test panel alone, but have since been independently confirmed as Sika specific (M. Bruford, pers. comm). Sika were also scored as 'sika' for any sika specific allele, though in fact only two sika alleles were found at the *BOVIRBP* locus and a single one at *OarFCB193* (Figs. 4.3.3a&b). The lack of polymorphism in the sika loci is explicable by the small number of founder animals. Individuals that gave indistinct banding patterns or failed to give a product were rerun and scored independently by a second observer. Some animals failed to give a reliable result, but these were a random sample of the population. Most of these samples failed to give a result at *any* locus and it was concluded that the DNA was damaged. The discrepancies in sample sizes between loci are accounted for by these technical problems.

4.3.4. Amplification, Restriction Digestion and Electrophoretic Separation of Mitochondrial DNA Haplotypes.

Overview

Mitochondrial DNA types were classified by species-specific restriction fragment length polymorphisms (RFLP's) from the ND genes of the NADH complex in the mitochondrial genome. This region has proved highly variable in several taxa (Hall & Nawrocki, in press), though the red and sika specific haplotypes found in this

study were previously unknown. The creation of an RFLP haplotype involved amplification of a 2kb fragment of mtDNA by PCR (Protocol 4B, below), cleavage of the product into fragments by endonuclease digestion (Protocol 4C, below), followed by agarose gel electrophoresis separation of those fragments (Appendix 4.3b). PCR techniques and applications have been previously discussed in section 2.2.4 and as they apply to the amplification of mtDNA in a similar fashion to genomic microsatellite loci, I will not replicate that discussion here, save comments specific to this reaction. Restriction digestion techniques applied only to mtDNA haplotype analysis in this study and are discussed below.

4.3.4.1. The PCR amplification of the 16s + ND1 region (ND genes in the NADH complex) of the cervid mitochondrial genome.

Reaction conditions were identical to those previously used on brown trout (1994). The primers are ORF 381 & ORF 563 (Georgiadis & Patton, unpub.)
Forward (16s): 5' ACC CCG CCT GTT TAC CAA AAA CAT 3' (24 mer) 381
Reverse (Met tRNA) : 5' GGT ATG AGC CCG ATA GCT TA 3' (20 mer) 563.
The fragment amplified is a 2 kilobase length.

4.3.4.2. The principle and application of polymorphic RFLP loci

Restriction endonucleases recognise specific sequence combinations of four, five or six bases and cut the DNA strand at those sites. The differing lengths of the resulting fragments give a characteristic banding pattern when separated by gel electrophoresis. Evolutionary changes within cleavage sites, involving base deletions, insertions or substitutions will prevent recognition and cleavage at the site, resulting in differing fragment lengths and banding patterns. In principle, base insertions and deletions *between* restriction sites also alter fragment lengths, but in practice resolution of fragments on agarose gels is insufficient to detect these. Effectively a restriction endonuclease is only capable of detecting sequence change in the tiny proportion of the DNA involved in restriction sites. RFLP patterns can be used to distinguish individuals at the family, population, species or genus level, dependent on the amount of sequence divergence present and amount of sequence exposed to digestion i.e. the size of the cleavage site multiplied by the maximum number of cuts made. The level at which differentiation between individuals is sought, i.e. family, population or species, determines how many potentially polymorphic digestions are required. The more DNA that is exposed to digestion, the more potential there is for variation being found, if it exists.

Protocol 4B: PCR amplification of a fragment of the mitochondrial genome

Preprepare Working dilutions of DNA samples (estimated from checking gels)
25pM/µl 'ORF' primers
1.25mM (per nucleotide) deoxynucleotide triphosphates (dNTPs)
10x *Taq* buffer
Taq polymerase @ 10 units/µl (in freezer)
dH_2O

a) Switch on PCR machine to warm up. In this case either a Perkin Elmer '9600' or a *Hybaid* 'Omnigene' was used.

b) Programme in reaction cycle.

Reaction cycle
i) 2 mins at 95°C
ii) 30 cycles of 60 secs at 55°C
90 secs at 72°C
30 secs at 94°C
iii) 10 mins at 72°C.

c) Calculate recipe for enough 'cocktail' for a 25µm reaction for each sample, plus a control.

Cocktail for 1 x 25µm reaction

On ice	*µl*	
dH_2O	15	
10x *Taq* buffer	2.5	
dNTPs	4.0	
primer 1	1.0	
primer 2	1.0	
Taq XL	0.125	(add last)

d) Make up appropriate cocktail **on ice**.

e) Add 1-3µl DNA to each PCR tube. NOTE ORDER AND LABEL.

f) Add cocktail up to 25µl.

g) If the PCR machine requires an oil seal on the sample to prevent evaporation, add 1 drop mineral oil to each tube.

h) Place in PCR machine and start reaction cycle immediately.

i) When reaction is finished, store products at -20°C until electrophoresis.

The likelihood of cleavage sites occurring in a given sized fragment of DNA can be calculated for four, five and six cutters, assuming equal proportions of the bases in that DNA sequence (Lansman *et al.*, 1981). This assumption may not be

valid, but provides a reasonable starting point for choice of cutters. Clearly the chance of a four base cutter site within a given piece of DNA is lowest. If there are too many sites, small fragments will be produced and resolving and scoring them on a gel is unreliable. If there are too few, the chance of polymorphism is low. A choice of cutters should be guided initially by the question being asked and the level of variation is being sought, and then by the likely size and number of fragments expected. An initial amount of trial and error is inevitable. Lansman *et al.* (1981) show that the expected fragment number can be calculated from the expression $m_t a$, where

$$a = (g/2)^{n_1} [(1-g)/2)]^{n_2} \quad [4.0]$$

letting g be the GC content of the mtDNA and n_1 and n_2 the number of guanines and cytosines (G and C) and the number of thymines and adenines (T and A) respectively in the restriction cutter fragment.

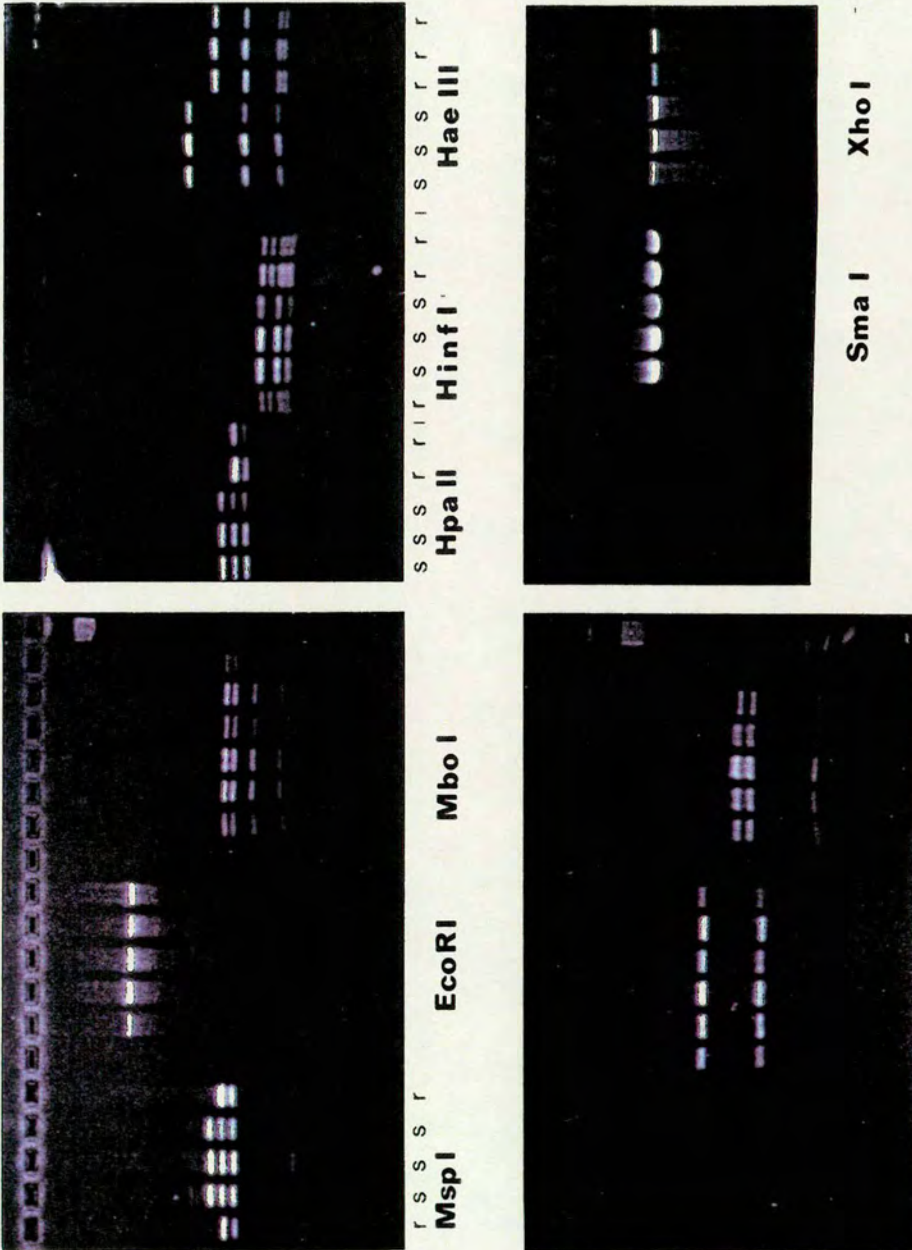
Enzyme	Recognition sequence	Cutter size	Expected no. cleavage sites
Alu I	AG/CT	4	7.8
Hae III	GG/CC	4	7.8
Hpa II	C/CGG	4	7.8
Mbo I	/GATC	4	7.8
Msp I	C/CGG	4	7.8
Rsa I	GT/AC	4	7.8
Xho I	G/TGAG	5	2
Hin fI	G/ANTC	5	2
Sma I	CCC/GGG	6	0.49
Eco RI	G/AATTC	6	0.49

Table 4.3.4a. The restriction endonucleases used to test for species-specific RFLP haplotypes. *Hpa II* and *Msp I* are isochizomers whose recognition of the sequence depends on methylation of the cytosine base. *Hpa II* will not cleave if the second C is methylated, *Msp I* will not cleave if methylation is on the first C.

Figure 4.3.4b

Mitochondrial DNA haplotypes.

Fragments from restriction digestions of a 2kb region of the mt genome with the endonucleases *MspI*, *EcoRI*, *MboI*, *HpaII*, *HinfI*, *HaeIII*, *RsaI*, *AluI*, *SmaI*, *XhoI*. Diagnostic digestions are labelled *r* or *s* for the species type they represent. *HaeIII*, *HinfI* & *MspI* were used in screening the populations. Identical haplotypes were found in Argyll & Great Glen populations.



In this case the aim was to find species-specific markers within the maternally inherited mitochondrial genome. Thus markers that were invariant at the population level, but variable to the species level were ideal. Eleven restriction endonucleases were initially used in digestions of the test panel of known red and sika DNA described above (section 2.3). Most of these were four cutters, expected to produce approximately 8 restriction sites in the 2kb fragment (Table 4.3.4a). Four of these produced species-specific haplotypes, and three, *Hae III, Hin fI* and *MspI* were subsequently used to screen the population. The others were monomorphic (Fig. 4.3.4a).

Protocol 4C: Restriction enzyme digests

Preprepare: Clean, PCR amplified DNA
Restriction enzymes and appropriate buffers
dH_20
Incubator @ 37°C
Agarose checking gel set-up

a) Add 11μl dH_2O to 7μl PCR reaction product.
b) Add 0.3μl restriction enzyme and 2μl relevant buffer.
c) Incubate for at least 5 hrs.
d) Load and run 8μl product with 4μl 5x loading dye on a 1.5% agarose DNA checking gel, as Appendix 2.2b.
e) Stain, illuminate and photograph bands on gel (Appendix 2.2b).
f) Store remaining product at -20°C (for up to 48 hrs) in case a rerun is required.

4.3.4.3. Scoring mtDNA gels

Gels were scored from a photograph of the ultra-violet illumaination after ethidium bromide staining. Direct scoring requires prolonged exposure to UV light, which is unsafe. Each animal was typed 'red' or 'sika' for each digest as only two haplotypes were recorded for each one. Band patterns were generally unambiguous, but if a digestion was thought to be incomplete, a second PCR product aliquot was digested. No samples failed to digest a second time. Some samples initially failed to give a PCR product. Three attempts were made at amplification, after which the sample was discarded.

4.3.5. Electrophoresis and staining of species diagnostic isozymes

4.3.5.1. Isozyme use in population genetics

Lewontin & Hubby (1966) and Harris (1966) first showed how natural variation in enzyme structure could be used in the study of Mendelian inheritance and evolution. Literature concerning the use of these isozymes (or allozymes) in population genetics has since become immense (reviewed by (Hames & Rickwood, 1990). As they have grown, techniques for gel electrophoresis of many mammalian isozymes have become readily available and they are an alternative to direct DNA analysis for assessing genetic variation. Laboratory procedures are generally simpler and less expensive than DNA methodologies, allowing larger populations to be screened for less time and effort, although PCR based procedures now rival these advantages.

Isozymes are enzyme proteins with identical functional groups and activity, but differing tertiary structures or sizes dependent on their genetic basis. Generally they are cell cycle proteins, involved in glycolysis or the citric acid cycle, and are present in the majority of taxa in similar form. The enzymes are composed of subunit polypeptide chains, each of which may be coded for by separate segregating loci or by alleles at one locus. Enzymes with a single polypeptide unit are known as monomers, those with two subunits as dimers and those with four as tetramers. Isozyme electrophoresis attempts to separate subunits of differing structure and to interpret the variation observed in terms of its genetic basis. This is essentially a four stage process encompassing extraction of the enzymes, separation of isozymes by gel electrophoresis, staining to visualise the resultant band pattern and interpretation of its genetic basis.

Extraction of enzymes in animals is usually a matter of lysing cells in a suitable buffer, as cell cycle proteins are soluble. Care must be taken to keep samples cold as they are usually thermo-labile. In fact sample storage is best at -80°C and only viable for about two years. A simple protocol for extraction is to homogenise tissue in a cold, pH 7.0 buffer and centrifuge down the solids. This leaves a supernatant suitable for electrophoresis.

Electrophoresis separates the polypeptide chains according to their size, structure and net electrical charge. A gel medium provides a sieving mesh through which the molecules are dragged. An electric current provides the impetus for

migration, with an aqueous buffer conducting the voltage through the gel. Starch, at a concentration of 10-15% w:v, is the most commonly used gel medium, although cellulose acetate, and polyacrylamide are alternatives. Polyacrylamide provides the densest gel and so resolves small differences in migration distance, especially in small molecules. However it is expensive, toxic and its high resolution is often superfluous or indeed too great to enable large molecules to separate. Cellulose acetate gels are ready-made with a cellulose gel matrix mounted on an acetate plate. This provides an extremely thin gel with little electrical resistance, allowing separation of isozymes in 20 to 40 minutes, as opposed to several hours on starch gels. Their disadvantage is that, unlike starch gels, they can only be stained for a single enzyme as they cannot be sliced into sections. This greatly reduces their worth in studies where many systems are being screened.

Observation of the bands produced by electrophoresis relies on the specificity of the enzyme catalytic activity. Enzyme specific substrate and co-factors are applied to the gel in the presence of a dye, producing a colour band where catalytic activity occurs. Banding patterns on the gel indicate the migration distance of the subunits of the protein.

Interpreting these bands is the most difficult part of the procedure. Artefact bands can be produced by degraded tissue, by buffering the system at the wrong pH, uneven gel density, contamination of the sample or non-specific staining. Nowadays the structures and therefore the expected band patterns of common cell cycle isozymes are well known (Harris & Hopkinson, 1976; Richardson, Baverstock & Adams, 1986) and artefact bands may be detected as not conforming to these expectations. However, artefacts can often occur where bands *would* be expected and are only detectable by rerunning samples, or finding inheritance mismatches between related individuals. Repeatability of results should always be checked. For all enzymes homozygotes for each coding locus give a single band, but heterozygotes produce complex patterns depending on the number of segregating loci coding for the protein, the polymorphisms present at each locus and the multimeric structure of the enzyme (May, 1992). Correct interpretation of heterozygote banding patterns can yield a lot of information about enzyme structure and genetic basis, but should be approached with caution.

4.3.5.2. Isozymes in this study

In this study I used isozymes and microsatellite DNA markers as complementary techniques, providing greater opportunity for detection of species-specific markers.

Previous studies of cervid taxonomy (Feldhammer *et al.*, 1982; Gyllensten *et al.*, 1983; Herzog, 1988; Linnell & Cross, 1991; Emerson & Tate, 1993) were reviewed to find loci likely to yield species-specific isozyme polymorphisms and these were screened on the test panel of known species (section 4.3.3) to check their applicability in this case. There was found to be a large amount of disagreement between studies in the literature (Table 4.3.5). Most studies seem methodologically robust although some have used sample animals derived from different subpopulations designated as species (Emerson& Tate, 1993). The variation in results probably arises from interpopulation differences in allele frequencies and highlights the danger of making taxonomic conclusions from small samples (see also Chapter 2). Three enzyme loci were chosen for population screening; 6-phophogluconate dehydrogense (*6pgdh*), Mannose phosphate isomerase (*MPI*) and Superoxide dismutase 1 (*Sod 1*). The *Sod 1* locus was diagnostic in the test panel employed in this study, though there has been some debate over the species-specific nature of polymorphisms at this locus in red and sika deer. Linnell & Cross's (1991) work on Irish deer and Emerson and Tate's (1993) study on New Zealand populations give different opinions, the former citing sika as fixed for a 'fast' allele and red deer as polymorphic at the locus ('slow' allele frequency = 0.913), the latter assigning the polymorphism to sika ('fast' allele frequency = 0.833) and showing red deer as fixed for a slow allele. The Linnell & Cross study, however, used samples from the sympatric red-sika populations in Kerry, Ireland and may have detected polymorphism as a result of prior hybridisation. The Emerson & Tate study is based on Manchurian sika (*Cervus nippon manchuricus*), rather than Japanese, as in this case. They find that red deer (of British and European origin) are fixed for the slow allele.

The monomeric *MPI* gave a two banded heterozygote, whilst in *6pgdh* and *Sod 1* heterozygotes a third heteromeric band was seen. This is produced by a mixture of the two subunit types that give the homozygote bands (Fig 4.3.5a). All samples were screened by starch gel electrophoresis of tissue homogenate, followed by enzyme specific staining. *Sod 1* and *6pgdh* yielded species-specific bands, *MPI* showed the presence of rare alleles in the hybrid zone, but was not species diagnostic as Emerson & Tate (1993) had found it to be.

Isozyme	Name	Study	Red	Sika	Comment
AAT-1	Aspartate aminotransferase 1	Linnell & Cross, 1991	Mono	Mono	
AAT-2	Aspartate aminotransferase 2	Linnell & Cross, 1991	Mono	Mono	
ADA-1	Adenosine deaminase 1	Gyllensten et al., 1983	Mono		
ADA-2	Adenosine deaminase 2	Gyllensten et al., 1983	Mono		
AK-1	Adenylate kinase 1	Gyllensten et al., 1983	Mono		
AK-2	Adenylate kinase 2	Gyllensten et al., 1983	Mono		
ALB	Albumin	Emerson & Tate, 1993	Poly	Mono	*Studies do not agree
ALB		Baccus et al., 1983	Mono		
ALD	Alcohol dehydrogenase	Herzog, 1988	Mono	Mono	
ALK	Alkaline phosphatase	Emerson & Tate, 1993	Mono	Mono	
AP	Acid phosphatase	Gyllensten et al., 1983	Mono		
C3	C3 complement protein	Emerson & Tate, 1993	Poly	Mono	
CA-1	Carbonic anhydrase 1	Emerson & Tate, 1993	Mono	Mono	
CA-2	Carbonic anhydrase 2	Emerson & Tate, 1993	Mono	Mono	
CK-1	Creatine kinase 1	Gyllensten et al., 1983	Mono		
CK-1		Linnell & Cross, 1991	Mono	Mono	
CK-2	Creatine kinase 2	Linnell & Cross, 1991	Mono	Mono	
DIA-1	Diaphorase 1	Emerson & Tate, 1993	Mono	Mono	
DIA-1		Herzog, 1988	Mono	Mono	
DIA-2	Diaphorase 2	Emerson & Tate, 1993	Poly	Mono	
EST-1	Esterase 1	Gyllensten et al., 1983	Mono		
EST-2	Esterase 2	Gyllensten et al., 1983	Mono		
EST-3	Esterase 3	Gyllensten et al., 1983	Mono		Studies do not agree
EST-4	Esterase 4	Baccus et al., 1983	Poly		
GAPDH	Glyceraldehyde phosphate dehydrogenase	Gyllensten et al., 1983	Mono		
GC	Vitamin D binding protein	Emerson & Tate, 1993	Poly	Mono	*
GDH	Glucose dehydrogenase	Herzog, 1988	Mono	Mono	
GDH		Gyllensten et al., 1983	Mono		
GDH		Linnell & Cross, 1991	Mono	Mono	
GLDH	Glutamate dehydrogenase	Herzog, 1988	Mono	Mono	

Isozyme	Name	Study	Red	Sika	Comment
GOT-1	Glutamate oxalate transaminase 1	Baccus et al., 1983	Mono		
GPD	Glucose 6 phosphate dehydrogenase	Baccus et al., 1983	Mono		
GPD		Gyllensten et al., 1983	Mono		
GPI-1	Glucose phosphate isomerase 1	Emerson & Tate, 1993	Poly	Mono	Studies do not agree
GPI-1		Gyllensten et al., 1983	Poly		
GPI-1		Herzog, 1988	Mono	Mono	
GPI-2	Glucose phosphate isomerase 2	Gyllensten et al., 1983	Mono		
GPT	Glutamate pyruvate transaminase	Gyllensten et al., 1983	Mono		
GUS	b-glucuronidase	Gyllensten et al., 1983	Mono		
Hb	Haemoglobin	Emerson & Tate, 1993	Mono	Mono	
HK-1	Hexokinase 1	Herzog, 1988	Mono	Mono	
HK-1		Gyllensten et al., 1983	Mono		
HK-2	Hexokinase 2	Gyllensten et al., 1983	Mono		
IDH-1	Isocitrate dehydrogenase 1	Emerson & Tate, 1993	Poly	Poly	Studies do not agree
IDH-1		Baccus et al., 1983	Poly		
IDH-1		Gyllensten et al., 1983	Mono		
IDH-1		Herzog, 1988	Mono	Mono	
IDH-1		Linnell & Cross, 1991	Mono	Mono	
IDH-2	Isocitrate dehydrogenase 2	Gyllensten et al., 1983	Poly		
IDH-2		Linnell & Cross, 1991	Poly	Mono	
LDH-1	Lactate dehydrogenase 1	Emerson & Tate, 1993	Mono	Mono	
LDH-1		Baccus et al., 1983	Mono		
LDH-1		Gyllensten et al., 1983	Mono		
LDH-1		Herzog, 1988	Mono	Mono	
LDH-1		Linnell & Cross, 1991	Mono	Mono	
LDH-2	Lactate dehydrogenase 2	Linnell & Cross, 1991	Mono	Mono	
LDH-2		Baccus et al., 1983	Mono		
LDH-2		Gyllensten et al., 1983	Mono		

Isozyme	Name	Study	Red	Sika	Comment
MDH-1	Malate dehydrogenase 1	Emerson & Tate, 1993	Mono	Mono	Studies do not agree
MDH-1		Baccus et al., 1983	Poly		
MDH-1		Gyllensten et al., 1983	Mono		
MDH-1		Linnell & Cross, 1991	Mono	Mono	
MDH-2	Malate dehydrogenase 2	Gyllensten et al., 1983	Poly		
MDH-2		Linnell & Cross, 1991	Poly	Mono	
ME	Malic enzyme	Emerson & Tate, 1993	Mono	Mono	* Studies do not agree
ME		Baccus et al., 1983	Mono		
ME		Gyllensten et al., 1983	Poly		
MPI	Mannose phosphate isomerase	Emerson & Tate, 1993	Mono	Mono	* Studies do not agree
MPI		this study	Poly	Poly	
MPI		Baccus et al., 1983	Mono		
MPI		Gyllensten et al., 1983	Mono		
MPI		Linnell & Cross, 1991	Mono	Mono	
MPI		Baccus et al., 1983	Mono		
NDH	Nothing' dehydrogenase	Linnell & Cross, 1991	Mono	Mono	*
PEP	Peptidase	Gyllensten et al., 1983	Mono		
PEPT-B	Leucylglycylglycine peptidase	Baccus et al., 1983	Mono		
PGDH	Phosphogluconate dehydrogenase	Emerson & Tate, 1993	Mono	Mono	*
PGDH		Baccus et al., 1983	Mono		
PGDH		Abernethy, in press	Mono	Mono	*
PGDH		Gyllensten et al., 1983	Mono		
PGDH		Herzog, 1988	Mono	Mono	*
PGDH		Linnell & Cross, 1991	Mono	Mono	*
PGI-1	Phosphogluco-isomerase 1	Linnell & Cross, 1991	Poly	Mono	Studies do not agree
PGI-1		Baccus et al., 1983	Mono		
PGI-2	Phosphogluco-isomerase 2	Linnell & Cross, 1991	Mono	Mono	
PGI-2		Baccus et al., 1983	Mono		

Isozyme	Name	Study	Red	Sika	Comment
PGM-1	Phosphoglucomutase 1	Emerson & Tate, 1993	Mono	Poly	Studies do not agree
PGM-1		Gyllensten et al., 1983	Poly		
PGM-1		Herzog, 1988	Mono	Mono	
PGM-1		Linnell & Cross, 1991	Poly	Mono	
PGM-2	Phosphoglucomutase 2	Linnell & Cross, 1991	Mono	Poly	Studies do not agree
PGM-2		Baccus et al., 1983	Mono		
PGM-2		Gyllensten et al., 1983	Poly		
PK	Pyruvate kinase	Gyllensten et al., 1983	Mono		
PLG	Plasminogen	Emerson & Tate, 1993	Poly	Poly	
PTF	Post-transferrin	Emerson & Tate, 1993	Mono	Mono	*
SDH	Sorbitol dehydrogenase	Gyllensten et al., 1983	Mono		
SDH		Baccus et al., 1983	Mono		
SOD-1	Superoxide dismutase 1	Emerson & Tate, 1993	Mono	Poly	Studies do not agree
SOD-1		Abernethy, in press	Mono	Mono	*
SOD-1		Gyllensten et al., 1983	Poly		
SOD-1		Linnell & Cross, 1991	Poly	Mono	
SOD-2	Superoxide dismutase 2	Emerson & Tate, 1993	Mono	Mono	
SOD-2		Gyllensten et al., 1983	Mono		
SOD-2		Linnell & Cross, 1991	Mono	Mono	
TO	Tetrazolium oxidase	Baccus et al., 1983	Mono		
TRF	Transferrin	Emerson & Tate, 1993	Poly	Mono	
TRF		Linnell & Cross, 1991	Poly	Mono	

Table 4.3.5 Isozymes in the study of *Cervus elaphus* and *C. nippon* systematics. Of the 24 loci examined by two or more groups, 11 (45.8%) produced different results in different studies. In all cases the discrepancy lies in the number of different alleles found rather than in the mobility of those alleles as without comparing banding patterns directly on the same gel, there is no certainty of identity of alleles designated in different studies. In some instances one study found a locus to be monomorphic whilst another found polymorphism, in others different numbers of alleles were found within a polymorphic locus. At *MPI* only one study found red deer fixed for one allele and sika for a different one (Emerson & Tate, 1993) whilst others found them fixed for the same allele (Linnell & Cross, 1991) and yet a third found polmorphism (this study).

4.3.5.3. Tissue preparation

Kidney tissue for isozyme extraction was stored at -70°C. An approximately 500mg fraction was cut from each sample and homogenised on ice in 0.5ml of 1 in 20 'AC' buffer (Appendix 4.3c). The homogenate was centrifuged for 15 secs at 6000g and returned to ice. Whatman No. 4 grade filter paper wicks (4mm x 10mm) for loading the gel were soaked in the supernatant and excess fluid blotted off.

4.3.5.4. Starch gel electrophoresis

In this case starch gel electrophoresis was used as it was inexpensive and protocols existed for the enzyme loci required. Hydrolysed starch was bought in powder form (Sigma Chemicals Co.) and mixed to a 10% gel in the lab. in a 0.04M Citrate buffer at pH 6.1 ('AC' buffer; Appendix 2.2c), diluted in the gel at 1:20. Gels were poured in perspex moulds $160mm^2$ x 5mm and allowed to set for 8 hrs at room temp. They were cooled at -4°C for 1hr prior to use. Samples were loaded on 10 x 4mm wicks inserted at a central origin. *Sod 1* ran cathodally, *6pgdh* and *MPI* ran anodally. Gels were run for 6hrs at -4°C, covered with polythene to prevent dessication. They were stained by agar overlay immediately after running (Protocol 4D).

4.3.5.5. Scoring isozyme bands

Bands were scored and immediately recorded as 'fast' or 'slow' according to the distance migrated. Homozygote individuals gave a dense single band and were scored FF or SS. Heterozygotes at *MPI* gave two equal bands, one fast, one slow. *6pgdh* and *Sod 1* heterozygotes gave a triple banded pattern, with a equal fast and slow bands and a stronger midway (heteromeric) band (Figs. 4.3.5a&b). All heterozygotes were scored FS. Each gel had a known marker individual run alongside the unknown samples. Samples that failed to produce a stained band or produced an indistinct result were rerun. Samples that failed three times were discarded. Forty random samples were double run to check repeatability of scores. This gave a reliability of 100% for *MPI*, 97.5% for *Sod 1* and 98% for *6pgdh*. This was deemed acceptable.

Protocol 4D: Staining for *6pgdh*, *Sod 1* and *MPI* activity

Preprepare Tris-HCl buffer pH 8.0
Tris-HCl buffer pH 9.0
$MgCl_2$
MTT @ 10mg per ml.suspension
NADP @ 10mg per ml suspension
PMS @ 10mg per ml suspension
6-phosphogluconic acid
D-mannose 6 phosphate
Glucose-6-phosphate dehydrogenase @ 1 unit /µl
Phosphoglucoisomerase @ 1 unit / µl
Agar powder
dH_2O

3 x 50ml glass beakers
3 glass stirring rods
500 ml conical flask
Gel slicer
Glass plate for second slice
Incubator at 37°C

Protocol 4D (continued)

a) For each stain mix up all ingredients **except MTT and PMS** in a LABELLED beaker.

6pgdh	*Sod 1*	*MPI*
25ml Tris-HCl pH 8	25 ml Tris-HCl pH 9	5ml Tri-HCl pH 8
		13mg D mannose 6-phosphate
50mg MgCl2		
2.5µl NADP		5mg NADP
15mg 6PGA		30µl G6PD
		5µl PGI
1mg MTT	1mg MTT	5mg MTT
2.5mg PMS	1mg PMS	3mg PMS

b) Place 2g agar powder in conical flask, make up to 100ml with dH_2O.

c) Cook in microwave or over bunsen until clear and not frothing.
(If in microwave cover with ventilated clingfilm and take care not to over-boil.)
Keep warm in incubator while gel is prepared.

d) When gel has run switch off and remove film and wicks.

e) **Label gel orientation**.

f) Slice gel into 2 x 2.5 mm slices.

g) Place second slice, sliced side up, on glass plate.

LABEL WHICH STAIN IS REQUIRED.

6pgdh and *MPI* stains on anodal halves.

Sod 1 on cathodal half.

h) Add MTT and PMS to stains and mix well.

i) Add equal volume of warm agar to each stain , mix and pour QUICKLY over gel half.

j) Place in incubator until bands appear.

k) After 5-10 mins put *Sod 1* under lamp to develop.

l) Score bands or photograph with labels.

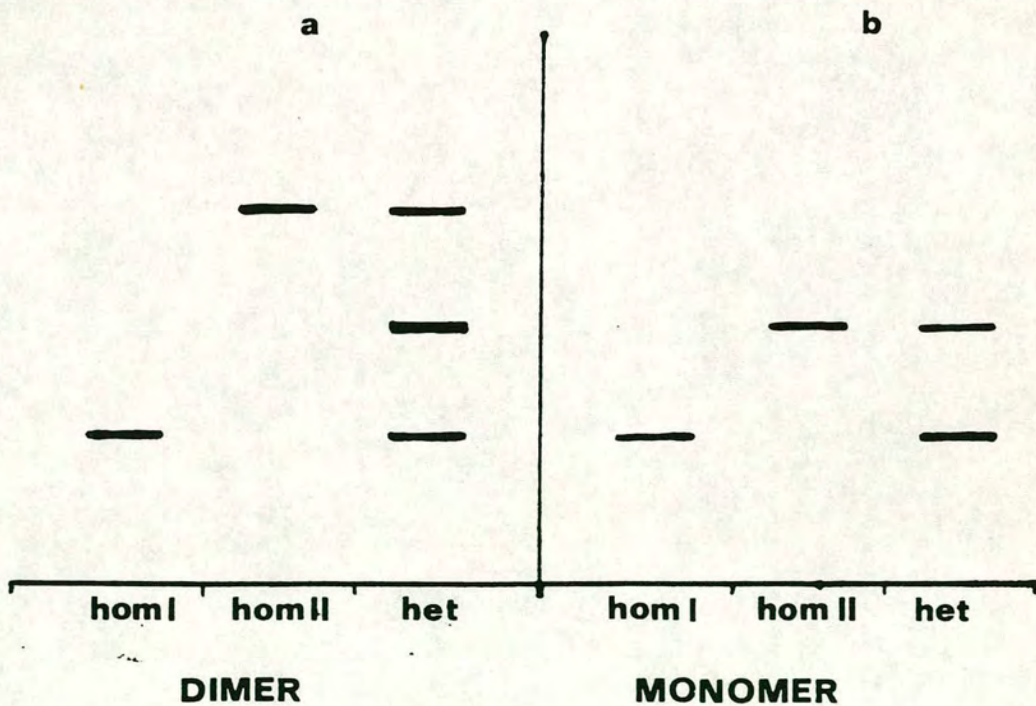


Figure 4.3.5a&b. Banding patterns on isozyme gels. a) shows the single fast and slow homozygote bands and the 3-banded heterozygote typical of dimers (*6pgdh and Sod 1*). b) shows the equivalent genotypes for the monomeric *MPI*.

4.4. Analytical Methods

4.4.1. Maximum Likelihood

Conventional statistics are based around the concept of a 'null hypothesis', against which the data is tested. Significance is assigned to the test statistic dependent on its distribution under the null hypothesis (usually derived from a normal distribution). Confidence in the estimate depends on the chance of obtaining that estimate if the null hypothesis were true. Generally significance is assigned to a result that gives a <5% chance of rejecting the null hypothesis if it is in fact true, though criteria for accepting a result as significant may change. This method of statistical testing requires prediction of each null hypothesis before the test and tests a single assumption at a time.

Likelihood analysis provides an alternative approach to hypothesis testing, in that it compares the observed data to a single, or a suite of, alternative hypotheses and assignes a value of likelihood to the match between the real data and the predicted values under each alternative, given certain starting parameters. Similar to customary testing, hypotheses are acceptable if the log likelihood of its match to the data is within 2 units of the maximum likelihood of achieving that set of results. A 2 unit drop in log likelihood (ΔlogL) corresponds roughly to the hypothesis being 7.4 times *less* likely to have produced the data than the alternative. As hypotheses become more complex, the perfect solution will become more precise and ΔlogL for data sets that are not in complete accordance with expected distributions will be larger. To account for this the limit of 2 units logL should account for the number of parameters being fitted. An approximation can be made using the χ^2 distribution for data sets with large sample sizes. In this case 2ΔlogL is approximately equivalent to χ^2 and hence conventional tables can be used to estimate significance levels for complex tests.

Likelihood tests are used throughout the analysis of the genetic data. The equations used in each calculation are given below and these are reiterated using the algorithms. These initiate the calculation with a set of arbitrary parameters in the equation and calculate a ΔlogL (L_2-L_1) after a random change is made. If this change improves the likelihood estimate, it is accepted, if not it is rejected with a probability of L_2/L_1. The process continues until the change in likelihood is consistently small, when a maximum likelihood estimate is found. Criteria for acceptance of a maximum

likelihood estimate can be set dependent on the sample size and likelihood of alternative explanations.

4.4.2. Cline width

In hybrid zones the most basic data acquired is in the form of a frequency cline between two parental populations in each of which the character is fixed for different states. This character may be a molecular marker such as an isozyme or a quantitative trait like eye colour. Analysis of cline shape and width can be used to infer the ratio between selection strengths and dispersal distances in hybridising populations (Barton, 1979b; Barton & Hewitt, 1985; Hewitt, 1988; Barton & Hewitt, 1989; Mallet, Barton & Lamas, 1990; Barton & Gale, 1993). Clines are generally described by a logistic or tanh curve of the form

$$p = 1+\tanh[2(x,y)/w]/2 \qquad [4.1]$$

or, equivalently

$$p = [1+ e^{-4(x,y)/w}]^{-1} \qquad [4.2]$$

The modelled parameters are y and w, the width and position of a cline, given allele frequencies (p) at distances from an origin, x.. The likelihood of the data is calculated assuming that all error results from sampling error of a binomial distribution around the expected curve.

$$\text{LogL} = \text{constant} + N_{alleles}[N_p \log p_e + N_q \log q_e] \qquad [4.3]$$

wherep_e andq_e are the expected frequencies of p and q. However, this is not a valid assumption as some variance around the model curve will be due to true deviations. These are caused by linkage disequilibrium, heterozygote deficit (F_{is}) and heterogeneity between loci (Sites, Barton & Reed, 1994; McCallum, in prep.). Linkage disequilibrium reduces the effective sample size because alleles are not sampled independently and heterozygote deficit will increase the error as there are effectively even fewer gametes free to recombine than the model assumes in its calculation of p_e andq_e. If F_{is}, R and heterogeneity between loci (Var (p/q)/ var p) have been previously assessed they can be used to calculated N_E the effective sample size of alleles, which then replaces $N_{alleles}$ in the above equation.

The assumptions that the tanh or logistic curve are good models for the cline shape and that the corrections for N_E are close approximations introduce possible errors. Ideally a simulation of the data including exact calculations of the effect of genic interactions without fitting a model curve will give the best estimate of cline width and position.

Initially cline width is calculated as a maximum likelihood estimate of w using eqn [4.3] without correcting for genic interactions. This provides an approximate first estimate, with support limits of values within 2 units logL, for a mean allele frequency across all loci p. In Chapter 8 the possiblities of applying computer simulation models to the data to describe cline width more accurately are explored. The usefulness of cline width estimates for a moving, unstable cline are then discussed.

4.4.3. F-statistics

When two alleles, p and q, are in Hardy-Weinberg equilibrium genotype frequencies are described by

$$p^2 + 2pq + q^2 = 1 \quad [4.4]$$

Wright's (1951) inbreeding coefficient, F_{is}, tests the chance of two alleles at a locus being 'identical by descent' i.e. sharing the same ancestry. A proportion F of gametes remain homozygous, whilst (1-F) combine at random. Deviations from Hardy-Weinberg in a hybrid zone may result from selection, migration or non-random mating reducing the heterozygote genotype and can be thought of in a similar way to alleles derived in different parents. In Hardy Weinberg genotype frequencies would be in the ratio

$$p^2+pqF \ : \ 2pq(1-F) \ : \ q^2+pqF \quad [4.5]$$

where if F is 0, there is no deviation from Hardy-Weinberg.

For a sample in which the observed number of homozygous genotypes are N_{pp} and N_{qq} and heterozygotes are N_{pq}, maximum likelihood estimates and support limits (<2 units logL) for F are generated by the equation

$$\text{LogL} = \text{constant} + N_{pp}\log[p^2+pqF] + N_{pq}\log[2pq(1-F)] + N_{qq}\log[q^2+pqF] \quad [4.6]$$

Heterogeneity between Fis estimates at different loci can be assessed by the change in logL between the individual locus estimates. If there is no significant difference between the estimates of Fis at each locus, they are behaving in a similar fashion.
Wright's F inbreeding coefficient (Wright, 1951) may be calculated by hand from the observed frequency of heterozygotes and the expected frequency calculated from the allele frequencies p and q as

$$F = [1-(N_{pq}/N)]/\, 2pq \quad [4.7]$$

which is equivalent to the maximum likelihood solution to equation 4.6. Significant deviation for Hardy-Weinberg is tested by comparison to the χ^2 distribution of

$$\chi^2 = 2NF_{is}2(k-1) \qquad [4.8]$$

where k = no.of alleles at the locus.As the red x sika system essentially reduces variation to a diallelic system, this becomes $4NF_{is}$, with one degree of freedom.

4.4.4. Linkage disequilibria of nuclear loci.

The concept of linkage disequilibrium is reviewed in Chapter 3. Measurements of linkage disequilibria in this analysis pool two components; the disequilibria within gametes and cross-gamete interactions (Weir, 1979; Hedrick, 1987). This is a necessary step as the derivation of the double heterozygotes cannot be deduced from the genoytpic data available; they may be they fusion of two heterozygous gametes or of the opposite homzygotes.

The effect of within locus heterozygote deficits, calculated as deviations from Hardy-Weinberg (F statistics above), can be used to strengthen the model by controlling for gametes effectively not available for recombination, but this does not fully solve the problem of disentangling the two contributing disequilibria. One should bear in mind the derivation of the disequilibrium measures when interpreting their biological significance.

For two loci with alleles P, Q and R, S at frequencies *p, q, r, s*, ten possible genotypes can be generated, the PQRS genotype occurring both from PQRS and QPSR unions.

The expected frequencies of these genotypes in the absence of disequilibrium or heterozygote deficit can be derived from the product of the frequencies of each allele. If both disequilibrium between loci and heterozygote deficits within each locus are taken into account the expected frequencies of these genotypes would be

which is equivalent to the maximum likelihood solution to equation 4.6. Significant deviation for Hardy-Weinberg is tested by comparison to the χ^2 distribution of

$$\chi^2 = 2NF_{is}2(k-1) \qquad [4.8]$$

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$$\text{Freq. PPRR} = [(p\text{P}r\text{R+D})^2(1+\text{F})] + \text{F}(p\text{P}r\text{R+D}) \quad [4.9]$$

$$\text{Freq. PPRS} = 2(p\text{P}r\text{R+D})(p\text{P}s\text{S-D}) \quad [4.10]$$

$$\text{Freq. PPSS} = [(p\text{P}s\text{S-D})^2(1+\text{F})] + \text{F}(p\text{P}s\text{S-D}) \quad [4.11]$$

$$\text{Freq. PQRR} = 2(p\text{P}r\text{R+D})(q\text{QrR-D}) \quad [4.12]$$

$$\text{Freq. PQRS/QPSR} = 2(p\text{P}r\text{R+D})(q\text{Q}s\text{S+D})+2(q\text{Q}r\text{R-D})(p\text{P}s\text{S-D}) \quad [4.13]$$

$$\text{Freq. PQSS} = 2(p\text{P}s\text{S-D})(q\text{Q}s\text{S+D}) \quad [4.14]$$

$$\text{Freq. QQRR} = [(q\text{Q}r\text{R-D})^2(1+\text{F})] + \text{F}(q\text{Q}s\text{S-D})$$

[4.15]

$$\text{Freq. QQRS} = 2(q\text{Q}r\text{R-D})(q\text{Q}s\text{S+D}) \quad [4.16]$$

$$\text{Freq. QQSS} = [(q\text{Q}s\text{S+D})^2(1+\text{F})] + \text{F}(q\text{Q}s\text{S+D}) \quad [4.17]$$

As the measure D has a dependence on the allele frequencies in the population, D measures between loci with different allele frequencies are non-comparable unless standardised (Hedrick, 1987). Several standardisations are possible, none of which acheive complete independence from allele frequency (Hill, 1974; Weir & Cockerham, 1978; 1979; 1987; Lewontin, 1988; Weir, 1990). In this analysis D values are standardised as

$$\text{R} = \text{D}/\sqrt{pqrs} \quad [4.18]$$

which is essentially a correlation coefficient and makes frequency-dependence negligible except at extreme allele frequencies (Hedrick, 1987).

Reiteration of the following equation allows maximum likelihood estimation of R for observed genotype frequencies. F is first estimated from the data as above, eqn. [4.6].

$$\begin{aligned}\text{LogL} = \; & \text{N}_{\text{PPRR}}\log\{[(p\text{P}r\text{R+D})^2(1+\text{F})] + \text{F}(p\text{P}r\text{R+D})\}+ \\ & \text{N}_{\text{PPRS}}\log 2\{(p\text{P}r\text{R+D})(p\text{P}s\text{S-D})\}+ \\ & \text{N}_{\text{PPSS}}\log\{[(p\text{P}s\text{S-D})^2(1+\text{F})] + \text{F}(p\text{P}s\text{S-D})\}+ \\ & \text{N}_{\text{PQRR}}\log\{2(p\text{P}r\text{R+D})(q\text{QrR-D})\}+ \\ & \text{N}_{\text{PQRS/QPSR}}\log\{2(p\text{P}r\text{R+D})(q\text{Q}s\text{S+D})+2(q\text{Q}r\text{R-D})(p\text{P}s\text{S-D})\}+ \\ & \text{N}_{\text{PQSS}}\log\{2(p\text{P}s\text{S-D})(q\text{Q}s\text{S+D})\}+ \\ & \text{N}_{\text{QQRR}}\log\{[(q\text{Q}r\text{R-D})^2(1+\text{F})] + \text{F}(q\text{Q}s\text{S-D})\}+ \\ & \text{N}_{\text{QQRS}}\log\{2(q\text{Q}r\text{R-D})(q\text{Q}s\text{S+D})\}+ \\ & \text{N}_{\text{QQSS}}\log\{[(q\text{Q}s\text{S+D})^2(1+\text{F})] + \text{F}(q\text{Q}s\text{S+D})\}+ \\ & \text{constant}\end{aligned} \quad [4.19]$$

4.4.5. Cytonuclear Disequilibria

Disequilibria between the cytoplasmic genome (in this case mitochondrial DNA) and nuclear genotype can also be quantified on similar grounds to the above measures of nuclear allele linkage disequilibria (Asmussen, Arnold & Avise, 1987). These measures look at associations between the exclusively uni-parentally inherited, haploid mitochondrial (mtDNA) genotype and the biparentally-inherited, diploid nuclear genotype. The analysis has been used to derive information on the direction of hybridisation that would be unavailable from either type of markers alone (Carr *et al.*, 1986; 1987; Hoffman & Turelli, 1988; Asmussen, Arnold & Avise, 1989; Baker *et al.*, 1989; 1989; Smith, Taylor & Brown, 1989; 1990; 1991; Paige, Capman & Jennetten, 1991; Sperling & Spence, 1991; Wayne & Jenks, 1991; Cronin, 1991a; 1992). Models of cytonuclear disequilibria are reviewed in Chapter 3.

The values for gametic (D), and residual disequilibria (d) are calculated using a maximum likelihood estimate, treating mtDNA as a nuclear gene. For nuclear alleles R and S and mtDNA types r and s:

$$D_1 = \text{freq. RRr} - (\text{freq.RR})*(\text{freq.r}) \quad [4.20]$$

$$D_2 = \text{freq. RSr} - (\text{freq.RS})*(\text{freq r}) \quad [4.21]$$

$$D_3 = \text{freq. SSr} - (\text{freq.SS})*(\text{freq r}) \quad [4.22]$$

$$D = D_1 + {}^1\!/_2 D_2 \quad [4.23]$$

$$d = (q - p)D - {}^1\!/_2 D_2 \quad [4.24]$$

The estimate takes into account the heterozygote deficit already known to be involved, estimating F directly from the data and assuming that a fraction (F) of the alleles are identical by descent and (1-F) are derived by random mating. 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, Arnold & Avise, 1987,1989). Both measures were estimated by maximum likelihood (Asmussen, Arnold & Avise, 1989) and 4 models of interaction between the F statistic, D and d were fitted to the data in a hierarchical model. The models were

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

$F\neq0, D=0, d=0$: no disequilibria, het deficit

$F\neq0, D\neq0, d=0$: gametic but not genotypic disequilibria, het. deficit

$F\neq0, D\neq0, d\neq0$: gametic and genotypic disequilibria, het. deficit.

Likelihood ratios were presented for the expected distribution of cytonuclear genotypes under each of the hypotheses, fitted to the observed data.

Chapter 5.

DESCRIPTION AND DISCUSSION OF CLINES IN *CERVUS* IN SCOTLAND

5.1. Phenotypic clines: data from censuses

5.1.1. Argyll

Frequencies of all red-like and sika-like animals in the 10 populations are given in Table 5.1.1. A disparity between the ranges of sika-like males and females was found. No sika-like females were seen beyond Birdfield forest, at 188kms along the transect (Chapter.4, Fig.4.1.2a), though sika-like males were seen further away on the Cowal peninsula. This is likely to be caused by the longer dispersal distances of males (Davidson, 1973). If these males are breeding successfully, as the genetic data later shows is the case, some hybrid offspring must be female, though these were not seen in the censuses. This is likely to be because the F1 offspring are at very low frequency and F2 and backcross offspring are censused as red deer. At very low frequencies the census method is unlikely to be accurate, most often underestimating numbers of infrequent types (see above, section 4.2). 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.

The censuses, carried out in winter (November - February), gave unexpectedly high stag: hind ratios. Generally stag proportions decline in high-density deer populations (Clutton-Brock, Guinness & Albon, 1982; Clutton-Brock & Albon, 1989). The even sex-ratio found here may illustrate decreased intersexual or overall competition in these populations, or may be a result of biased censusing if males are more likely to leave forest cover than females. The census method has been previously tested in forestry and did not indicate a sex-bias in sampling, but with low sample sizes the possibility should not be dismissed.

Forest	Distance from intro. (kms)	$\bar{N}$	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	0.18
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	0.35	0.25	0.25	0.15
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

Table 5.1.1. The proportions of each population with predominantly sika-like or red-like phenotype in Argyll forests (see Fig. 4.1.2a). Animals were not classified as 'hybrid' as visibility was often insufficient to make detailed observations reliable, and the phenotypic expression of mixed characters is known to be very variable (Harrington, 1973, 1982).

5.1.2. Great Glen

In the Great Glen, censuses were carried out as far as possible in pre-thicket forestry, even though some of these proved very small areas (Farigaig, Laddie Wood). Censuses within closed-canopy conifer plantations were impossible and so densities are based on the (not ideal) small-area censuses for the woodland areas. The open hill areas were censused above Farigaig and Laddie woods in addition to the forestry in an attempt to compensate for this. However, these showed extremely low densities of sika-like animals (Table 5.1.2). This is contrary to the population seen on an *ad hoc* basis within the woodlands and was not thought to be representative of the Farigaig and Laddie Wood populations. The difficulty of censusing population phenotypes in dense forestry prevented accurate visual assessment of the effect of forestry on the distribution of the deer on this fine scale. However, these census differences inspired further work in analysing sika spread in relation to forestry distribution. This will be discussed in Part 4.

Forest	Distance from intro. (kms)	N	sika-like females	sika-like males	red-like females	red-like males
Farigaig	13.75	12	0.83	0.17	0	0
Farigaig hill	13.75	34	0.06	0.12	0.56	0.26
Knockie	27.5	26	0.54	0.42	0	0.4
Inchnacardoch	36.75	58	0.22	0.14	0.38	0.26
Glen Urquhart	41.5	35	0	0.06	0.57	0.37
Glen Affric	42.5	51	0	0	0.62	0.38
Laggan Wood	48.5	26	0	0	0.62	0.38
Laddie Wood	53	6	0	0.17	0.64	0.17
Laddie hill	53	16	0	0.06	0.69	0.25

Table 5.1.2. The proportions of each population with predominantly sika-like or red-like phenotype in Great Glen forests. Animals were not classified as 'hybrid' as visibility was often insufficient to make detailed observations reliable, and the phenotypic expression of mixed characters is known to be very variable (Harrington, 1973,1982).

5.2. Clines in gene frequencies: data from nuclear loci.

For all loci (except *BOVIRBP* which had two sika alleles) the sika alleles were unique. For the isozyme loci the red allele was also unique, but in the microsatellite loci several red alleles were present. The lack of polymorphism in the sika population is likely to be a result of the small number of founder animals (Hartl & Pucek, 1994). Animals were scored as 'red' or 'sika' for any of the possible, relevant alleles present (see Figs. 4.3.3a&b, 4.3.5a&b). The complete fixation of the 'fast' and 'slow' species-specific alleles in the Great Glen populations supports the belief that *Sod 1* is diagnostic in these populations.

5.2.1. Argyll

A clear cline across the transect was discovered at each locus. Frequencies and sample sizes are reported in Table 5.2.1. Allele frequencies plotted against minimum distance overland from the introduction point are shown in Fig. 5.2.1a. The great asset of the peninsular form of Argyll is that the transect can reasonably be considered as linear. Although the area at the head of Loch Fyne allows greater variation in dispersal paths, sampled forests are still necessarily encountered

Forest	Distance from intro (kms)	Sika Allele Frequency									
		6-pgdh	N	*Sod 1*	N	*BOVIRBP*	N	*Oar FCB 193*	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	*8*	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*

Table 5.2.1. Nuclear loci allele and mitochondrial DNA haplotype frequencies across the transect in Argyll. Values are for the Sika allele. As there was no intra-animal variation across the 3 mtDNA haplotypes screened, they are scored as one.

Forest	Distance from intro (kms)	Sika Allele Frequency									
		6-pgdh	N	*Sod 1*	N	*BOVIRBP*	N	*Oar FCB 193*	N	mt DNA	N
Farigaig	13.75	1	*24*	1	*22*	1.000	*23*	1.000	*24*	1.000	*22*
Knockie	27.50	1	*10*	1	*10*	1.000	*10*	1.000	*10*	1.000	*9*
Inchnacardoch	36.75	0.242	*29*	0.23	*15*	0.297	*32*	0.234	*32*	0.300	*30*
Glen Affric	41.50	0	*14*	0	*15*	0.125	*16*	0.091	*17*	0.180	*17*
Glen Urquhart	42.50	0.143	*7*	0.143	*7*	0.357	*7*	0.286	*7*	0.290	*7*
Laggan	48.50	0.143	*7*	0.143	*7*	0.167	*6*	0.243	*7*	0.000	*5*
Laddie	53.00	0	*34*	0	*31*	0.067	*33*	0.067	*34*	0.000	*32*

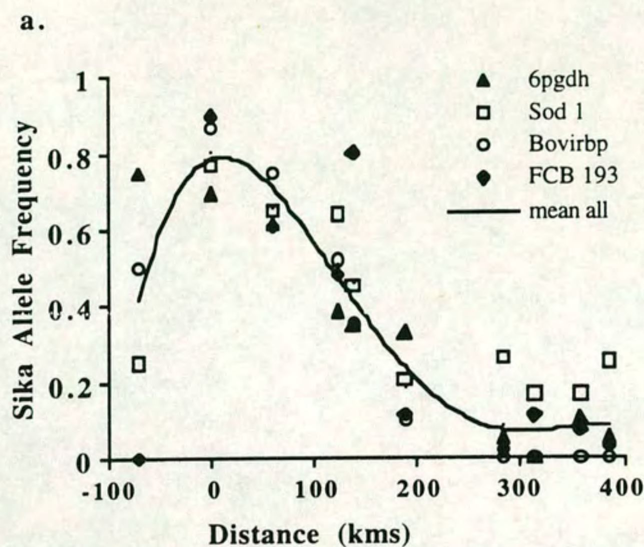
Table 5.2.2. Nuclear loci allele and mitochondrial DNA haplotype frequencies in the Great Glen forests. As above the mtDNA haplotypes are scored as one locus

sequentially by a population founded at Carradale. Disruption of this linearity could be caused by sika swimming Loch Fyne at its narrowest point as well as dispersing overland. They have very occasionally been seen to do this (Stuart & Stuart, 1848). Dispersal across the loch could contribute to the relatively high sika allele frequencies in the southern Cowal sites as it effectively brings them closer to Carradale, but the contribution is likely to be tiny in comparison to overland dispersal and is ignored in subsequent analysis.

At a given mean sika allele frequency (p), frequencies at individual loci (p_i) were similar (Fig.5.2.1b). Heterogeneity of linear concordance slopes was tested by anova (Sokal & Rohlf, 1981, Ch. 17), a=9.73, *n.s.* This indicates that cline widths must also be approximately equal for all loci. Mean cline width was estimated by fitting a tanh curve by maximum likelihood (Sanderson, Szymura & Barton, 1992, and see Chapter. 4). This gave a mean best estimate of 367kms, with support limits of 341 - 398kms. Linear concordance does not, however, test for shifts in the shape or geographical location of individual clines either compared to the mean, or to any other locus. Many variations in cline shape will fit the same straight line, but have different implications for the selective forces forming those clines (Barton & Gale, 1993). The concordance of non-linear fits can be estimated by fitting polynomial regressions to each cline by maximum likelihood, and subsequently comparing the concordance of these regressions, also by maximum likelihood (MacCallum, 1994.). In this case a set of nine sampling locations was insufficient to fit cline shape meaningfully. The linearity of the system does, to a certain extent, ameliorate the need to know cline shape as the position and width can only vary in one dimension. Improving knowledge of cline shape may allow better insight into the behaviour of individual loci, but in this case the data are insufficient for detailed cline shape analysis.

5.2.2. Great Glen

In the Great Glen there was no obvious way of identifying a sequentially colonised set of forests. Allele frequencies from sampled forests were plotted directly against their minimum overland distance from the introduction site (Fig. 5.2.2a). Differences in actual dispersal rates in various directions will alter the effective distance of forests from the introduction site, but as there are no data on relative dispersal rates in this area, this could not be accounted for. The most important difference would be in the relative amounts of dispersal past the north and south ends of Loch Ness. Inverness city is at the north end and could make this a less attractive



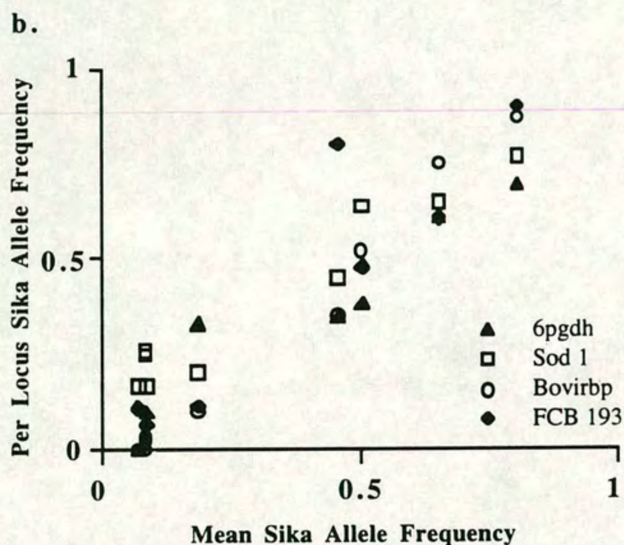


Figure 5.2.1a&b. Sika allele frequencies at each locus in the Argyll study area. a) plotted against minimum overland distance along the transect. The curve is fitted to the mean frequency. b) plotted against mean sika allele frequency across all loci. The cline for each locus is linearly concordant with all others. Heterogeneity of linear slopes, $a = 9.73$, n.s.

route for the deer. If dispersal was only via the south, it would make Glen Urquhart and Glen Affric much more distant sites than they are currently thought to be, stretching the cline 'tail' in a similar way to the Argyll cline shape. Dispersal is unlikely to be only round the north end as there is continuous forest down the east side of the loch, from the introduction site at Aldourie to the southern end and so no obvious barrier in this direction. Whitehead (1964) reports deer colonising south along the east shore. There is the possibility that deer swim Loch Ness, making all sites on the west side effectively closer than stated. As in Argyll, dispersal by swimming is assumed to be rare and is ignored in the analysis

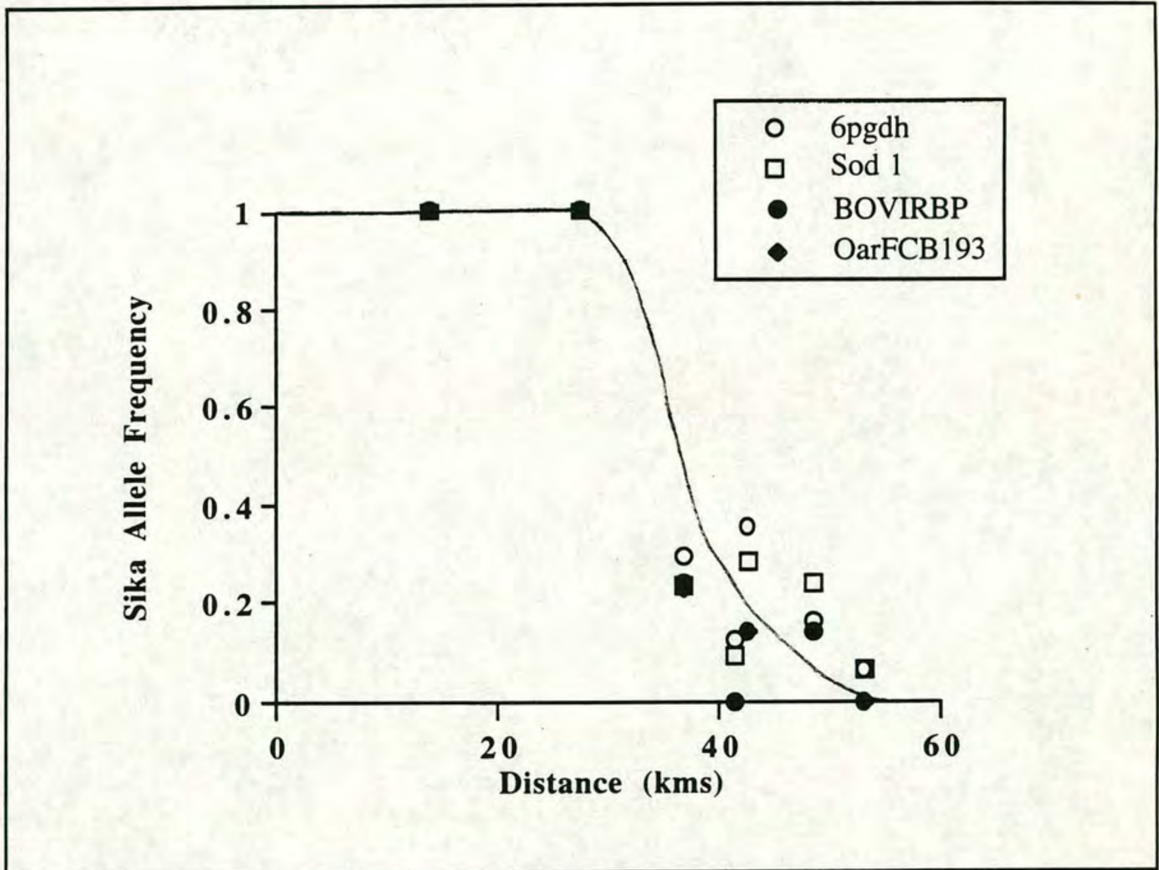


Figure 5.2.2a. Sika allele frequencies in the Great Glen samples. The cline appears much steeper than that in Argyll, although the lack of populations with intermediate sika frequencies makes fitting a cline difficult. The line is fitted by eye for clarity, but is *not* a statistically fitted cline shape. Depending on actual dispersal route, the furthest two sites, Glen Urquhart and Glen Affric, could be further than they are placed here (minimum distance overland). This would increase the cline width.

Except for the Inchnacardoch forest, populations in the Great Glen were either strongly red-like or sika-like, with the other genotype at extremely low frequency. This gave rise to a potentially very steep cline, in contrast to the Argyll system.

However as no forests were found with intermediate sika frequencies, it is difficult to fit a cline to so few sites with a high degree of confidence.

In other hybrid zone studies, the position of a cline in both one and two dimensions has been solved using a maximum likelihood solution to fit a logistic or tanh curve, including terms for the effects of heterogeneity between loci, heterozygote deficits and pairwise linkage disequilibrium (Szymura & Barton, 1986). In this case the small number of sites prevented a statistically acceptable solution being achieved in this way.

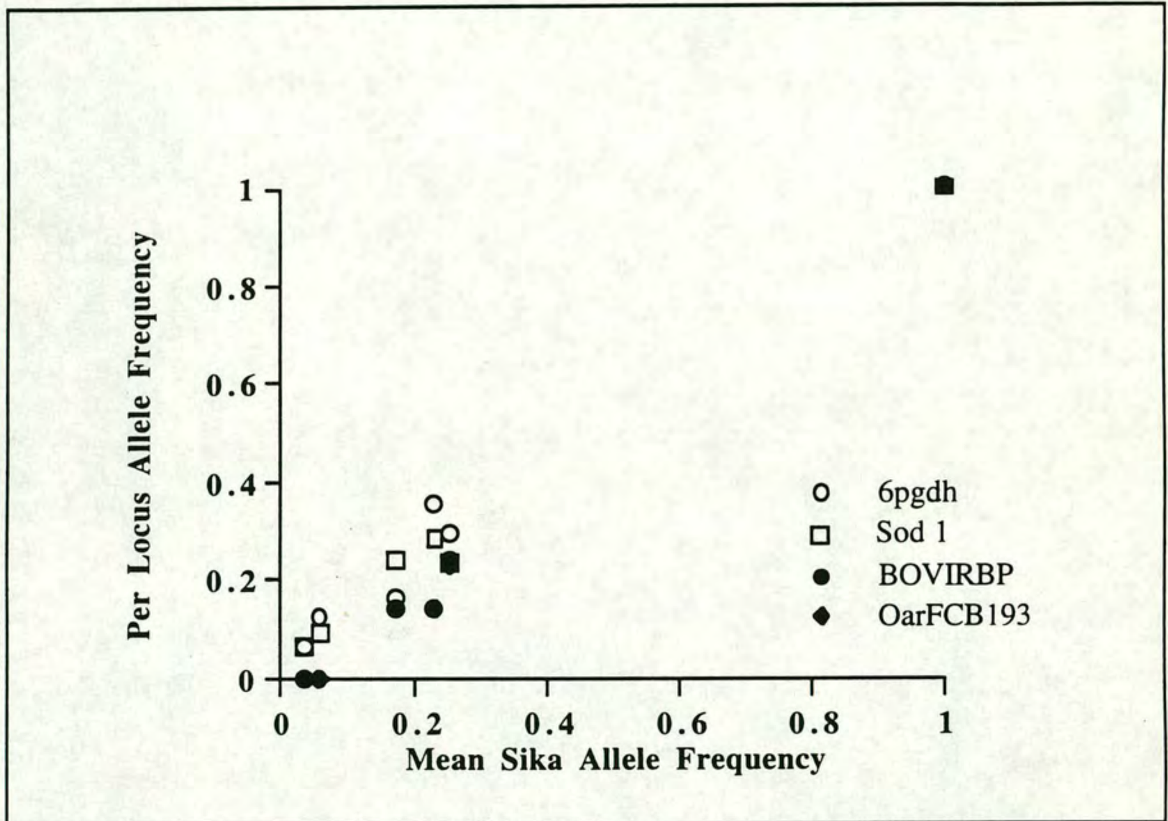


Figure 5.2.2b. The linear concordance in the Great Glen cline. Concordance appears high, but is largely dependent on the Farigaig and Knockie forests where the sika allele is fixed. It is impossible to say anything about concordance in intermediate populations.

5.3. Population structure: heterozygote deficits

5.3.1. General remarks

Hardy Weinberg equilibrium predicts that under random mating in a closed population, in the absence of selection genotypes at a locus with alleles *p* and *q*, will be in proportions such that $p^2+2pq+q^2=1$. Therefore for known allele frequencies expected genotype frequencies can be calculated. A significant heterozygote deficit implies that fewer heterozygote animals are observed than expected from the predictions of Hardy-Weinberg equilibrium (see Chapter 4 for description of analysis).

5.3.2. Argyll

All loci showed similar patterns in F_{is} values (Wright, 1965) across the transect up to the East Cowal forest (313kms from Carradale). Significant heterozygote deficits were found in all loci in forests between Carradale and Kilmichael (123kms from the introduction) and for *Sod 1* at sites up to the end of the transect (Table 5.3.2). Overall log likelihood of heterogeneity between loci, calculated by maximum likelihood was 10.53 (3df, $p< 0.01$). At Kilmichael samples sizes were too low to calculate F_{is}. In sites close to the edge of the sika-like female range (Birdfield, at 188kms), the trend is toward weakly negative F_{is} values, indicating equilibrium or possibly heterozygote surpluses. F_{is} is greatest where allele frequencies are close to 0.5. Fig. 5.3.2 shows F_{is} plotted against mean sika allele frequency.

5.3.3. Great Glen

In the Great Glen heterozygote deficits were profound in all populations (Table 5.3.3). There were no populations showing heterozygote surplus. In most populations at least one locus was fixed for either the red or sika allele, making the calculation of F_{is} values impossible.

Forest	Distance from intro. (kms)	Mean Sika Allele Frequency	6pgdh F_{is}	Sod 1 F_{is}	Bovirbp F_{is}	OarFCB193 F_{is}
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 5.3a. F_{is} values for the populations in Argyll. Sample sizes at Kilmichael were too small to calculate F_{is} values are calculated using Wright's (1965) method to assess deviations from Hardy-Weinberg proportions. 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 intro. (kms)	Mean Sika Allele Frequency	6pgdh F_{is}	Sod 1 F_{is}	Bovirbp F_{is}	OarFCB193 F_{is}
Inchnacardoch	36.75	0.254	0.62 *	0.56 *		0.81 *
Glen Urquhart	41.5	0.232	0.69	0.3		
Glen Affric	42.5	0.057	0.43	0.63	-	-
Laggan	48.5	0.174		-	0.15	0.15
Laddie Wood	53	0.034	-	0.58	-	-

Table 5.3b. F_{is} values for the populations in the Great Glen. Values vary between -1 and 1; large values indicate strong deviations, positive values showing a heterozygote deficit.. Where an allele is fixed at the locus F_{is} cannot be calculated, Farigaig and Knockie forests are therefore omitted as are some values for Glen Affric and Laddie Wood. Values differing significantly from Hardy-Weinberg expectations ($p<0.05$) are marked with an asterisk.

5.4. Associations between loci: Linkage disequilibria

5.4.1. General remarks

As discussed in chapter 4, section 4.4, the value 'R' is a measure of the association between alleles at different loci, standardised by gene frequencies in the population. Patterns of linkage disequilibria were similar across all locus pairs across both transects. R values were calculated from raw data of individual allele scores, standardised to sum to 2 at each locus (Barton & Gale, 1993). They were also calculated using a maximum likelihood analysis with the 'ANALYSE' hybrid zone analysis programme (N. Barton, unpublished; Chapter.4.). These gave similar results for most loci pair and forest combinations, although the maximum likelihood method was unable to fit a reliable estimate to sites with low sample size. As this was the case, the following interpretations use the former set of R values, though results and significances (>2 units likelihood) obtained in the maximum likelihood analysis are given below.

5.4.2. Argyll

Strongly positive values for R, indicating non-random associations favouring parental combinations, were obtained from sites 0-138kms from Carradale. Zero values or weakly negative ones, indicating a trend toward random associations or even favouring recombinants, were obtained at Birdfield forest and beyond (Table 5.4.2a). These results echo the within-locus patterns of heterozygote deficit revealed by F-statistics above (Fig.5.3.2a). Most likely values are shown in Fig. 5.4.2a.

South Cowal again seems to show characteristics consistent with a site closer to the introduction point. For sites farthest from the introduction point, the red allele was fixed in some loci, and R values could not be calculated for those pairs.

Forest	Distance from intro. (kms)	6pgdh Sod1	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	0.795	-	-	-	-0.100	-	0.201
S. Cowal	386	-0.17	-	-	-	-0.100	-	-0.025

Table 5.4.2a. Linkage disequilibria R values across the Argyll transect. R values are standardised by allele frequencies in the population. Values marked with an asterisk are significantly different from random associations (Maximum likelihood values >2, tested using the 'ANALYSE' programme for hybrid zone data (Barton, unpublished). Populations toward the Carradale end of the transect, i.e. those where sika have been resident longest, have high R values, indicating non-random associations between alleles. Reasons for these patterns are discussed in section 5.7.

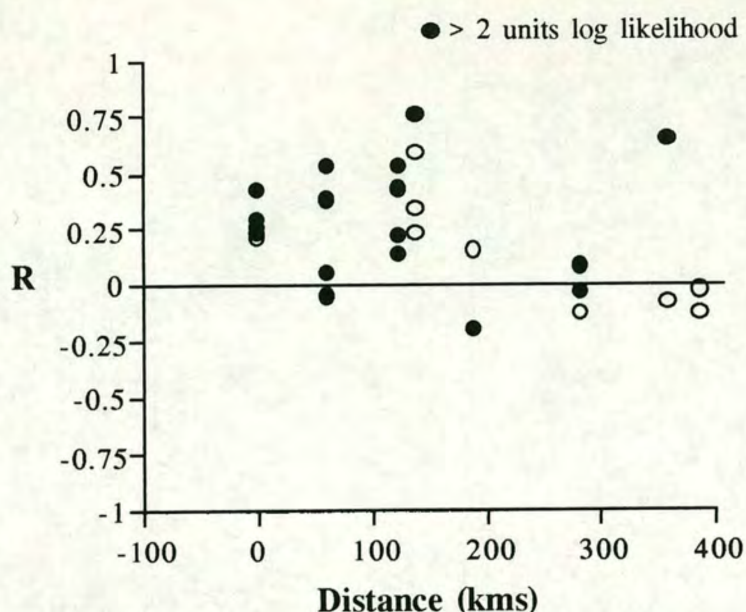


Fig. 5.4.2a. Linkage disequilibrium values calculated using a maximum likelihood analysis. Values assigned greater than 2 units of likelihood are marked as solid circles. The results differ very little from those calculated by hand (above, Table 5.4.2a) except in sites where samples sizes were small (Kilmichael). In these cases the likelihood analysis failed to give a reliable result.

5.4.3. Great Glen

In the Great Glen sites only samples from Inchnacardoch forest showed significant disequilibrium, but this was consistent across locus pairs. Table 5.4.3 shows the R values and log likelihood estimates. A log likelihood of 2 is approximately equal to 95% confidence in the estimate. In the other forests small sample sizes (Brown, 1975) and/or the fixation of alleles at one or both loci prevented calculation of disequilibrium.

	Locus pair					
	6pgdh *Sod 1*	*6pgdh* *BOVIRBP*	*6pgdh* *FCB 193*	*Sod 1* *BOVIRBP*	*Sod 1* *FCB 193*	*BOVIRBP* *FCB 193*
R	0.480	0.782	0.334	0.701	0.580	0.963
-logL	11.163	10.682	4.58	10.66	4.707	0.373
p	<0.001	<0.001	<0.01	<0.001	<0.01	<0.01

Table 5.4.3. Linkage disequilibrium in the Inchnacardoch forest population. There was no significant disequilibrium found in any of the other forest sites of the Great Glen.

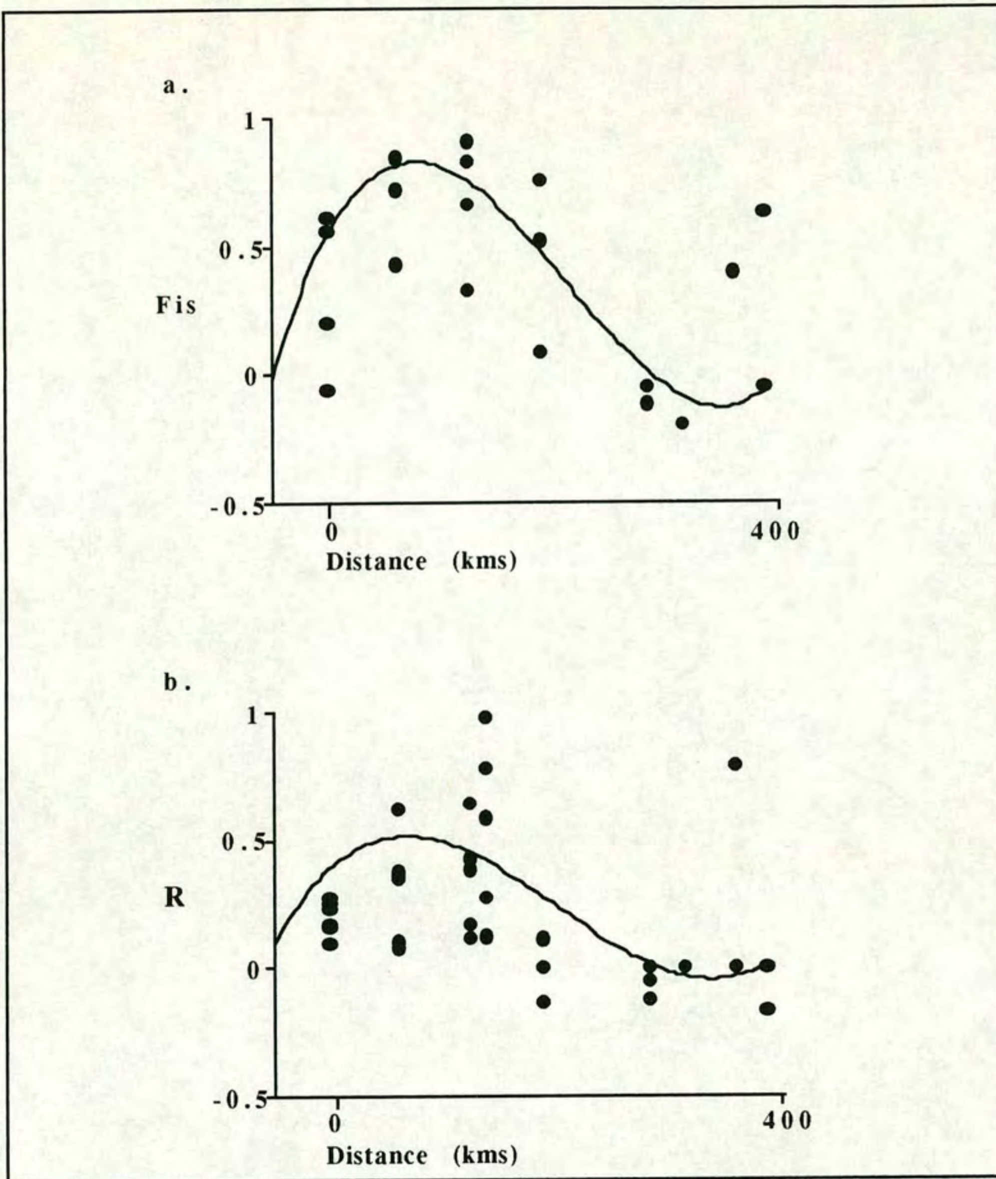


Figure 5.4.2a&b. The patterns in a) F_{is} and b) Linkage disequilibrium (R) across the Argyll transect. Both within-locus heterozygosities and between-locus recombinants decline with increasing age of sika residency and increasing sika allele frequencies, towards Carradale. Both forests at the Cowal end of the transect show a trend toward increased heterozygosity in the *Sod 1* locus. The apparent outlier R value at the Glendaruel forest (358kms) is a non-significant result due to small sample size. The south Cowal forests show values in both cases more consistent with a forest closer to Carradale. Heterogeneity between loci, measured by maximum likelihood gave a log likelihood of 4.11 (df= 5) which is not significant. This shows that the patterns are similar across all loci pairs.

5.5. Cytonuclear Disequilibrium

Cytonuclear disequilibrium was only considered in the Argyll population. Current models of cytonuclear disequilibria (Asmussen, Arnold & Avise, 1987, 1989, and see Chapters 3 & 4) are not applicable to these data as the population is not closed and disequilibria can be generated by forces other than the mating system. A simple model of relationships between gametic and residual disequilibria was tested by maximum likelihood analysis (described in Chapter 4) to examine possible deviations from non-random mating, but results were inconclusive and their interpretation unclear. A model that includes the effects of dispersal of hybrids, heterozygote deficit and also linkage disequilibrium between nuclear loci is required to fully understand the patterns of cytonuclear associations found. As yet this is not available.

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 estimates are too large to draw robust conclusions from the patterns (Table 5.5). 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 are 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 none of the models was a significantly better fit to the data than the others (Table 5.5). Cytonuclear disequilibria are tested by pairwise interactions as a maximum likelihood estimate of homogeneity proved impossible to fit reliably (N. Barton, pers. comm.).

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
	Best fitted model F, D,d.	all > 0	none	all > 0	none
	ΔlogL	2.29		2.48	
Achaglach-gach	*D*	0.19	0.121	0.063	0.058
	d	0.069	-0.05	0.021	0.016
	Best fitted model F, D,d.	all > 0	none	none	none
	ΔlogL	9.06			
Knapdale	*D*	0.136	0.144	0.08	0.195
	d	0.0009	0.019	0.054	0.0004
	Best fitted model F, D, d.	all > 0	none	all > 0	none
	ΔlogL	4.88		2.92	
Birdfield	*D*	0.145	-0.008		
	d	0.088	-0.001		
	Best fitted model F, D, d.	none	none		
	ΔlogL				

Table 5.5. 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 (RR, RS, SS) and the sika 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 too small to fit to a model of mating preference (see text) as sample sizes are reduced by the heterozygote deficit in these populations. ΔlogL shows the decrease in logL resulting from fitting other models. The best fitting model is indicated. A decrease of < 2 units of logL is insignificant and precludes designation of a best fitting model.

5.6. Mitochondrial DNA introgression

5.6.1. Argyll

Frequencies of sika mtDNA in the populations closest to the introduction site were high, though sika mtDNA was absent beyond Birdfield (188kms). The cline in mitochondrial DNA haplotypes was different to the mean cline in the nuclear markers; sika nuclear DNA extending farther than mtDNA and existing at lower frequencies in the Carradale and Achaglachgach populations (Fig.5.6a).

Genetically, hybrids were identified as animals possessing any combination of genotypes, across all loci scored including mtDNA, that was not consistently 'red' or 'sika'. The proportion of all hybrids possessing sika mtDNA i.e. from a sika matriline, declined to zero beyond Birdfield, indicating that hybridisation at the edge of the sika female range must be facilitated by the dispersal of sika (or hybrid) stags into the red population (Fig. 5.6c).

The frequency of sika mtDNA in any population where sika-like females are resident is not significantly different from the frequencies of the sika nuclear alleles (χ^2= 1.89, df=1, n.s.). This indicates that there is no difference in the chance of hybrids being produced from females carrying either mitochondrial type.

5.6.2. Great Glen

In the Great Glen the cline in mtDNA was very similar to that in nuclear DNA (nDNA). The two populations furthest from the introduction were fixed for red mtDNA, but still contained low frequencies of sika nDNA (Fig. 5.6b). This is a similar trend to that found in Argyll, but the differences here are much less both in the magnitude of the outstanding sika nuclear DNA frequencies and in the distance they persist beyond the cline in sika mtDNA.

Again the participation of both red and sika hinds in hybrid matings is clear. Figure 5.6d shows the proportions of hybrids mothered by red and sika hinds. In Farigaig forest no hybrids were identified, but beyond this site hybirds are found from each matriline, as in Argyll. Sample sizes are smaller in the Great Glen, which may account in part for the greater variability in the proportions, or this may be a real effect of different mating patterns within the hybridising populations in the two areas. Habitat differences between the areas may influence mating system (see Chapter 1).

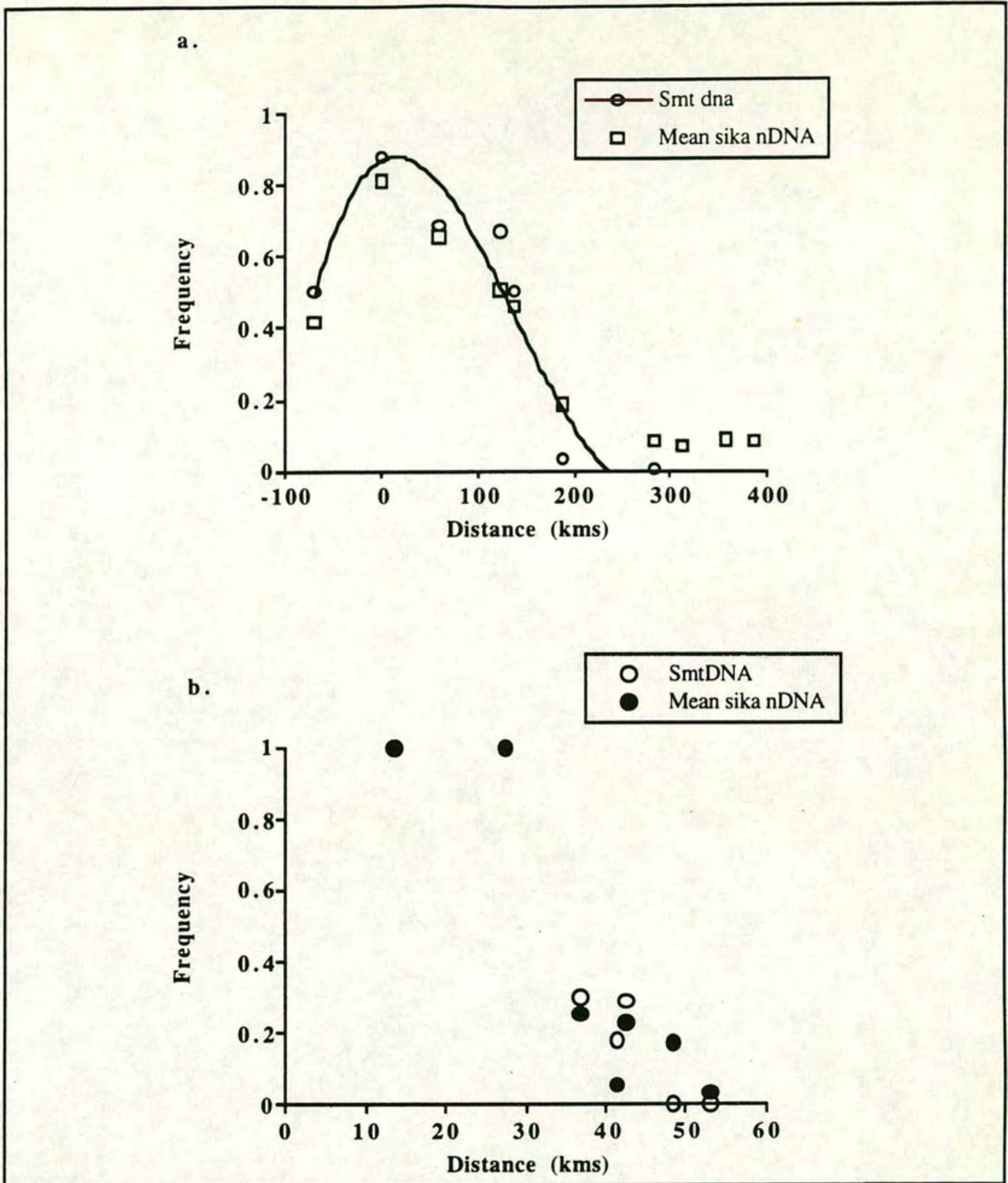


Figure 5.6a&b. The clines in mtDNA and (mean) nuclear DNA over a) the Argyll transect and b) the Great Glen transect. The mtDNA cline is steeper and shows higher frequencies at the introduction end of the transect in Argyll. In the Great Glen, this trend, though present, is not so pertinent. In order for the cline in nDNA to be wider than that in mtDNA, males must disperse (and breed) more widely than females, which is consistent with expectations from cervid behaviour in other studies.

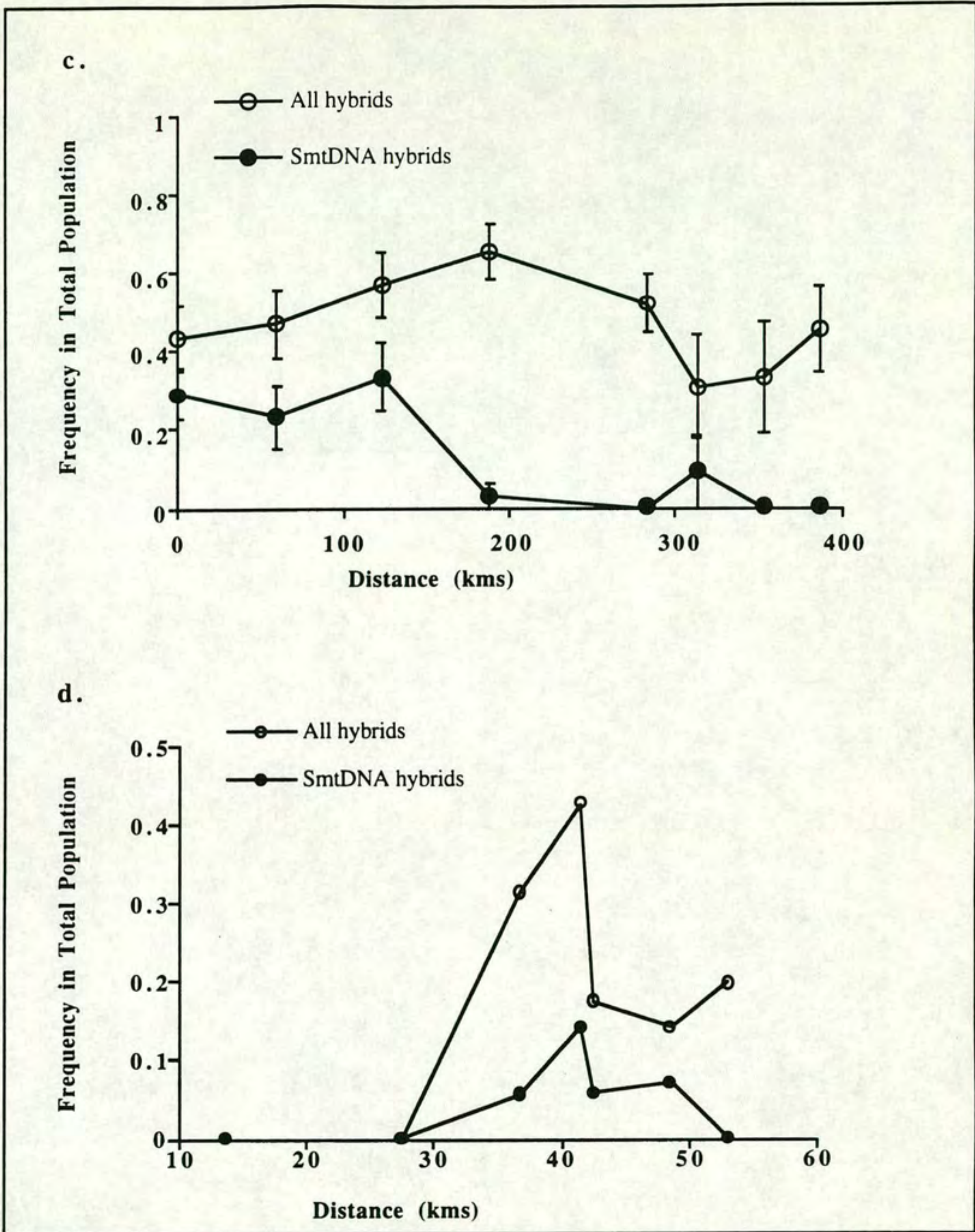


Figure.5.6c&d. Hybrid descent. Hybrids were assessed as those animals possessing alleles from both taxa or a mtDNA haplotype differing from their nuclear genotype. The total proportion of hybrid animals in each population is shown by the open circles. Hybrids possessing sika mtDNA are shown in closed circles, also as a fraction of the whole population. In Argyll (c), no hybrids with sika mtDNA were found beyond Birdfield (138kms), indicating that in this region sika or hybrid stags mating red hinds are responsible for the hybridisation events. In the Great Glen (d) the pattern is more stochastic, however it is still clear that both sika and red hinds are participating in hybrid matings.

Although this data is a preliminary assessment of the population structure, potential differences deserve further attention.

An important trend illustrated in both populations is the wider dispersal of nuclear DNA. In a hybridising population the increase of the founder type mtDNA and nDNA frequencies should be equal if selection and dispersal are equal between the sexes. If selection favours females of one type more than males, then the frequency of mtDNA may be expected to increase beyond that of nDNA in those populations, as the nDNA contribution in males is squandered. However, for the cline in nDNA to be wider than that of mtDNA, sika-like males *must* disperse further than females.

5.7. Distribution of genotypes

5.7.1. General remarks

A hybrid index scoring method has been used in hybrid zone studies to quantify the amount of backcrossing occurring to each parental form (Yang & Selander, 1968; Barton & Szymura, 1986). This is a slightly different approach to the measures of linkage disequilibria and heterozygosity which do not distinguish between the parents, but simply measure the frequency of heterozygotes and recombinants relative to homozygotes at each locus or pair of loci.

Individuals were each awarded a score on an index of 0-1, giving red alleles as a proportion of all alleles scored across all loci, including mitochondrial haplotype. 'Pure' red individuals scored 1, 'pure' sika scored 0. Distributions were pooled over four areas of the transect. These corresponded either to quartiles of mean sika allele frequency or to geographical location. In both areas these gave similar though not identical groupings of the forests. In neither site was the distribution pattern across the index radically altered by the grouping method (i.e. Fig 5.7.2a&b). In both areas the distribution across the index became biased toward sika-like scores with increasing sika allele frequency near to the introduction site.

5.7.2. Argyll

The distributions of individual scores pooled over populations in four areas of the transect are shown in Fig 5.7.2a&b. These areas correspond to quartiles of mean sika allele frequency, and are also sequentially more distant from the introduction. 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 populations where sika frequency = 0.749-0.5 (60-138kms) region.

5.7.3. Great Glen

In the Great Glen populations there were fewer hybrids overall and the distribution across the index was therefore less continuous than in Argyll. Again the trend in backcrossing to sika reflected the overall sika allele frequency in the population, which increased with increasing proximity to the introduction site (Fig.5.7.3).

5.8. Rare Alleles

The isozyme locus *MPI* was not red-sika diagnostic and was only screened in the Argyll samples. Most populations were fixed for a 'slow' allele, however in populations in the centre of the cline a rare 'fast' allele was present at low frequency (Fig. 5.8). The appearance of rare alleles in hybridising populations zones has been previously documented(Slatkin, 1985).

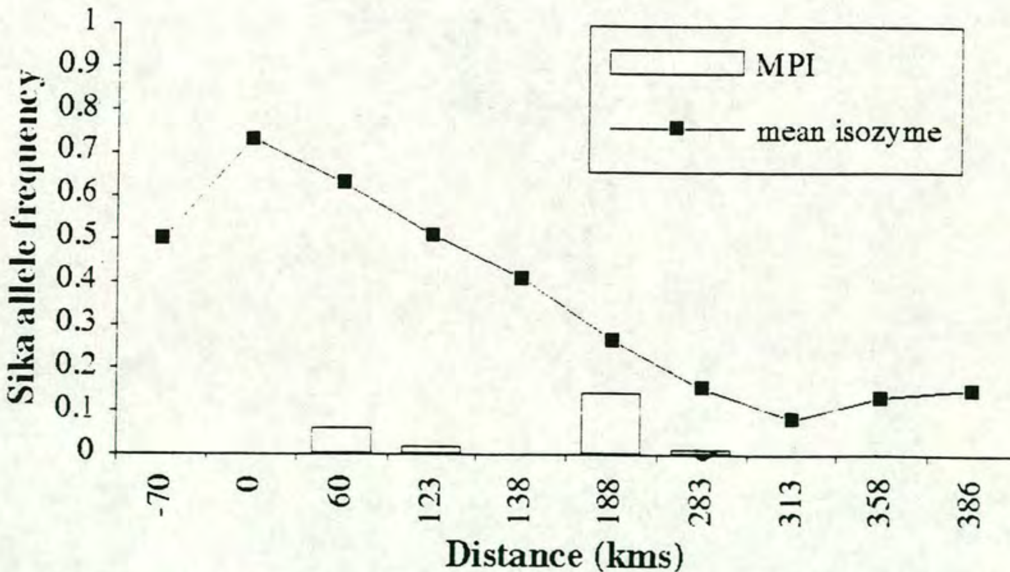


Figure 5.8. Rare alleles at the *MPI* locus. Rare alleles were found in populations in the centre of the cline, but not in those at the edges. This distribution of rare alleles in hybrid zones has been documented before (see Barton & Slatkin, 1985, for cited examples). The effect of recombination may allow expression of rare alleles, but the pattern is not fully explained.

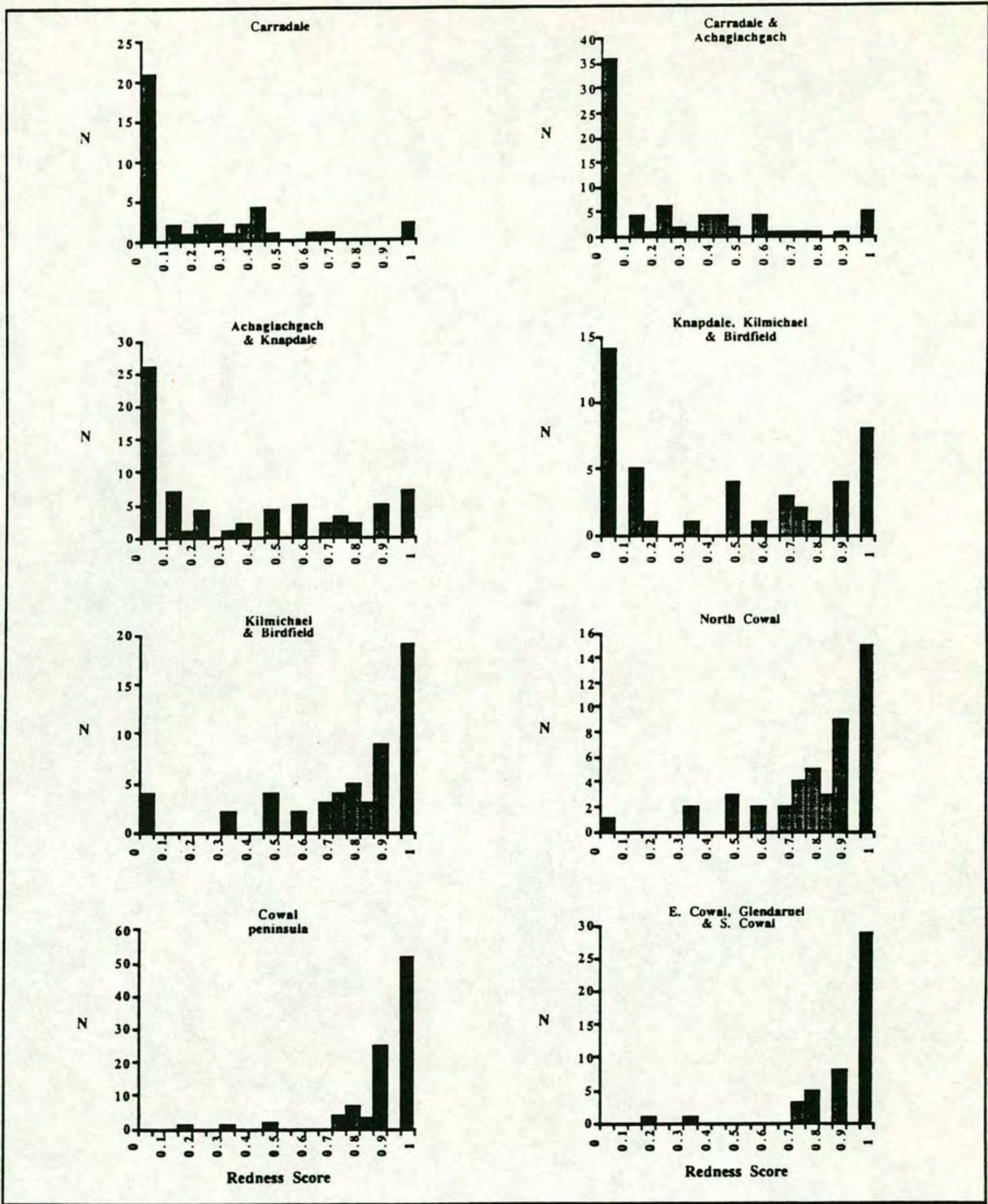


Figure 5.7.2a&b. The distribution of individuals from the Argyll populations across a hybrid index, described above. 'Pure' red animals score 1, 'pure' sika score 0. The scores are pooled across the transect either by **a**) geographic location (left column) or **b**) mean sika allele frequency (right column). These groupings result in different forests being combined, though the overall pattern is preserved. A shift in backcrossing from red to sika is seen with increasing sika frequency closer to Carradale. This suggests that backcrossing frequency is not biased toward either genotype, but reflects the allele proportions present.

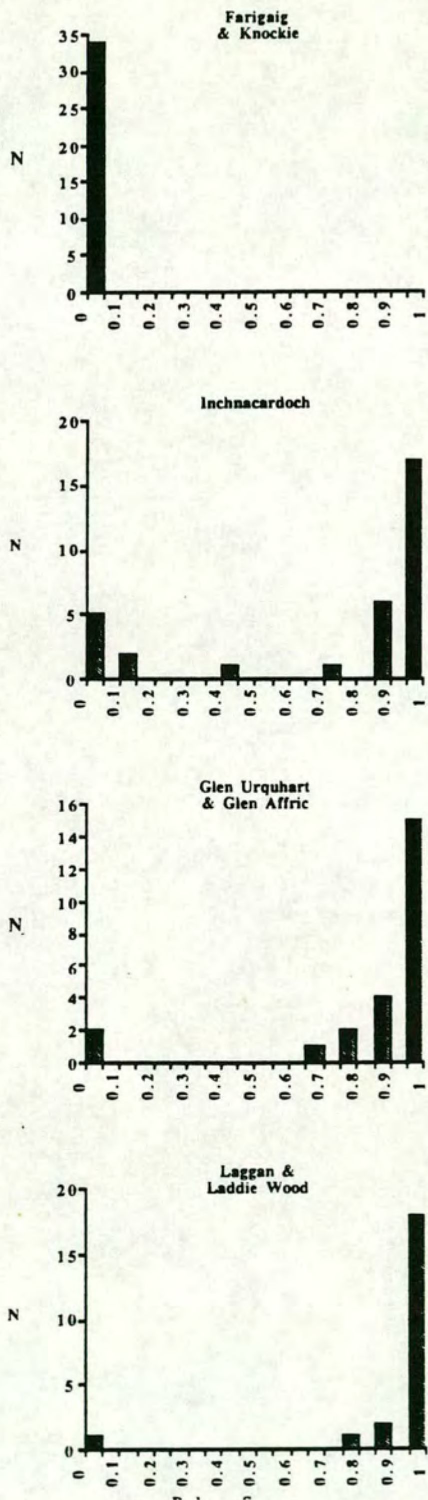


Figure. 5.7.3. The distribution of hybrids across a genotype index. Scores from individuals in each geographic region around Loch Ness are pooled. Farigaig and Knockie = east Loch Ness, Inchnacardoch = south west, Laggan and Laddie Wood = south and Glen Urquhart and Glen Affric = west. There is a lack of F1 type hybrids in all populations. This may indicate that hybridisation evnts between 'pure' parental types are a rare event, or that selection against hybrids is strong. These issues are further discussed in the sections on heterozygosities and linkage disequilibria (5.3, 5.4).

5.9. Discussion of cline characteristics

5.9.1. Summary of results

Concordant and coincident clines in sika allele frequencies occur at all loci studied in Argyll. Mean cline width was shown to be approximately 367km (341 - 397kms), 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 hybridisation 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, Arnold & Avise, 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.

5.9.2. 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 and 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 markers are unlikely to be linked as deer have 66-68 chromosomes on which they may be situated. No evidence for linkage was found in 229 deer typed for the two microsatellite loci used (J. Pemberton, pers. comm.). The randomly chosen markers are also 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 subsequent analysis (Baird & Abernethy, in prep).

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. 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 is most unlikely to be the result of genetic drift. Overall the large and consistent increases 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 implies selection for one or more sika genes.

5.9.2. Multi-locus 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 multi-locus genotype rather than single genes. If quantitative traits under multi-locus 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, non-random 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 multi-locus traits from uniform selection on independent loci, which would not be expected to produce persistent, strong linkage disequilibria in hybridising populations. Coherent and species-specific phenotypes will be likely to persist as a result of strong disequilibria (e.g. Szymura & Barton, 1986; Mallet *et al.*, 1990).

In the cervid populations of Scotland we find concordant and coincident clines, heterozygote deficits and linkage disequilibrium. At the multi-locus level, the pattern of heterozygote and recombinant deficit behind the initial advance of sika could be produced by

- differential dispersal of genotypes
- hybrid disadvantage or selection favouring some genes only
- by assortative mating in the hybridising populations
- or by a combination of 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, simple immigration of parents is unable to account for the pattern.

The range of backcrosses 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 backcrossing 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. The patterns found in the hybrid index, and in F_{is} values suggest that there is a hybrid disadvantage.

As well as indicating the contribution of males in propagating the initial wave of hybridisation, 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 backcross to either parental type. If mating is so strongly assortative that hybrids will only backcross to one taxon, then

introgression will be uni-directional (e.g. Hoffman & Turelli, 1988; 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 bi-parentally inherited (nuclear) markers, they can be used to test hypotheses about mating patterns. Cytonuclear disequilibria analyses have been used successfully to infer directionality of hybridisation and strength of assortative mating preferences in *Hyla* tree frogs (Lamb & Avise, 1986; Asmussen, Arnold & Avise, 1987) and North American cottonwoods (Paige, Capman & Jennetten, 1991) and in some cases mitochondrial data alone have been sufficient to detect strong directionality in mating preferences of hybridising deer populations (Cronin, Vyse & Cameron, 1988). Maximum likelihood comparisons of the cytonuclear genotype distributions in this 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 5.4.2a and 5.5). Genotypic disequilibria (*d*) values which would potentially yield information on the directionality and strength of assortative mating in the sexes (Asmussen, Arnold & Avise, 1987, 1989; Arnold, Asmussen & Avise, 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 hybridising deer populations (Cronin, 1991; Carr *et al.*, 1986; Stubblefield, Warren & Murphy, 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 colonised populations, however 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, Capman & Jenetten, 1991). Models of disequilibrium expectations are based on mate fidelity being independent of frequency and immigration being solely of parental types (Asmussen, Arnold & Avise, 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 colonisation by hybrid immigrants make interpretation of backcross frequencies in the style of previous cytonuclear disequilibrium measures extremely difficult (Asmussen, Arnold & Avise, 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 prep, Chapter 8.).

In populations at the edge of the range of sika-like females, 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 hybridising 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 a heterozygote excess for the given sika allele frequency in these populations, compared to Hardy Weinberg expectations that assume the alleles present are spread between males and females.

Although clear in theory, this effect is hard to perceive when populations are sampled across generations as in the present study, because the hybrids backcross to the resident red deer reducing the heterozygosity in their offspring. This counteracts the effect of the original hybridisation 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.

5.9.3. Conclusions

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

It is clear from the above data that Japanese sika do hybridise with red deer in Scotland, and that genetic introgression occurs in *both* directions. This supports the conclusions of Ratcliffe (1987a). Chronologically, the first hybridisation events are the result of sika or hybrid stags invading red female ranges, but thereafter hybrids are produced from either female or backcross. Selection pressure appears to be acting on multi-locus 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 likely to be 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, Arnold & Avise, 1987, 1989; Arnold, Asmussen & Avise 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 (Chapter 8). If it proves to be the case that a selectively advantaged sika phenotype, involving many loci, remains coherent and is able to outcompete red deer in Scottish woodlands, the ability of the red deer phenotype to persist in this habitat must be questioned.

Chapter 6.

ECOLOGICAL SIGNIFICANCE OF GENOTYPE: A STUDY OF WINTER DIET

6.1. Diet as a functional response of genotype

Diet selection is likely to be important to herbivore survival and reproductive success. In conjunction with the genetical survey (Chapters 3-5), the relation between genotype and diet selection was examined. This chapter first briefly states the possible responses to diet selection in mixed populations, and outlines methods used in this study of diet in red-sika populations. The reasoning behind the choice of methods over alternatives for each analysis are outlined below. As in the genetical analysis methods (Chapter 4) I have relegated widely published standard procedures and reagent recipes to appendices, but have given in full procedures that are less familiar, or have been adjusted specially for this study. All the work relied on samples of rumen contents obtained from culled animals. These were analysed in three different ways; firstly for particle size distributions within the sample, then for botanical composition and finally for neutral-detergent fibre (NDF), acid-detergent fibre (ADF), lignin and ash contents.

6.1.1. A brief theory of herbivore diet selection

6.1.1.1 Body size

The body size of a herbivore has been the most successful parameter used to date in explaining diet choice. The relationship first described in the herbivore guilds of the Serengeti by Bell (1971a,b) and Jarman (1974) was that, as gut capacity scales to body weight at unity (Demment, 1982) but basal metabolic rate scales to body weight at less than unity; BMR = $W^{0.75}$, larger animals which have a low basal metabolic rate but need a large total intake, can utilize lower quality foods, whereas smaller animals with higher BMR need less bulk of a higher quality food (see also Chapter 1; 1.1.3). Larger animals can retain more bulk for longer, increasing the digestibility of fibre-rich food stuffs. Demment (1983) shows that particle retention in

the rumen-reticulum is also related to body weight and that for very large animals (600-1200kg) maximum digestibility is attained for all forages, as passage rate is so low. Large animals are limited by their ability to find plentiful high quality foods and so process larger quantities of lower quality. Small animals are limited by their capacity to process bulk and so gain low levels of nutrients from fibre-rich diets, forcing them to seek higher quality feed. The rumen prolongs passage time, allowing smaller animals to utilize higher fibre foods than they could as hind gut fermenters. The evolution of ruminants and comparison to other herbivory strategies is well-studied (Bell, 1971; Hofmann, 1982; Owen-Smith, 1982; Van Soest, 1982; Demment & Van Soest, 1985) but beyond the scope of this work and I will limit myself to the discussion of diet choice within ruminants.

6.1.1.2. Jaw shape and body allometry

In several instances the inability of large herbivores to satisfy their total metabolic requirements in poor or depleted habitats has been cited as the mechanism for smaller competitors excluding larger species (or sexes) from mutually preferred areas during times of scarcity (Kiddie, 1962; Bell, 1971; Clutton-Brock, Major & Guinness, 1985; Putman, 1986; Gordon & Illius, 1989; Illius & Gordon, 1990; Putman, Culpin & Thirgood, 1993; Klein & Bay, 1994). When sward height falls below a critical minimum, animals with a large bite volume cannot take a full bite and so, for the same bite effort, gain relatively less nutrition than small-mouthed animals. The flattening of the incisor arcade can enhance the ability to use short swards, but reduces the ability to select plants or plant parts within the sward (Illius & Gordon, 1987, 1990; Gordon & Illius, 1988). Gordon & Illius (1988) found that within ruminants, grazing species (as classsified by Hofmann, 1985) have significantly broader, flatter incisor arcades than intermediate or browsing ones, though there is no significant difference in allometry within intermediate and browsing species. They made interspecific comparisons over a wide range of ungulate body sizes and found that this relationship deteriorated in animals below 91kg. They conclude that "animals below this size can feed with sufficient selectivity not to require adaptation of dental structure", though doubtless this will depend on the dispersion of food items within the sward and the relative nutritional value of the forage an animal intakes involuntarily, as a result of a broad and unselective mouth. They also note that intermediate feeders have dentition more similar to that of the browser than the grazer and suggest that this is because 'browsing' dentition is more likely to be under positive selection in times of scarcity and will not significantly inhibit grazing intake when resources are abundant. The grazer would be limited in selectivity if forced to

browse in lean periods. Of course, if times of scarcity equate to enforced use of short swards rather than browse, grazers with flattened incisor arcades will be advantaged. Gordon & Illius point out that dental structure is the result of phylogenetic history and past selection as well as present conditions.

In later work Illius and Gordon (1990) show that for a single species, red deer, even for animals of less than 91kg weight, allometric relationships between weight and jaw morphology can explain differential selection on juvenile males and females: females, with blunter muzzles and lower body weights than male calves, being better able to exploit depleted short swards. The advantage on short swards being less in the ability to select, but in the ability to get maximum intake volume per bite. As discussed above, for an animal will a relatively narrow jaw, this is hard to achieve below a critical sward height. A critical time for survival, especially for males, occurs as body weight increases faster than incisor breadth in the first year, before the eruption of the second pair of adult incisors. The different constraints of life history strategy between male and female deer (males selected for earlier faster growth than females) may mean that selection acts differently on jaw ontogeny in the two sexes, preventing males from maintaining the same body size to jaw size ratio as in females. The problem of differential selection on common traits in the two sexes will be discussed further below.

6.1.1.3. Nutritional requirements of the sexes

In Chapter 1 I briefly reviewed the different selective pressures on the sexes in deer resulting from their differing life history strategies. Male reproductive success is linked to early growth and total body size in middle age (5-10yrs). Female success is linked to annual fat reserves and in fact inversely correlated to body size. These different pressures, along with the more general effects of body size differences, mean that male and female have differing nutritional requirements through life and through the year. Over winter males need little more than maintenance requirements, although fat reserves are very low after the rut and so they cannot survive for long on less than maintenance. Pregnant females require more than barren females, but both may have some fat reserves at the onset of winter and have lower total requirement than males, as they are smaller. Daily weight loss over winter is normal in both sexes of wild Scottish red deer (Blaxter *et al.*, 1974; Illius & Gordon, 1990). These different nutritional requirements may obviously affect diet choice.

6.1.1.4. Social organisation

The social organisation of herbivore groups can affect their use of habitat and thus the plant communities that are available for feeding. This was most elegantly described by Jarman (1974) in his paper on the social organisation of African antelope guilds. Jarman suggests that group sizes are dictated by constraints of feeding style and that feeding style may be related to body size. Hence group size is related to body size and this has been found to be true for a wide range of mammals (Damuth, 1981). However group sizes may also be affected by anti-predator strategies, water availability, breeding strategy or physical characteristics of the environment, in which case diet choice may be constrained. Social organisation may only affect feeding ecology seasonally, for instance during the breeding season, but the effect on diet selection may be profound.

6.1.2. Existing knowledge of red and sika diet selection

6.2.2.1. Feeding ecology of red deer in Scotland

The diet of moorland Scottish red deer has been well-studied (Mitchell, Staines & Welch, 1977; Staines & Crisp, 1978; Clutton-Brock, Guinness & Albon, 1982; Staines, Crisp & Parish, 1982; Clutton-Brock, Iason & Guinness, 1987; Clutton-Brock & Albon, 1989) and agreement between studies has been high. Red deer on moorland range have a diet composed of 75-90% *Calluna vulgaris*, grasses and sedges, but include also broadleaved trees, conifers, woody shrubs and heaths, forbs, rushes, mosses, lichens, fungi, fruits and nuts and these can occasionally account for large portions of the rumen fill. Red deer from Rum and Glenfeshie are found to eat predominantly heather (*Calluna*) and grasses (Charles, McGowan & East, 1977; Mitchell, Staines & Welch, 1977; Watson & Staines, 1978; Clutton-Brock, Guinness & Albon, 1982; Staines, Crisp & Parish, 1982). Staines, Crisp & Parish (1982) and Clutton-Brock, Iason & Guinness (1987) find that hinds eat less heather than stags and tend to include more herb-rich short sward in their ranges. Watson & Staines (1978) find that this correlates with mineral rich soil patches which suport nitrogen rich vegetation communities. Staines and Crisp (1978) find, interestingly, that rumen nitrogen (a crude measure of diet quality) does not differ between stags and hinds, except just prior to calving when hinds increase rumen nitrogen. This may be because hinds include more grasses in their ranges which improve in quality early in the year, prior to the 1st June mean calving date.

The observation that larger stags tend to use more fibre-rich heather and less grass than smaller hinds is in keeping with predictions based on body size (Bell, 1971; Damuth, 1981; Clutton-Brock & Harvey, 1983; Demment, 1983; Gordon & Illius, 1989; Weckerly, 1993; Klein & Bay, 1994). Illius and Gordon (1987, 1990) further refine this relation to include data on jaw shape and find that predictions of sward use by the two sexes on moorland on Rum (Clutton-Brock, Iason & Guinness, 1987) are upheld by their model.

Compared to studies of red deer in other countries (Kiddie, 1962; Prins & Geelen, 1971; Kossack, 1976; Dzieciolowski, 1979; Mann, 1982; Putman, Culpin & Thirgood, 1993) there is reasonable similarity, but comparisons are often confounded by the differences in habitat use; most red deer outside Scotland being woodland creatures whilst Scottish data is largely from moorland populations.

6.1.2.2. Feeding ecology of sika deer in Japan

Most studies of sika deer in their native Japan have used very high density study populations and may not be typical of all Japanese populations (Takatsuki, 1980, 1983, 1984; Kawahara, 1983; Doi, 1989; Koga & Ono, 1994). Nor may they be comparable to introduced populations faced with a new habitat. However it is useful to review what is known of their natural ecology to give an idea of similarities or differences between red and sika.

Similar to red deer, sexual segregation in spatial dispersion has been noted by Takatsuki (1980) and in diet composition by Koga & Ono (1994). However, in the latter study, which concentrated only on grassland use, stags were found to be more selective within a winter sward than hinds. Stags chose more forbs and hinds took more grasses, which at this time of year are quite high in fibre. This is converse to red deer populations where stags would be expected to be less selective than hinds. As sika have small body weights (females 30-50kg; males 70kg in this population) and both sexes are below the 91kg that Gordon & Illius (1988) propose to be the lower limit for enhanced selectivity through jaw curvature, it is reasonable to assume that selectivity is not limited by morphology. This is a very high density population (>77/km^2) and so may be expected to have a low density of males (Clutton-Brock & Albon, 1989), perhaps reducing intrasexual competition and allowing increased selectivity if resources are spatially segregated to prevent competition from females. Unfortunately data on relative densities and dispersion of the population are not given.

All studies of sika in Japan designate them as grazers, but many have looked at impoverished ranges of habitat where little browse is available (Kawahara, 1983; Takatsuki, 1983; Doi, 1989). In populations in England and New Zealand (Davidson,

1973; Mann, 1982; Putman, 1986) sika are found to browse more. In these populations, however, other herbivores are present and may alter the feeding niche of sika through competition (Putman, 1986).

6.1.3. Competition and selection in mixed populations.

Three possible ecological interactions between red and sika are first outlined and then discussed in terms of phenotypic and genotypic changes predicted by each of them. Diet choice is initially considered as choice of botanical components.

6.1.3.1. Three possible interactions between individuals in a mixed population

1. Similarities between red and sika diet choice may result in competition between the two in mixed populations, intensifying selection on genes that influence digestive capability or other physiological factors involved in exploiting food intake efficiently (e.g. Mould & Robbins, 1982). If parental diet choices are very similar it is unlikely that hybrid diets will differ from them, increasing the probability that selection will act on physiological processes that affect nutritional gain from the same herbage intake. Competition will only act to drive selection if resources are scarce. In times of abundance dietary overlap may be complete without provoking competition.
2. If competition is strong between the two parental phenotypes we may expect that diets will diverge, especially in times of food scarcity, to reduce interspecific competition (Schoener, 1986; Gordon & Illius, 1989). It is unclear how quickly these effects would be seen in populations being colonised, as intensity of competition will ultimately be dependent on local deer densities and local resource abundance. Presumably there is a strong element of learning in diet choice, perhaps retarding changes in mean population behaviour as competition differs through the lifetime of an individual. Divergence of diets post-mixing would only correspond to genotype segregation if a common character of sika or red allowed exploitation of the new resource, for instance height allowing red deer to browse trees out of reach of sika. In this case hybrids, being of intermediate morphology, would therefore be in partial competition with both parents. However, if one parent is at low frequency, and therefore under-exploiting the new resource, hybrids, who can partially exploit it, may be better off than the parent at high density, which is experiencing higher levels of intraspecific (intra-phenotypic) competition. This situation is unstable, in that the less

frequent parent is advantaged, resulting in increased densities and increased competition for the new resource, but could lead to strong selection for a rare type at initial, low densities.

A character that was neither red nor sika in origin, for instance a learnt behaviour, may be selected in the population but not necessarily in animals of a particular genotype.

3. Alternatively differences in behaviour or phenotype may separate the two taxa into exploiting different food resources from the time of introduction, and result in habitat-dependent selection on traits that maintain that niche separation, driven by habitat availability and intra-specific competition. In this case it is likely that all hybrids would be selected against, being inferior competitiors in each niche (Rand & Harrison, 1989).

Both scenarios two and three describe similar situations to that envisioned in adaptive radiations, where new variants are advantaged by exploiting new resources for which competition is (initially) low. The newly arrived sika phenotype could exploit a previously underutilized resource in Scotland, either without competition from red deer or any other herbivore, or because they are excluded from resources red deer are utilizing.

6.1.3.2. Predictions for behaviour of the Scottish red and sika populations

Considering first a simple situation where behavioural factors other than feeding strategy are equal and all members of the population have the same nutritional requirements, the first type of interaction would predict selection on a physiological trait that significantly improved the efficiency of exploiting a resource, presuming such an advantage exists. If so, animals could continue to select a botanically similar diet, but genes that influenced digestive physiology would be strongly selected. These genes are unlikely to be marker genes, but may affect the behaviour of the markers, if they are linked. We may expect to see evidence of strong selection on a red or sika locus in populations experiencing scarcity of resources. The intensity of selection will decline with increasing resource abundance. If the advantage is sika (which is likely, given the increase in sika frequencies; Chapter 5) then strong linkage disequilibrium would also be expected as the genome is unlikely to break down by neutral recombination in only 20 generations (see Chapter 5).

The second scenario, of competition between animals selecting a similar diet, would promote differences in diet composition between phenotypes that could exploit

different resources. This would be probably, though not necesssarily, correlate to red and sika-like phenotypes, though it is less clear how hybrids would behave, as fitness is likely to be dependent on selection of phenotype allometry, relative densities of the parents and relative abundance of resources. Divergence of diets may occur over several generations, perhaps requiring more time than sika have been resident in the studied population (c. 20 generations). We would expect to find dietary differences initially related to genotype.

The last scenario of initial occupancy of different feeding niches, would also predict differences in diet between phenotypes, this time corresponding closely to genotypes, with all hybrids likely to be disadvantaged. We may also expect an element of habitat-genotype association in the hybrid zone.

Of course, life is not so simple as to allow us to consider a population of such behaviourally and metabolically similar animals. In deer populations it is well known that males and females have differing nutritional requirements throughout the year (Watson & Staines, 1978; Mann, 1982; Kay, 1985), and that dimorphism puts different constraints on the relationship between bulk and quality of their intake (Clutton-Brock, Guinness & Albon, 1982). Male and female responses should be considered separately in this case.

There are also problems in considering the interaction between genotype and response. Response (diet choice) is influenced by genotype through sex, social behaviour, phenotype and but also through learning, competition and available forage. In comparing responses of genotypes, even accounting for sex and forage availability, phenotypic and possible social and cultural influences on the animal are unknown. Hybrids may be disadvantaged by having an intermediate morphology, unable to compete with either parent. Genes involved in control of morphology in this case are under epistatic selection, with fitness of individual genes defined by their genetic background. Epistatic selection is a barrier to gene flow and would tend to result in a population where the taxa remain morphologicaly distinct, despite exchange of neutral, genetic material or genes that are universally favoured in some other way (Barton, 1983; Barton & Bengtsson, 1986).

Social and cultural effects are extremely hard to predict without a long observational study, however in deer they may be more extreme in females, who are known to be highly influenced in range choice by their mothers (Guinness, Hall & Cockerill, 1979). Local densities of the various genotypes may vary widely, though the previously discussed measures of linkage disequilibrium and heterozygote deficits indicate that recombinant genotypes are always likely to be less frequent than parental

types. Where social factors influence ranging behaviour and available diet, genotype of the individual may be less important than learnt behaviours in diet choice.

6.2. Methods in diet analysis

The following sections outline the methods used in the diet study of a mixed red - sika population in the centre of the Argyll cline. Differences in botanical composition (as a measure of selectivity), fibre composition (as a measure of nutritional quality; Mould & Robbins, 1981; Van Soest, 1982) and particle size distribution (a possible indicator of physiological differences in the rumen; Hofman, 1985) are assessed. The results are presented and summarised in Chapter 7 and discussed in relation to the genetical composition of the population and the predictions above in Chapter 8.

6.2.1. Sampling and storing rumen contents

In the central area of the Argyll transect (Ch. 4, Fig. 4.1.2a); Knapdale forest, whole rumen contents were collected from each adult (>2 year old) animal shot. These animals were also tissue sampled for genotyping (see Part 2). Animals were culled in either early morning or late afternoon, usually during feeding. Approximately equal numbers of male and females were culled each week, though as females predominate in the annual forestry management cull from which these samples were taken, females tended to be more numerous. In the overall sample $N_{males} = 30$, $N_{females} = 38$) Animals were not included in the sample if they were classed as diseased by Department of Agriculture standards on wild shot venison, now the British Wild Game Meat Regulations, in force on 1st January, 1994.

The entire rumen contents from each animal were transferred to a 20 litre bucket to which 2 litres 10% Formalin were added before sealing. This made a minimum 1% Formalin solution in the sample. Prior to sampling, fresh rumen content had been tested with serial dilutions of Formalin for the strength required to stop fermentation at room temperature. A 1% solution was found to be the minimum strength to preserve the sample effectively.

The samples were stored outdoors (<10°C) at the field sites and collected weekly to be analysed in the laboratory in Edinburgh. The maximum storage time was 6 weeks, but most samples were processed within 2 weeks of collection.

6.3. Particle size distribution in the rumens

6.3.1. Overview

The rumens were initially separated through a sieve tower to investigate the particle size distribution in the rumen. The exact method is given in Appendix 6, Protocol 6A. This treatment also yielded samples suitable for the subsequent botanical and fibre-content tests outlined in Appendix 6, Protocols 6B-6E.

Forage materials have been shown to have qualities of size and density that affect their elutriation through sieves of various meshes (Waldo *et al.*, 1971). This means that when the "2mm" fraction is referred to the particles are not necessarily 2mm in every dimension, but simply those that are retained by a 2mm sieve. For brevity the term '2mm fraction' (or relevant size) is used.

Early trials in this study showed that matting of large particles could significantly hamper separation. The dimensions of a sieve mesh affected the mass of the fraction it retained and that directly below, such that if the mesh size were small, matting of the material within it resulted in an increased fraction in that sieve and a corresponding decrease in the sieve below. The fraction in the third sieve was generally less affected, the ratio of the mesh of the first sieve to that of the third being large enough to allow particles to separate. The effect was undetectable in the fourth fraction. Trials were made with increasing mesh size ratios of the top two sieves until the lower fractions separated repeatably. The top two fractions were then pooled, as distributions between them could be ascribed to sieving artefacts.

6.4. Botanical composition

6.4.1. The use of rumen analyses to assess diet

The botanical composition of the rumens can be assessed using two methods. Both methods suffer different biases, discussed below, and it was unclear which gave the most accurate picture of the rumen content. The first method estimates the volume

of dietary components and the second measures their dry weight. Each is outlined below, with comments on the errors involved. As the relationship between weight and volume is not constant between plant species in the diet, the two methods give different pictures of the diet (Gaare, Sørensen & White, 1977).

For both techniques the final data describes each rumen content at the moment of death and is *not* necessarily representative of the diet of the individual or population over a longer term (Hanson & Graybill, 1956). As only larger particles are identifiable, the description is yet further restricted to the fragments recently ingested or particularly slow to degrade and does not neccesarily represent the proportions of plants ingested in the same feeding bout (Bergerud & Russell, 1964; Staines, 1976b). The interpretation of the data and any quantitative inferences about diet structure are discussed in Chapter 7.

Generally there are six potential biases that should be considered in inference of botanical diet composition from rumen contents.

1. *Individual variation in ingestion.* The more diverse the habitat the more likely that individual disparity will complicate inference of a mean intake. Increasing sample size can ameliorate this effect, though often this is impractical as time to collect and process samples is so great (Hanson & Graybill, 1956).

2. *Differential digestion rate.* Plants are digested at different rates dependent on their fibre content, structure and the other plants in the diet (Van Soest, 1982). This will affect their representation in the large particle fraction at a given time (Hanson & Graybill, 1956; Bergerud & Russell, 1964). This is probably the most difficult bias to control for as digestion rates of wild forages are usually unknown for individual plants (Gaare, Sørensen & White, 1977) and anyway passage rates can be altered by the composition of the diet as a whole (Van Soest, 1982). Staines (1976) shows that the representation of *Calluna* and grass in the 1mm fraction of red deer rumens declines by around 80%, 24 hours after last ingestion, but that *some* grass particles persist for over 50 hours after last consumption and *Calluna* persists up to 100 hrs.

3. *Biases due to separation and quantification method.* Plant proportions may either be assessed by dry weight or by volume (Staines, 1976b; Mann, 1982). The assessment by volume is quicker than that by dry weight, but generally woody plants are more dense and appear in greater proportion in the dry weight analysis than succulent plants and fungi, which appear relatively greater in volumetric analysis (Gaare, Sørensen & White, 1977).

4. *Variation in sampling time*. Ruminants must have a periodicity to their food intake to allow for rumination. During rumination particles are masticated and broken down, altering the composition of the large (visually recognisable) fraction. If rumens are sampled at differing times after ingestion it may markedly affect the proportions of plants in the 'large' fraction (Dirschl, 1962; Staines, 1976b). Ideally animals would be sampled at known times since feeding, but failing this (most wildlife studies) increasing sample size will compensate for the variation (Hanson & Graybill, 1956).

5. *Variation in sampling season*. Many deer species are shown to have seasonal variability in the diet (Mitchell, Staines & Welch, 1977). Pooling of samples should take into account seasonal variation and ideally sampling should be over as short a time interlude as possible.

6.*Biases due to sieve mesh sizes*. Around 30% of the rumen can be in tiny particles, < 0.1mm, which are lost through sieving even for microhistological analysis. The fraction of the rumen analysed should be considered in drawing conclusions from the analysis. (Bergerud & Russell, 1964).

In this study samples of whole rumen content, unsieved, were used for fibre analysis in order to assess the small particle fraction size and contribution to fibre content. Sieved (1mm+) fractions were used for botanical separation only as visual identification of small fractions is impossible. There may be a bias introduced if the recently ingested meal is very different from previous intake.

The method of separation was a complete separation of dry matter. This was chosen as previous trials (Abernethy, Ratcliffe & Chadwick, unpubl.) on scottish rumen samples showed high proportions of herbaceous material and fungi which are difficult to estimate accurately by the regression method.

Samples were collected from all animals culled and so sample sizes could not be increased. This was unfortunate as individual variation in diet choice was high and probably means that biases due to individual variation are the most serious consideration. Samples were collected from December to February when there was little variation in plant availability and quality. Digestibility of grasses declines slightly during this period but the effect is small (Staines, Crisp & Parish, 1982). In order to get the largest sample size possible it was essential to take samples over the entire winter. Passage rate differences in different plants are not accounted for as they are unquantified for this type of diet (Staines, 1976b).

6.4.2. Volumetric regression method

The following method was used to estimate the botanical composition of 58 rumens from Inchnacardoch forest in the Great Glen in a pilot study in 1991. It is essentially that of Staines, Crisp & Parish, (1982) There are two parts to the technique; the direct analysis of the sample (Appendix 6, Protocol 6B) and the creation of regression curves for interpreting these data (Appendix 6, Protocol 6C). Analysis of the sample provides a count of the number of times fragments of each plant touch a grid line in a standard sample tray. To interpret this score in terms of true plant volumes, the number of 'hits' counted must correspond in a predictable way to the volume of plant present in the whole sample. Known volumes of fragments of each plant species or category (i.e. conifer needles) are scored in the same way as the unknown samples and a regression equation calculated to relate 'hits' to volume. Thus unknown volumes can be inferred from hit scores in the standard tray. As fragment size is known to affect the relationship (Putman, 1984), fragments for calculation of regressions were collected from the rumens in this study, after the initial scoring trials. Some material was collected from each of at least 10 animals wherever possible. For plants occurring in less than 10 rumens the collection involved as many animals as possible. In cases where the plant was very rare in the sample, the volume was measured directly by collecting each fragment, blotting off surface water and measuring the displacement of liquid in a measuring cylinder. This more direct method was also used for plants with widely varying fragment sizes in the sample.

The volumetric method has advantages in that it is quick to process samples relative to methods involving individual identification of all fragments. However, the calculation of regression lines for each plant *is* time consuming, yet is necessary for each group of animals studied, or the method becomes unreliable. If there are few animals to be studied, or many plants present, the time saved in the scoring of rumens may be outweighed by that taken in calculating usable regressions, though the method is still likely to be faster than other alternatives if replicate samples are analysed from each rumen. Its main disadvantage is that it is an *estimation* of volume, rather than measurement and is subject to error at many stages. Firstly the initial volume of the sample is hard to determine accurately as removal of all surface water from a large, mixed sample is tricky. Entirely drying the sample would reduce this, but would also damage plant fragments, making identification more difficult. It would also require the drying of the plant fragments prior to regression calculations. Accurate determination of the total sample volume is not actually essential to the technique as, if everything else is accurate, this should be the sum of the individual plant volumes. However, it

does provide a useful check on this accuracy, as realistically experimental error must be expected to creep in.

Secondly there is error in the calculation of the regression values. This is likely to be the most serious source of error. Regressions are calculated as above from a number of single species trials, from which a best-fit line is taken. The number of trials made, the range of volumes used, and the size and variation of fragments used in the trial can all have bearing on the accuracy of the regression line, but surprisingly, the geometry of the fragments does not seem to affect the hits per volume relationship (S. van Wieren, pers. comm.). Accuracy should be increased by minimising the differences between the regression material and that in the samples. In this case regressions were calculated from pooled material from animals in the same population and, if sufficient samples contained the plant, this was divided between 'species' as classified by phenotype. Further division of material into that from males and females could also be considered if enough animals had been sampled and sufficient material existed of each plant to make this possible.

The method has been used in many studies with simple diet compositions and provides robust results if applied carefully (Bergerud & Russell, 1964; Staines, 1976b; Gaare, Sørensen & White, 1977; Jackson, 1977; Staines & Crisp, 1978; Mann, 1982).

6.4.3. Dry weight separations

The procedure for dry weight assessment of diet components is given in Appendix 6, Protocol 6D. This method was used to analyse 68 rumens from central Argyll. The method requires only the separation of plant fragments in the sample into species groups followed by drying to constant weight. Components can then be expressed as a proportion of the total dietary mass. The main errors arise from variation between subsamples and inaccurate drying and weighing of components once separated. The former is likely to be the greater problem as the time involved in

A
D
E
F
G
B
H
I
C

making a complete separation means that few replicates can be analysed. As some components plants are scarce and may dry to a mass of less than 0.001g, weighing inaccuracies may be a source of error in estimation of trace elements in the diet. This is the major difference between estimations of volume and dry matter. Staines, Crisp & Parish (1982) suggest that a correction factor may be applied to convert volumes to dry matter which would increase comparability of the methods, but cannot account for weighing error of low mass elements.

For each animal two 10g (wet weight) subsamples of the 1mm+ fraction of mixed rumen contents were fully separated to constituent plants. Figure 6.4.3. shows a typical rumen sample after separation.

6.4.4. Comparisons of the two methods

In all methods of reconstructing diet from digested or damaged plants, the problem of accurate identification of those plants should be considered. Here, in both cases, the sample was initially sieved through a 1mm mesh to discard all particles too small to readily identify through a 12x magnifying lens. A 5-40x magnification binocular microscope was used for first identification of fragments, but usually the 12x lens was sufficient thereafter. A reference collection of common herbs, grasses and leaves was made prior to the analysis and identification of fragments for comparison of venation patterns and cross-sections of leaves and stems. Field guides to the major plant groups (Dallimore & Jackson, 1948; Hubbard, 1984; Rose, 1986; Fitter, 1987) were used as references for general plant form, especially leaf-edge or stem surface characteristics. Examples of leaf cross section identification for two plant groups are shown in Fig. 6.4.4. Colouration often provided a means to grouping fragments within a sample, but was not consistent between samples containing the same plant and was not used as a diagnostic character. Most flowering plants were identified to species, but this was not possible for most of the monocotyledonous species, though where identification was reasonably unambiguous (i.e. the grass *Deschampsia flexuosa* or the woodrush *Luzula sylvatica*), these were scored separately. If a plant could not be identified to species it was classed by family or genus, or occasionally as 'unknown'. Within a sample some individual fragments could not be identified at all and were scored as 'other'. Other fragments could not be identified to species, but could be grouped within a sample. For instance small woody twigs which could be identified as being ericaceous, either *Calluna vulgaris, Erica cinerea* or *E. tetralix,* but could not be assigned to a single species. These were divided

between the represented species of the group in proportion to the leaf and other known fragments in the sample. This may have introduced a bias, but was felt to be more representative of the sample content than to classify these pieces as either 'unknown' or to lose information by lumping together two species likely to represent different fractions of the total mass.

The dry weight measurement of plant components is a more direct approach than the volumetric method. The treatment, though slow per sample, which is its main disadvantage, but does not require the calculation of regression coefficients to allow interpretation of the data as the volumetric method does. As it measures the amount of each plant present exactly it is more accurate within the subsample than the volumetric method, but the time involved prevents analysis of as many replicate subsamples.

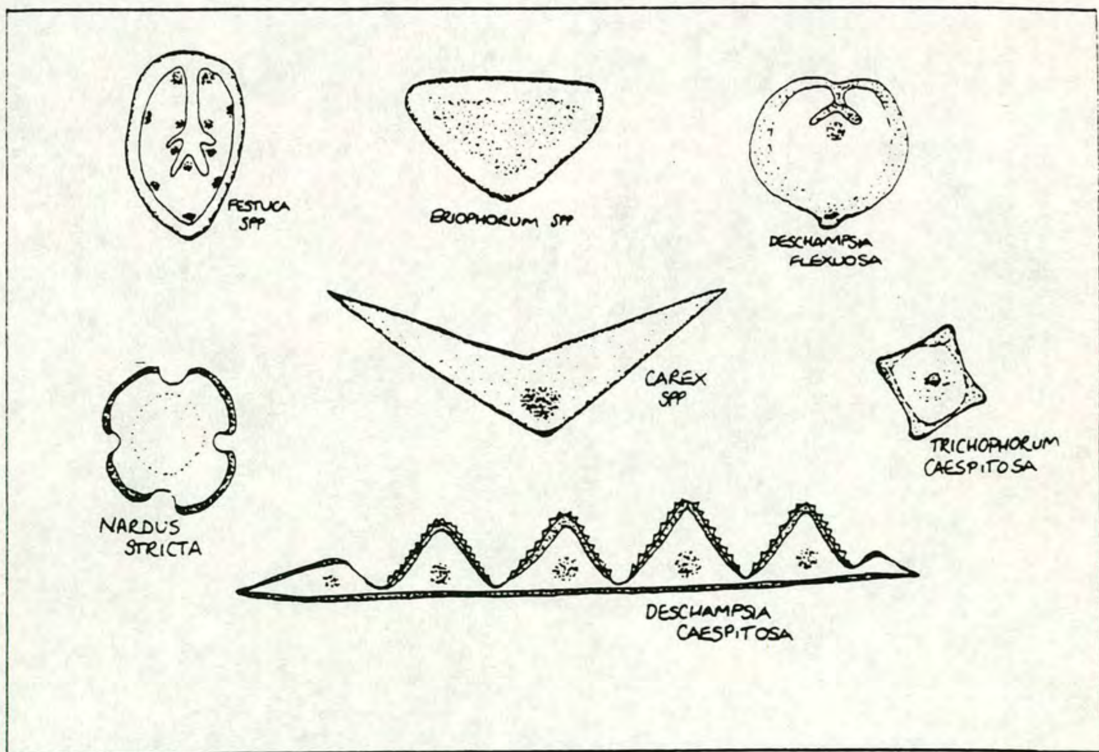


Figure 6.4.4. Cross sections of common grasses and sedges in red and sika rumens. Cross sections of fragments are useful identification tools.

6.5. Assessing diet quality

6.5.1. Overview

The nutritional value of ingested plant forage can, roughly speaking, be summarised by two relationships: the ratio of soluble to structural components and the levels of phenolics and other structurally complex binding moelcules within the plant (Van Soest, 1982). *In vitro* chemical analysis (simulated digestion) of forages can be used to assess these relationships and estimate diet quality (Mould & Robbins, 1981), although *in vivo* digestibilities may differ between animals dependent on diet botanical composition, passage time and microbial flora (Robbins *et al.*, 1975; Milne *et al.*, 1978). Generally in wild populations true *in vivo* digestibilities cannot be measured and *in vitro* assessment of diet quality is the best measure.

Early measures of diet quality used crude fibre, determined by ashing a dried residue of indigestible material after digestible fat and nitrogen-free compounds (NFE) were extracted, and comparing proportions of these fractions. This has since been found to be unsatisfactory as digestibility is related only to available, soluble, cell contents and not to those bound structurally to cell wall fractions. The crude fibre analysis did not differentiate these, tending to over estimate the NFE proportion and underestimate true lignin, thus inflating true digestibilities, though the error was found to be highly variable (Ely & Moore, 1955). Lucas (1964) developed a system which took into account the relationship between available and unavailable components of the diet. He described three forage classes: Class 1 components are digestible cellular contents including sugars, starch, proteins, organic acids, pectin and lipids; Class II, structural carbohydrates including cellulose and hemicelluloses; Class III, indigestible substances such as lignins, cutins, tannins and other polyphenols and ash. This system is a vast improvement on the crude fibre reckoning as it has a better functional reasoning behind it. However in Class II there is still variable digestibility, dependent on tannins and structural binding proteins, which remains a problem to *in vitro* digestibility estimates (Robertson & Van Soest, 1981).

Current methods use a system of detergent solutions in which forages are refluxed to dissolve particular components. Comparison of residue weights can then be used to calculate the proportion of various fibre classses. An initial treatment with neutral detergent (ND) dissolves Class I components, including pectins although these are involved in cell wall structure. Treatment of either the ND residue or a second sample with acid detergent (AD) removes Class II hemicelluloses and cell wall

complexed proteins (Van Soest, 1967, but see Mould & Robbins; 1981 Gauthier, Huot & Picard, 1991) leaving indigestible Class III lignins, some celluloses and lignified nitrogen. True digestibilities have been measured *in vivo* and using rumen bacterial digestion trials and are related linearly to lignin and to $\log_{10}$ [lignin/AD residues] measured in this way (Van Soest, 1964) justifying their use as measures of diet quality.

6.5.2. Calculation of cell components from detergent residues.

The sequential detergent fibre analysis yields four residues from which various components of plant cell structure can be determined.

6.5.2.1. NDF and cell solubles

The neutral detergent fibre (NDF) residue retains all structural components of the cell wall except pectin, and the loss of weight is equivalent to cell soubles; lipids, sugars, organic acids, tannins, pectin, starch, non-protein nitrogen, soluble silica, and soluble protein. These are termed '*cell solubles*' and are deemed to be 100% digestible (Van Soest, 1982).

6.5.2.2. ADF and hemicelluloses

A sequential acid detergent fibre (ADF) residue contains cellulose, lignin and lignified nitrogen. Although Van Soest (1982) states that all hemicellulose is retained in NDF residue and lost in ADF, facilitating a simple assessment of hemicellulose, there is in fact disagreement in the literature as to the amount of hemicellulose retained in the NDF residue and the amount of fibre bound protein that is retained in NDF and lost by ADF (Mould & Robbins, 1981; Gauthier, Huot & Picard, 1991). Estimates of true hemicellulose calculated from NDF-ADF vary from 100% (Van Soest, 1982), through 77-88% dependent on forage type (Mould &Robbins, 1981) to 44% regardless of forage type (Gauthier, Huot & Picard, 1991). For comparison of fibre composition between forage types and in circumstances where absolute fibre contents are required these problems are serious, however for comparative studies where the error is applicable to each sample the consequences are less, unless as Mould & Robbins (1981) suggest the error is not constant between forage types. In the following analysis the fraction NDF-ADF is termed '*hemicellulose*' for convenience, but the implications of mismeasure of true hemicellulose are discussed in section 7.4.

6.5.2.3. Lignin, cellulose and ash

After concentrated sulphuric acid treatment, the residue contains crude lignin and ash (insoluble minerals). The loss in weight from the ADF residue is the estimate of *cellulose*. A final loss in weight after a step to ash the sulphuric acid residue gives the estimation of crude *lignin*. Insoluble *ash* is measured directly, but was insignificant in the samples measured here, most weights being <0.001g, within the weighing error of the balance.

6.5.2.4. Diet quality

Both crude lignin and $\log_{10}[(\text{lignin/ADF})*100]$ have been directly related to fibre digestibility in *in vivo* trials (Van Soest, 1967). The measure ADF has little biological significance but reduces error in percentage estimation of crude lignin in sequential trials as this is the residue from which lignin is estimated. Crude lignin will generally suffice as an approximate estimate of digestibility and thus diet quality. In this analysis both measures are used. The term $\log_{10}[(\text{lignin/ADF})*100]$ is shortened to logL/ADF.

6.5.3. Sequential analysis of fibre composition of the rumen contents

The analysis of rumen contents in this study involved a sequential assay of NDF, ADF, lignin and ash components. The tests were run on samples of mixed, preserved rumen contents, as in Appendix 6, Protocol 6A, dried to constant weight and ground to 0.5 mm uniform particle size in a hammer mill. Samples were initially dried at 40°C as conventional 100% drying procedures at 100°C have been found to create artificially high NDF residues and increase hemicellulose estimation (Mould & Robbins, 1981). Figure 6.5.3 gives a summary of the 4-day process (Appendix 6, Protocol 6E).

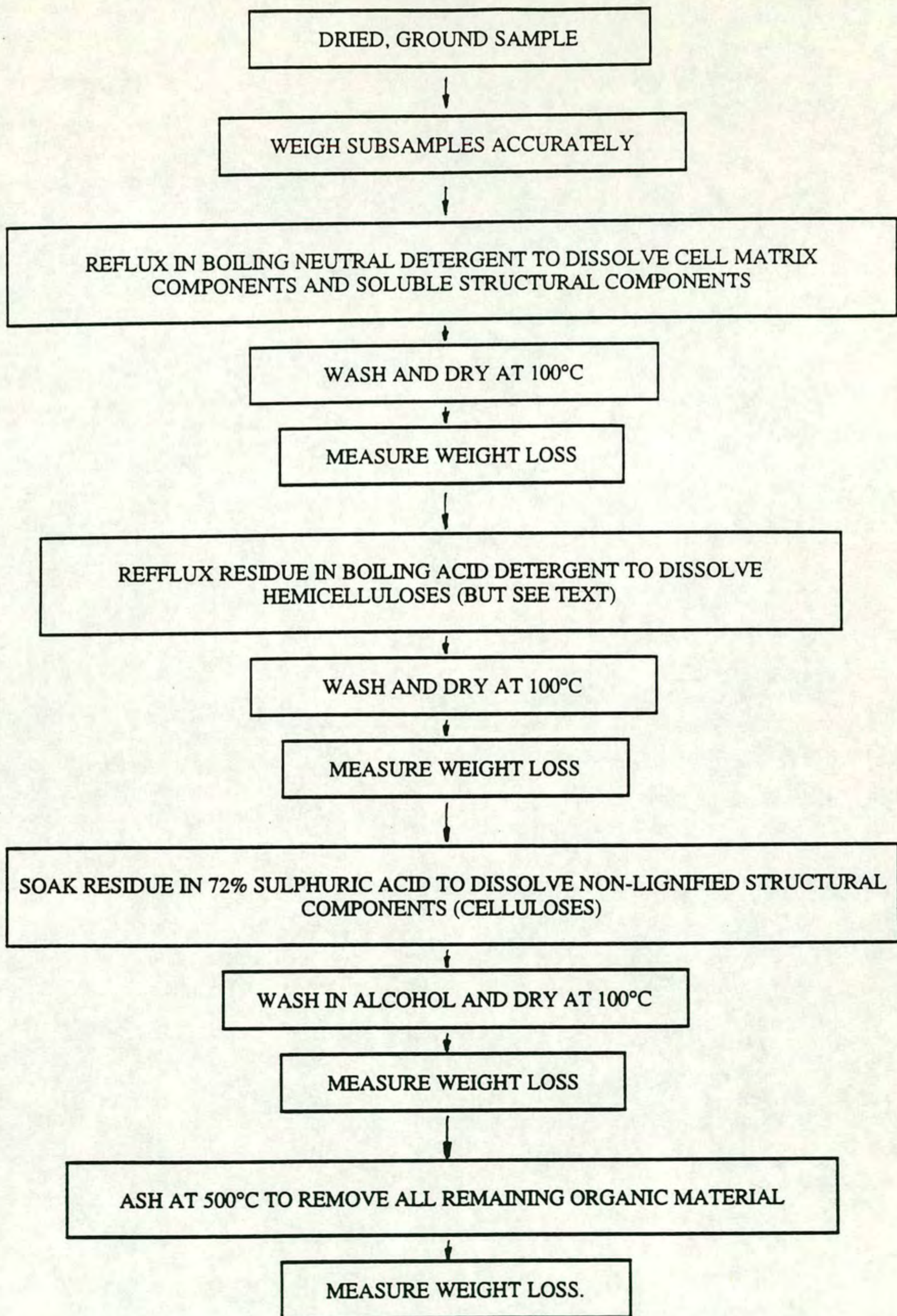


Figure 6.5.3. Sequential fibre analysis. Details are given in protocols in Appendix 6.

Chapter 7.

WINTER DIET SELECTION IN A SCOTTISH RED - SIKA POPULATION

This chapter presents the results from the diet analysis of the mixed populations. Diet was examined at three levels; botanical constitution, fibre composition and particle size distribution within the rumen contents (see Chapter 6). Animal characteristics of genotype, sex, maternal lineage and interactions between sex and genotype were used to explain variance in these three aspects of diet selection. The effect of botanical constitution on fibre components and particle breakdown and the effect of fibre composition on particle breakdown were also investigated, using a general linear regression model.

The results are compared to prior work on diet selection in deer. Points where predictions from previous work are *not* upheld are discussed in the light of genotypic variation and other factors influencing this population.

7.1. Botanical composition of the diet

7.1.1. Diversity of the diet

Winter diets of deer in Argyll were found to be highly diverse. A species list of all plant groups recorded is given in Table 7.1.1a. Assessing the importance of individual food plants was difficult as some plants occurred in a large number of rumens, but in small amounts. Others were in large amounts when they did occur, but the frequency of ocurrence was low. "Top ten" tables of plant use for males and females were collated based on the overall frequency, frequency of >10% dry weight contribution and maximum dry weight contributions of each plant category. These are given in Tables 7.1.1b&c.

Argyll (this analysis)	Group	Inchnacardoch (data Forestry Commission, unpublished.)	Group
Dryopteris spp.	Fern	Dryopteris spp.	Fern
Pteridium aquilinum	Fern	Pteridium aquilinum	Fern
Unidentified fern spp.	Fern	Unidentified fern spp.	Fern
Deschampsia flexuosa	Grass	*Deschampsia caespitosa*	Grass
Festuca ovina	Grass	*Deschampsia flexuosa*	Grass
Festuca rubra	Grass	*Festuca ovina*	Grass
Molinia caerulea	Grass	*Festuca rubra*	Grass
Nardus stricta	Grass	Festuca spp.	Grass
Unidentified grasses	Grass	*Molinia caerulea*	Grass
		Nardus stricta	Grass
		Unidentified grasses	Grass
Calluna vulgaris	Heath	*Arctostaphylos uva-ursi*	Heath
Erica cinerea	Heath	*Calluna vulgaris*	Heath
Myrica gale	Heath	*Erica cinerea*	Heath
Vaccinium myrtillus	Heath	*Myrica gale*	Heath
		Ulex europaensis	Heath
		Vaccinium myrtillus	Heath
		Vaccinium uliginosum	Heath
		Vaccinium vitis-idea	Heath
Galium saxatile	Herb	*Cardamine flexuosa*	Herb
Oxalis acetosella	Herb	*Cornus suecia*	Herb
Solcus spp.	Herb	*Crepis paludosa*	Herb
Taraxacum spp.	Herb	*Galium saxatile*	Herb
Trifolium spp.	Herb	Geranium spp.	Herb
		Moehringia trinervis	Herb
		Oxalis acetosella	Herb
		Ranunculus spp.	Herb
		Sonchus oleraceus	Herb
		Stellaria media	Herb
		Taraxacum spp.	Herb
		Trifolium spp.	Herb
		Veronica officinalis	Herb
Cladonia spp.	Lichen	Cladonia spp.	Lichen
Unidentified lichens	Lichen	Unidentified lichens	Lichen
Raconitrum spp	Moss	Raconitrium spp.	Moss
Sphagnum spp.	Moss	Sphagnum spp.	Moss
Unidentified mosses	Moss	Unidentified mosses	Moss
Luzula sylvatica	Rush	*Luzula sylvatica*	Rush
Juncus spp.	Rush	Juncus spp.	Rush
Carex spp.	Sedge	Carex spp.	Sedge
Eriophorum angustifolia	Sedge	*Eriophorum vaginatum*	Sedge
Eriophorum vaginatum	Sedge	*Trichophorum caespitosa*	Sedge
Trichophorum caespitosa	Sedge	Unidentified sedges	Sedge
Unidentified sedges	Sedge		
Betula pubescens	Tree	*Abies* spp	Tree
Betula verrucosa	Tree	*Betula verrucosa*	Tree
Larix spp.	Tree	*Juniperus communis*	Tree
Picea abies	Tree	Larix spp.	Tree
Picea sitchensis	Tree	*Picea abies*	Tree
Pinus sylvestris	Tree	*Picea sitchensis*	Tree
Unidentified broadleaves	Tree	*Pinus contorta*	Tree
		Pinus sylvestris	Tree
		Unidentified broadleaves	Tree

Table 7.1.1a. Plants identified in rumens of Argyll deer compared to those from Inchnacardoch forest, the Great Glen, in a previous study (Forestry Commission, unpub.).

b	Top 10 botanical components categorized by % individuals with plant present in rumen		Top 10 by % of individuals with plant representing >10% of rumen content		Top 10 by maximum recorded mass in an individual rumen	
	Plant	% of inds	Plant	% of inds.	Plant	% of plant
1	*Calluna vulgaris*	100.0	Grasses	92.1	*Calluna vulgaris*	89.8
2	Grasses	97.4	*Calluna vulgaris*	86.4	Grasses	74.4
3	Mosses	89.5	*Vaccinium myrtillus*	15.8	Fern root	61.6
4	Fern leaf	76.5	Fern root	7.9	*Betula* spp.	42.3
5	Lichens	76.4	Fern leaf	5.3	*Myrica gale*	33.3
6	*Vaccinium myrtillus*	71.9	*Luzula sylvatica*	5.3	*Vaccinium myrtillus*	31.3
7	Herbs	71.9	*Myrica gale*	5.3	Fern leaf	31.3
8	*Conifer*	57.9	*Betula* spp.	2.6	Conifer	22.1
9	Narrow sedges	50.0	Bark	2.6	*Juncus* spp.	21.4
10	*Juncus* spp.	39.5	Conifer	2.6	*Luzula sylvatica*	17.3
			Erica cinerea	2.6		
			Juncus spp.	2.6		
			Narrow sedges	2.6		

c	Top 10 botanical components categorized by % individuals with plant present in rumen		Top 10 by % of individuals with plant representing >10% of rumen content		Top 10 by maximum recorded mass in an individual rumen	
	Plant	% of inds.	Plant	% of inds.	Plant	% of plant
1	*Calluna vulgaris*	100.0	*Calluna vulgaris*	80.0	Fern root	87.7
2	Grasses	96.7	Grasses	80.0	*Calluna vulgaris*	76.2
3	Mosses	90.0	Fern root	20.0	Grasses	72.1
4	Lichens	76.7	Lichens	13.3	*Myrica gale*	59.2
5	Herbs	63.3	*Myrica gale*	10.0	*Vaccinium myrtillus*	29.6
6	*Vaccinium myrtillus*	63.3	Fern leaf	6.7	*Luzula sylvatica*	17.3
7	Fern leaf	56.7	Herbs	6.7	*Betula* spp.	16.5
8	Conifer	56.7	*Vaccinium myrtillus*	6.7	Lichens	15.7
9	*Juncus* spp.	40.0	Wood	6.7	Conifer	13.4
10	*Erica cinerea*	36.7	*Luzula sylvatica*	3.3	Herbs	12.9

Table 7.1.1b&c. Botanical constituents if the diet.**b**) females, **c**) males. There is wide variation in occasional use of individual plants, indicated by the appearance of certain plants in the third list, maximum recorded percentage in the rumen, but not on the first two measures of use throughout the population. Mosses are found in all samples account for a maximum of 2% in any one and are not considered to be being actively selected. They are not included in subsequent analysis.

7.1.2. Plant community designation and use

Kossack (1976) showed that use of plant communities is a more meaningful way to interpret the botanical composition of red deer diets, and reduces the problem of accounting for large variation in individual plant use due to large community diversity. Probably the most difficult decision of the analysis of botanical diversity, concerned the pooling of plant categories to reduce individual variation without losing information on selectivity. Choice of too broad a community membership will obscure selectivity within it, choice of too narrow a community will reduce representation of communities in many samples to zero, and so reduce statistical power. A principal components analysis of diet variation, using all individual plant categories, confirmed that the very large loadings given to a few, relatively rare plants (i.e.found in only a few animals, but in large quantities where present), obscured underlying patterns in use of more widespread plants (see Appendix 7). Implications of community choice should be clear when interpreting the data.

The plants present in the samples were each assigned to a community on the basis of their occurence together in published vegetation classifications for Argyll (McVean & Ratcliffe, 1964; Rodwell, 1991a-c). Four communities were identified as follows, and are represented in Figure 7.1.2:

Grasslands: grasses, herbaceous plants

Heaths: *Calluna vulgaris, Erica spp*, narrow sedges

Woods: *Vaccinium myrtillus, Luzula sylvatica,* Ferns, Lichens, Conifers and Broadleaf trees incl. bark, wood.

Mires: *Juncus* spp., broad sedges, *Myrica gale*

In later analysis woods and mires were combined as they generally each accounted for very little of the diet. The Woods category contained *Vaccinium myrtillus* and lichens although these occurr in heath communities also. In Argyll *Vaccinium* is more often associated with woods than heath and was thought to be more representative of the wood community (SNH, unpubl. data; McVean & Ratcliffe, 1964). A second heath community was originally tested, containing *Vaccinium*, but this explained less of the variance in diet than the heath category containing only narrow sedges, *Calluna* and Ericas. Basing plant communities on "constants"; plants that always occur in the community and rarely elsewhere, has been discussed and used by Rodwell (1991a-c) as a fairly robust method of vegetation community classification.

Percentage dry weight scores for use of each plant community were created by summing scores across the constituent plants. Community scores were used in all subsequent analysis.

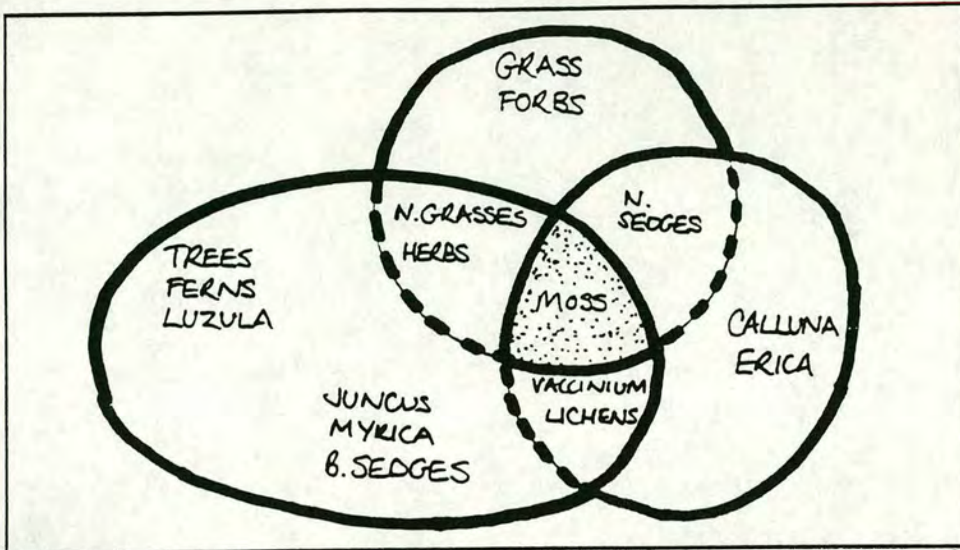


Figure 7.1.2. The plant communities identified and used in analysis of sika and red deer diets. Bold lines show inclusion in a community. Dashed lines show potential overlap between communities. Communities were tested both including and excluding potential overlap and the community group best able to describe variation was chosen.

7.1.3. Do animal characteristics of genotype and sex explain community use?

Analysis

A general linear regression model was used in the 'GENSTAT' statistical package to fit genotype, scored across the continous hybrid index from 0-1 where 0 = sika and 1 = red (see Chapter 5), with sex as a factor and an interaction term. Variations on this basic model are later used to test relationships between animal characters and fibre composition and particle size distribution and between the various diet measures themselves.

Scores for each community use for the 68 animals in the sample were used in five separate analyses, the last regression combining the wood and mire communities as neither explained any variance alone. Results of the analyses for heath, grassland and wood-mire communities are shown in Table 7.1.3a.

Results

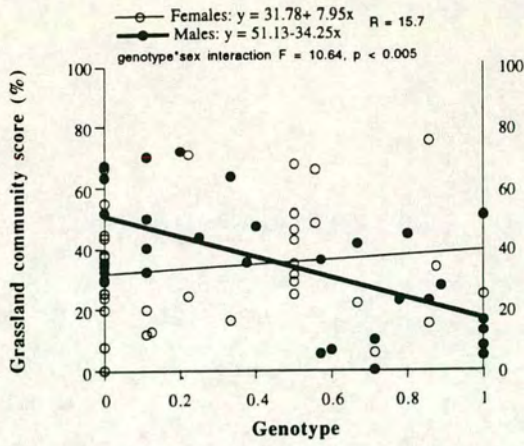
Genotype significantly influenced both heath and grassland use, though not the use of wood and mire plants. Red deer use more heath and less grassland than sika. Sex also affected the use of heath communities, with females using more than males.

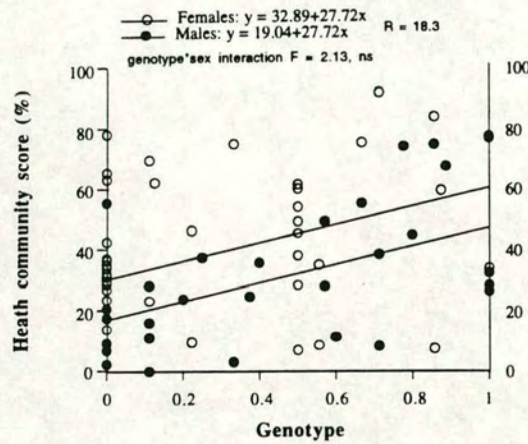
Sex alone had no effect on use of grasslands. The interaction between sex and genotype was highly significant in the use of grasslands, but not heaths, with sika males using grassland heavily (51% of diet) and red males making little use of it (17%). Within females, no effect of genotype was found in grassland use.

Use of woodland varied between 0.11% and 62.7% of the diet, but genotype and sex explained only 4.6% of this variance, which was not significant for genotype or sex above the 10% significance level (Figures 7.1.3a-c). The results are presented diagrammatically in Figure 7.1.3d, showing the differences between the sexes at the extreme genotypes. Predictions of community use, based on regressions of the 68 individual scores for each community, accounted for approximately 100% of the diet of the extr - me genotypes (Table 7.1.3b).

	Grassland		**Heath**		**Wood - Mire**	
	$F_{(1, 64)}$	*p*	$F_{(1, 64)}$	*p*	$F_{(1, 64)}$	*p*
Genotype	4.26	*<0.05*	10.27	*<0.005*	1.21	*ns*
Sex	0.56	*ns*	7.07	*<0.025*	2.89	*<0.1*
Genotype.sex	10.64	*<0.005*	2.13	*ns*	2.16	*ns*
$R^2_{(3, 64)}$	15.7	*<0.005*	19.7	*<0.001*	4.6	*ns*

Table 7.1.3a. The effect of genotype and sex on plant community use. A general linear model was used to regress genotype against community use scores, fitting sex as a factor and a genotype-sex interaction term. Genotype has a significant effect on use of grassland and heath, with sika using more grassland and red deer more heath. Sex alone has no effect in grassland use though it significantly affects heath representation in the diet. Females use more heath. In use of grassland the fit is improved by the interaction term. This is largely through differences in use of grass by stags (see Fig. 7.1.2a), sika stags feeding heavily on grassland, whilst all hinds use similar amounts of grassland communities. Although the use of wood and mire communities is affected by sex, only 4.6% of the total variance is accounted for. The use of wood and mire plants was extremely varied (see text).





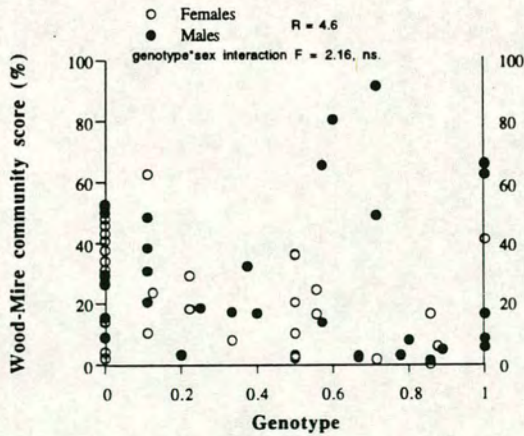


Figure 7.1.3a-c. Plant community selection by genotype and sex. Genotype ranges from 0= 'pure' sika to 1= 'pure' red. Genotype and sex individually explain significant variation in use of heath, with red using more than sika and females more than males. The interaction between sex and genotype explains variation in grassland use, with sika males using most and red males least, whilst female use of grassland does not vary with genotype. The presence of wood and mire plants in the diet was extremely variable and neither genotype or sex explain their use.

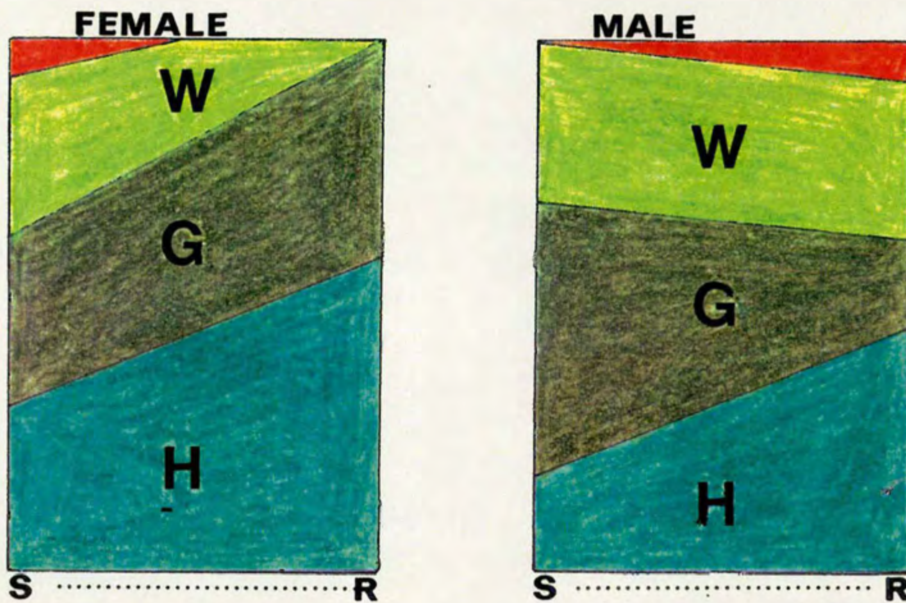


Figure 7.1.3d. Proportions of diet from grassland communities (**G**), heath communities (**H**) and wood-mire communities (**W**) for red and sika deer. The relationship between genotype and community use is shown for males (right) and females (left).

SIKA FEMALES		SIKA MALES	
grassland	31.78 ± 4.02	*grassland*	51.13 ± 5.34
heath	32.89 ± 4.02	*heath*	19.04 ± 5.17
woodmire	29.60 ± 4.64	*woodmire*	30.25 ± 6.17
TOTAL	94.27	TOTAL	100.4
RED FEMALES		**RED MALES**	
grassland	39.72 ± 7.20	*grassland*	17.01 ± 5.74
heath	60.61 ± 6.23	*heath*	46.46 ± 5.46
woodmire	7.07 ± 8.31	*woodmire*	29.58 ± 6.63
TOTAL	107.4	TOTAL	93.05

Table 7.1.3b. The diet composition of sika and red in terms of plant community use. Estimates of diet of 'pure' red and sika are made by linear regressions of community use against the continous variable of genotype from all animals including hybrids (see Chapter. 5). Standard errors of the estimate are given.

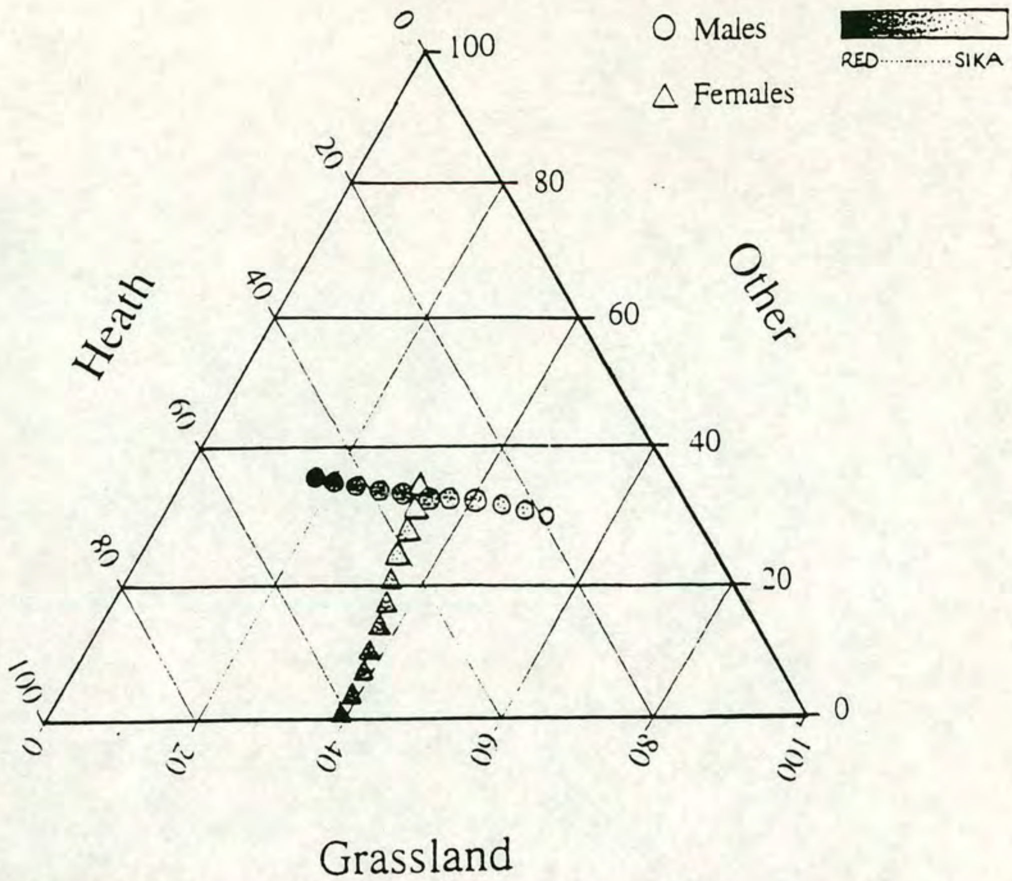


Figure 7.1.3e. The change in diet composition across the genotype index for males (circles) and females (triangles). Genotype scales from 0 = sika (open symbols) to 1 = red (closed symbols), divided into increments of 0.1 'redness'. See Ch. 5, 5.6.1. for full explanation of genotype index use. As the genotype index is based on only 5 loci, the extreme ends of the regression do not correspond to 'pure' red and sika, but to the most sika-like and red-like animals identified. Diet composition for animals of each genotype was predicted from previous linear regressions of heath and grassland components against genotype, fitting sex as a factor (Fig. 7.1.3a-c). The remainder of the diet (not heath or grassland plants) is classed 'other' but corresponds closely to the proportions of wood and mire communities used, shown in Fig. 7.1.3d. Clearly the greatest overlap in diet selection is between sika females and hybrid males, though how far overlap extends across the genotype index is difficult to test. Although errors can be calculated on the estimate of each component in the diet, changes in proportions must covary and the distribution of the error is complex. Significant differences between the sexes and extreme genotypes are found in use of heath and between the extreme genotypes of males in the use of grassland. Significant differences between extreme genotypes exist for female use of woodmire (other), but only account for 4.6% of the total variance. Overlap estimates (MacArthur & Levins, 1967) show most segregation between red males and red females and least (equal) between sika females and sika males or sika females and red females (see text).

Scores for use of each community were predicted from the original regressions at genotype indices from 0 - 1 in 0.1 increments. These are plotted for males and females in Figure 7.1.3e. The greatest overlap in diet selection is clearly between sika females (0 - 0.1 red) and hybrid males (0.5 - 0.6 red). Sika females and males are less separated than red females and males.

7.1.4. Dietary niche overlap

Potential competitive overlap was analysed using both symmetrical (Pianka, 1973; Schoener, 1974; Dunbar, 1978) and asymmetrical formulae (MacArthur & Levins, 1967). Measures of niche overlap are not statistically testable and should be interpreted as relative measures of *potential* competition within a community. Many measures of niche overlap have been proposed and comparisons between studies must also be made with caution (Pianka, 1973; Schoener, 1974, 1986; May, 1975; Emlen, 1975 ; Dunbar, 1978; Diamond, 1978; Slobodkin & Schultz, 1980; Putman, 1986). The symmetrical measures indicate the level of overlap in use of a resource, asymmetrical measures attempt to show the effect of replacing an individual of species A with one of species B and so calculate the likely direction of selection in times of resource scarcity. As May (1975) elegantly demonstrates, overlap along one resource axis, say prey size, is **not** evidence of actual niche overlap (competition), as use of another resource, say habitat type, may separate the two populations. Biological intuition must be used to decide whether enough parameters of an ecological niche have been used in the equation to allow inference of a competitive interaction.

Symmetrical overlap was calculated after Dunbar (1978) who uses a simple measure α, the sum of percentage points of each community common to both species.

For asymmetrical overlap, α_{12} is the effect on the rate of increase of species 1 if an individual of species 1 is replaced by a member of species 2.

$$\alpha_{12} = \Sigma_h \; p_{1h} \, p_{2h} / \Sigma_h \; (p_{1h})^2 \qquad [7.0]$$

after MacArthur & Levins (1967), where α is the niche overlap, p_{1h} is the proportion of species 1's total diet represented by use of component h, and p_{2h} is, likewise, the use of h by species 2. A multivariate overlap can be calculated by expanding the formula to include other components of the diet.

$$\alpha_{12} = \Sigma_h \; p_{1g} \, p_{2g} p_{1h} \, p_{2h} / \Sigma_h \; (p_{1g})^2 \, (p_{1h})^2 \qquad [7.1]$$

For any niche 'macrodimension', like habitat use, the categories of habitat within that dimension, i.e. woodland, open space, hilltop etc., can be classed as 'microdimensions' (Slobodkin & Schultz, 1980). Overlaps for the extreme genotypes

of each sex and are shown in Table 7.14 for the macrodimension of diet composition, with microdimensions of grassland, heath and wood-mire.

a:symmetric	Sika female	Red female	Sika male	Red male
Sika female	-	71.7	80.4	79.5
Red female		-	65.8	70.9
Sika male			-	65.6

b:asymmetric	Sika female	Red female	Sika male	Red male
Sika female	-	0.068	0.066	0.063
Red female	0.038	-	0.039	0.038
Sika male	0.065	0.053	-	0.050
Red male	0.056	0.060	0.058	-

Table 7.1.4a&b. Competitive overlap in use of grassland, heath and wood-mire communities between sexes and extreme genotypes, calculated after a) Dunbar (1978) and b) MacArthur and Levins (1967). For asymmetrical overlap, species 1 is shown on the left, species 2 at the top. For the symmetrical measure, least overlap is between sika and red males, reflecting their differential use of grassland and heath. The measures of asymmetrical overlap suggest that sika females have most affect on the other genotypes and red females least. Surprisingly, in comparison to the data on the inverse relationship between male and female density (Clutton-Brock & Albon, 1989), red males have a large effect on red females. It is likely that other resource dimensions, such as habitat use, time of feeding of choice of plant part, also affect competition actually experienced (see May, 1975).

In the symmetrical measures of overlap, greatest overlap is between the sexes of sika and least between the males of the two taxa. For the asymmetrical measures, sika females have the greatest effect on other genotypes and red females the least. These measures do not necessarily equate to competition as scarcity of resources and relative densities of the genotpes are unknown, however areas of potential competition are highlighted and sika females appear to be the most effective potential competitor. This is consistent with predictions from the body size concept dicussed in Chapter 6.

7.2. Fibre composition

7.2.1. Does the botanical composition of the diet affect fibre composition?

Analysis

The four fibre components of the whole rumen contents; cell solubles, hemicellulose, cellulose and lignin, and diet quality (as indexed by logLignin/ADF) were each regressed against each plant community to test whether plant community content could explain variation in fibre composition. The results are presented in Table 7.2.1. The whole fraction was thought to represent a (weighted) average of the diet more accurately than the 2mm+ fraction which only contains recently ingested material.

Results

Grassland and heath community use explained significant amounts of variation in the amounts of lignin (18.1%, 26.8% respectively) and hemicellulose (7%, 23.4%) in the diet. High heath use and low grassland use resulted in high lignin scores. Low heath use and high grassland use gave high hemicellulose content. Mould and Robbins (1981) do, however, suggest that grass forages may result in a higher estimation of hemicelluloses from an NDF-ADF subtraction than browse forages, which would give a similar trend. In the absence of a better test for the behaviour of mixed heather and grass diets in sequential hemicellulose estimates, these results for hemicellulose should be interpreted with some caution.

Only 7.3% of the variation in cellulose content was explained by grassland use. Increasing grassland use resulting in increased cellulose content. This may be a pronounced effect in winter samples (as in this study) where cellulose content of grasses is high due to the increased ratio of dead to live material.

Diet quality (logL/ADF) was significantly affected by community use, with high heath and low grassland use giving a lower quality diet.

Variation in cell soluble content was not explained by any of the plant communities. Wood-mire communities did not explain any variance in any of the fibre measures.

	Heath						Grassland						Wood-mire					
	R^2	$F_{(1,64)}$	p	b	t	p	R^2	$F_{(1,64)}$	p	b	t	p	R^2	$F_{(1,63)}$	p	b	t	p
Cell solubles	*	0.60	ns	0.012	0.77	ns	*	0.66	*ns*	0.02	0.81	ns	*	0.[illegible]	ns	0.01	-0.77	ns
Hemicellulose	23.4	20.87	<0.001	-0.09	-4.56	<0.001	7.0	5.87	<0.05	0.06	2.42	<0.05	*	0.52	ns	0.04	0.72	ns
Cellulose	3.5	3.37	ns	-0.05	-1.83	ns	7.3	6.09	<0.05	0.07	2.47	<0.05	*	0.12	ns	-0.02	-0.34	ns
Lignin	26.8	24.48	<0.001	0.14	4.95	<0.001	18.1	15.16	<0.001	-0.13	-3.89	<0.001	*	0.46	ns	-0.06	-0.67	ns
log(lignin/ADF)	14.4	11.77	<0.005	0.001	3.43	<0.001	13.5	10.95	<0.005	-0.001	-3.31	<0.001	*	0.01	ns	0.00	-0.91	ns

Table 7.2.1. Regression analysis of each fibre constituent in the whole fraction against each of the plant communities individually. (b) indicates the slope of the regression, and is given together with significance. In all cases except cellulose, heath accounted for more of the variance than grassland use and in no case was fibre composition influenced by use of wood-mire communities. Rumens with high heath content had high lignin, low hemicellulose and a high lignin: ADF ratio. Those with high grassland scores had high hemicellulose, lower lignin and a low lignin: ADF ratio (see Fig. 7.2.1).

7.2.2. Do animal characteristics of genotype and sex affect fibre composition of the diet?

Analysis

Animal characteristics were used to explain variance in fibre components of the whole and 2mm fractions of the rumen contents in a similar model to that in section 7.1.2. The whole fraction and 2mm fraction components were tested separately.

Results

Genotype significantly explained variation in amounts of lignin and hemicellulose in the diet (but see above), but did not explain variation in diet quality (logL/ADF). Sika rumens had less lignin and higher hemicellulose contents than red deer rumens.

Sex only affected diet quality measured in the 2mm (recently ingested fraction), and this only at 10% significance level, explaining only 5.3% of the variance. The interaction term was not significant in any test (Table 7.2.2a).

The model was extended to fit plant community and then the animal characteristics of genotype, sex and a genotype.sex interaction term, in order to test whether, within the group of animals selecting a plant community, genotype affected the quality of the diet chosen. The order of fitting plant community (heath or grassland fitted first) was determined from relative values of F for the regression coefficients calculated in Table 7.2.1 above. Heath was fitted first for all components except cellulose.

After fitting the plant communities, animal characteristics could not explain any further significant variation in fibre composition or diet quality (Table 7.2.2b).

7.3. Particle size distribution

Particle size distribution was originally assessed in 6 size classes; >2mm, 1-2mm, 0.5-1mm, 0.25-0.5mm, 0.15-0.25mm and <0.15mm. These classes were pooled to form 3 larger classes; large particles, >1mm; medium particles, 0.15-1mm and small particles, < 0.15mm. Ratios between the small and medium fractions were also calculated. The large fraction was assumed to be recently ingested material (Dirschl, 1962; Bergerud & Russell, 1964; Staines, 1976b) and was equivalent to that analysed in the botanical separations. Medium and small particle fractions were

	Cell solubles				Hemicellulose				Cellulose				Lignin				log(Lignin/ADF)			
	whole		2mm+		whole		2mm+		whole		2mm+		whole		2mm+		whole		2mm+	
	$F_{(1,62)}$	p	$F_{(1,54)}$	p	$F_{(1,62)}$	p	$F_{(1,54)}$	p	$F_{(1,62)}$	p	$F_{(1,54)}$	p	$F_{(1,61)}$	p	$F_{(1,54)}$	p	$F_{(1,6i)}$	p	$F_{(1,54)}$	p
Genotype	0.03	*ns*	0.23	*ns*	6.75	*.025*	2.14	*ns*	0.05	*ns*	1.13	*ns*	3.83	*0.1*	3.68	*0.1*	0.21	*ns*	2.46	*ns*
Sex	0.59	*ns*	0.67	*ns*	1.6	*ns*	1.09	*ns*	0.05	*ns*	2.12	*ns*	0.97	*ns*	1.16	*ns*	1.25	*ns*	2.79	*0.1*
Genotype.sex	0.17	*ns*	0.48	*ns*	0.59	*ns*	1.03	*ns*	1.97	*ns*	0.32	*ns*	2.36	*ns*	1.68	*ns*	0.85	*ns*	0.96	*ns*
R^2 (3, 60)			*		8.4		2.2		*		1.0		6.1		5.8		*		5.3	

Table 7.2.2a. The effect of genotype on fibre composition of the whole rumen contents and 2mm+ fraction. Only hemicellulose in the whole contents is significantly affected, being more abundant in sika then in red deer rumens. Lignification of the diet is only affected by genotype at 10% significance. Sex affects diet quality as measured by the log (L/ADF) at the 10% level.
* residual variance > response variance.

	Cell solubles		Hemicellulose		Cellulose		Lignin		log (lignin/ADF)	
	$F_{(1, 60)}$	p	$F_{(1, 60)}$	p	$F_{(1, 62)}$	p	$F_{(1, 59)}$	p	$F_{(1, 60)}$	p
Heath	**0.58**	*ns*	**<u>20.21</u>**	*0.001*	**0.63**	*ns*	**<u>24.59</u>**	*0.001*	**<u>11.91</u>**	*0.005*
Grassland	**1.72**	*ns*	**0.34**	*ns*	**<u>5.88</u>**	*0.025*	**<u>4.02</u>**	*0.01*	**3.74**	*ns*
Genotype	**0.13**	*ns*	**1.58**	*ns*	**1.02**	*ns*	**0.11**	*ns*	**0.87**	*ns*
Sex	**0.20**	*ns*	**0.06**	*ns*	**0.63**	*ns*	**0.03**	*ns*	**0.06**	*ns*
Genotype.sex	**0.02**	*ns*	**0.00**	*ns*	**0.2**	*ns*	**0.11**	*ns*	**0.07**	*ns*
R^2 (5, 59)	*	*ns*	**<u>20.9</u>**	*0.001*	**4.0**	*ns*	**<u>27.2</u>**	*0.001*	**15.4**	*0.001*

Table 7.2.2b. The effect of genotype and sex on the fibre composition of the diet, within animals selecting a plant community. Genotypic variables are fitted after plant community has been accounted for. The model fitted depends on the variance accounted for and confidence in regressions previously calculated for each plant community variable alone. Heath content accounted for most of the explained variance in all fibre categories except cellulose which was best explained by grassland score (Table 9.2.2). Thus in the model for cellulose grassland was fitted first and in all others heath was fitted first. Hemicellulose and lignin contents and diet quality (logL/ADF) are significantly affected by plant community, with higher hemicellulose and lower lignin percentages found in grassland type diets and *vice versa* in heath type diets. Within animals selecting a plant community there is no effect of genotype on the diet quality and fibre composition (see also Table 7.2.2a.), though genotype does affect the choice of plant community. Although cellulose is also affected by the amount of grassland in the diet. the total variance accounted for is only 4%, precluding firm conclusions drawn from this result.

analysed in the botanical separations. Medium and small particle fractions were assumed to be the result of digestive processes, which could be influenced by botanical constitution, fibre levels and characteristics of the animal. General linear models of these relationships were tested in a similar strategy to that applied in sections 7.1 and 7.2.

7.3.1. Does lignification affect particle distribution in the gut?

Three measures of lignin, in the whole rumen, the 2mm fraction and the calculated lignin in the < 2mm fraction were used to explain variance in particle size class proportions.

No significant variance was explained by any measure of lignification (Table 7.3.1).

7.3.2. Do animal characteristics affect particle size distributions?

The effect of genotype and sex on particle size distribution was tested in the same model as section 7.1.3.

Genotype affected the size of the medium particle class, though the ratio of small to medium articles was only explained at 10% significance. This may be because the estimate of small particles accumulates the experimental error incurred in estimation of medium and large particles and may increase 'noise' in the ratio estimate.(Table 7.3.2)

7.3.3. Once botanical composition is accounted for, do animal characteristics affect particle distribution?

Once again the model was extended to include plant community and fit genotype subsequently (see section 7.2.2).

Plant community use was insufficient to explain any variance in particle size distribution and fitting plant community did not improve the estimate of genotype effect on the medium particle class size, although an extra 2.9% of the total variance was explained. (Table 7.3.3.)

	Large			Medium			Small (<0.15mm)			Medium: Small		
Lignin measure	*F* (1, 54)	*p*	R^2	*F* (1, 54)	*p*	R^2	*F* (1, 54)	*p*	R^2	*F* (1, 54)	*p*	R^2
Whole content	0.01	*ns*	*	3.80	*ns*	4.8	2.18	*ns*	2.1	3.57	*ns*	4.5
2mm+ fraction	1.28	*ns*			-							
<2mm fraction	-	-	-	0.66	*ns*					0.29	*ns*	

Table 7.3.1. The effect of lignification on particle size distributions in the rumen. Comparisons are made between relevant fraction and lignin measures. No significant effects are found.

	Large (1mm+)		Medium (1mm - 0.15mm)		Small <0.15mm		Medium: Small ratio	
	F	*p*<			*F*	*p*<	*F*	*p*<
Genotype	**1.54**	*ns*	**4.98**	*0.05*	**0.37**	*ns*	**2.96**	*0.1*
Sex	**0.2**	*ns*	**0.33**	*ns*	**1.94**	*ns*		
Genotype.sex	**1.08**	*ns*	**1.25**	*ns*	**0.1**	*ns*		
R^2 (3, 52)	*	*ns*	**6.1**		*	*ns*		

Table 7.3.2. The effect of genotype on particle distribution in the rumen. Only the medium band of particles are affected by genotype.

	Large (1mm+)		Medium (1mm - 0.15mm)		Small <0.15mm		Medium: Small ratio	
	F	*p*<			*F*	*p*<	*F*	*p*<
Grassland	**0.16**	*ns*	**0.9**	*ns*	**1.07**		**1.22**	*ns*
Heath	**0.01**	*ns*	**1.95**	*ns*	**1.53**		**2.23**	*ns*
Wood - Mire	**2.62**	*ns*	**2.31**	*ns*	**0.12**		**0.20**	*ns*
Genotype	**3.26**	*0.1*	**4.3**	*0.05*	**0**		**1.57**	*ns*
R^2 (4, 51)	**3.0**	*ns*	**9.0**	*0.1*	*		**1.3**	*ns*

Table 7.3.3. The effect of plant community and genotype on particle size distribution within the rumen. No effect of plant community is found in any particle class, although once plant community is accounted for genotype has a small effect within the medium class; red deer have more medium particles than sika. This is not found in the ratio of small to medium particles. The relationship between genotype and medium particle fraction is not improved by fitting plant community characteristics, although more total variance is explained.

7.4. Comparisons to prior work

7.4.1. Botanical composition.

The results obtained here correlate well to those found in previous studies of red and sika populations (Mitchell, Staines & Welch, 1977; Watson & Staines, 1978; Staines & Crisp, 1978; Staines, Crisp & Parish, 1982; Mann, 1982). The rumen contents of most animals were dominated by *Calluna vulgaris* and grasses, with other plants occurring occasionally and in small volumes. One notable aspect of this study was the occurrence of some plants, e.g. ferns, in large quantities in some animals, but being rare in the population (see Tables 7.1.1b&c). This type of variation was also found in rumens of red and sika deer in Inchnacardoch (Abernethy, Chadwick and Ratcliffe, unpubl.) and, to a lesser extent, in sika in Mann's 1982 study. Data for European red and sika populations tend to show higher diversity in the plants accounting for > 10% of the diet than Scottish populations do (Kossack, 1976; Dzieciolowski, 1979; Bartos & Zirovnicky, 1982). The slightly increased diversity of the diet in this population may reflect differences in habitat diversity from other Scottish populations studied or may reflect an aspect of sika behaviour, however it is also likely that the published data on Scottish red deer may understate the occurrence of rare plants in the diet as data are often only presented for major constituents of the diet.

The use of more heather by females than males is a surprising result. In most other studies on Scottish deer (Mitchell, Staines & Welch, 1977; Watson & Staines, 1978; Staines, Crisp & Parish, 1982; Clutton-Brock, Guinness & Albon, 1982) males have been found to use more heather than females. The difference between this and other studies may reflect the importance of other influences, such as shelter, culling pressure, or social organisation on the use of moorland by the two sexes, or may reflect differences in the nutritive value of grasses and heaths in this area. The increased use of heather is not accompanied by a lower diet quality for females as has been the case for males consuming bulk heather in other studies (e.g. Staines, Crisp & Parish, 1982). If diet quality is not reduced by heather intake, there is little pressure to choose to eat grasses.

7.4.2. Diet quality and lignification

In the Argyll animals, differences in diet quality between the sexes and genotypes were not convincingly demonstrated. Males did choose a slightly lower quality diet (as indexed by the lignin: ADF ratio) than females ($p < 0.1$) and reds chose a slightly more lignified diet than sika ($p < 0.1$). These differences could be entirely accounted for by plant community use. Several studies have previously documented selection of a higher quality diet by hinds (Watson & Staines, 1978; Staines & Crisp, 1978; Staines, Crisp & Parish, 1982; Beier, 1987) generally through increased use of grasslands, and theory on body size would predict selection of a higher quality diet by smaller animals, in this case females and sika. The trends in the data uphold these predictions although the differences are not highly significant. It is interesting that the interaction between sex and genotype was not able to explain variation in diet quality, as from prior work this would have been expected to be a strong influence. However, diet quality is difficult to measure well (Van Soest, 1982) and error in estimation may have resulted from concentration of lignin in the rumen, as high quality food is digested quickly, or measures may not be sensitive enough to register small differences in quality .

The fact that variation in diet quality can be explained by use of different communities may reflect the importance of differences in dispersion of the sexes and genotypes, however as the plants within the designated 'communities' could in fact be obtained from several habitats and do not necessarily imply that feeding is all within one geographical area, the implications for spatial separation are unclear.

7.5. Limitations of the data.

7.5.1. Community use

The use of plant community use to describe combinations of plants in the rumen has a value in reducing the level of individual variation observed, and in translating a long and unwieldy list to an ecologically sensible unit. However, without observation of the animal feeding prior to sampling, it is not possible to be sure that the plants found in the rumen did in fact originate from the same geographical location. In this case there was much overlap between dispersion of communities, for instance, grasses are found throughout the area, except under closed-canopy conifers, though they form a constant and abundant part of the communities found in meadows and

along verges, which are spatially discrete units. The presence of a plant in the rumen does mean that the animal selected it to forage on, whether this was through selection of a spatial area dominated by a community as described in Chapter 6, or through selection of individual plants within a different community. Conclusions about plant community use do not necessarily imply use of a particular spatial area and cannot be used to make inferences about population dispersion through the area.

7.5.2. Body sizes in relation to genotype

One serious shortcoming of the data here is the lack of known body weights for genotyped animals. As hybridisation will produce an intermediate-sized animal (Harrington, 1979), and this will be influenced by sex, there is likely to be a range of body sizes generated in the population. However, the genetic structure of the hybridising populations (see Chapter 5) indicates that there may be selection against hybrid types, perhaps weighted toward intermediate phenotypes. This will skew the distribution of body sizes across the genotype index, probably into a bimodal distribution for each sex, with the sexes overlapping. Unfortunately the relationship between the genotype and phenotypic traits is unknown. Conclusions can be drawn about the influence of size on behaviour using relative body sizes of red, sika and hybrids from the literature and for broad, population level comparisons these are likely to be adequate, but for fine scale comparison of the behaviour of individuals this is unlikely to be sufficiently detailed.

7.5.3. Genotype index

The use of the genotype index reflects the character of only a small part of the genome and does not mean that animals scoring equally on the genotype index will be equivalent in phenotype. Although across the loci represented (4 diallelic combinations and one haplotype) there are 648 possible genotypes, only 27 categories are represented on the index in this population, as each locus scores equally, regardless of which it is. Even excluding environmental influences on phenotype during development, there are likely to be more than 27 phenotypic variants found in the population, as the rest of the genome will exert an influence on phenotype. The genotype index is an indication of how hybrised an animal is, it is not an absolute scale and does not represent'pure' types at its extremes, purity of an animal could only

be truly confirmed by typing of the entire genome, in which case each individual would be unique making 'purity' an unattainable goal (see Chapter 2). These limitations of the genotype index should be borne in mind in drawing conclusions from the interaction of genotype and quantitative traits.

7.6. Summary of results.

- Diet was botanically very varied. Occurrence of individual plants changed by as much as 0% to 89.8% between rumens.
- Genotype was found to significantly affect plant community choice. Sika-like deer use less heath and more grassland than red-like deer.
- Sex affects use of heath with, surprisingly, females using more than males.
- Sika males use grassland heavily and red males very little, whilst there is no significant variation in grassland use within females.
- Greatest overlap in community use is predicted between sika-like females and hybrid males. This could result in competition if densities are high. Sika females are likely to be the superior competitor.
- Diet *quality*, as measured by logLignin/ADF, varied very little across the sample, though red deer and males chose a slightly poorer diet.
- Plant community use significantly affects lignin, diet quality and possibly hemicellulose contents of the rumen, though within the group of animals choosing a community, there is no further significant variance between genotypes or sexes.
- Genotype, but not plant community or lignification significantly affects the size of the medium particle class, but only a small proportion (6.1%) of the total variance is accounted for.

Chapter 8.

THE INTERACTION BETWEEN ECOLOGY AND POPULATION GENETICS.

In this final chapter I discuss the theoretical implications of the feeding ecology study in the light of genetic variation known to exist in the population. In the results and analysis chapters (Ch. 4-7) I have stated that existing theory does not adequately explain the behaviour of an advancing hybrid zone and that interpretations of the genetic processes and quantitative traits observed are limited by uncertainty of what patterns would be expected under various regimes of selection, dispersal and mating system. In Chapter 1 I introduced the difficulties in applying theoretical concepts, developed to look at ecological differences between species, to populations where animals cannot be individually assigned to species groups. Here I explore the possible interpretations of the ecological data, the limitations of existing theory in this case and the way in which these quantitative trait differences may be useful in understanding the hybrid population.

A strategy for modelling the future behaviour of the hybrid zone through computer simulation is suggested in section 8.3., together with an outline of the further data required to construct an adequate model.

The chapter, and the thesis, concludes with a brief review of current management policy for red deer populations, the concerns that have been expressed over colonising sika populations and possible management strategies for mixed populations.

8.1. The selective pressures on individual fitness of red and sika deer.

In all organisms, fitness is ultimately measured in terms of lifetime reproductive success - the number of genes surviving in the next generation. Hybrid zones highlight the fact that individual genes can be passed on independently from the

rest of the original parental genome, and fitness varies gene by gene. However, the survival and reproductive success of an animal depends on the combined effects of its entire genome, together with an element of chance in the way environmental stochasticity affects phenotype.

Red and sika deer have similar life histories and are likely to be subject to similar selective pressures on reproductive success (Bunnell, 1987). These pressures on red deer are reviewed in Clutton-Brock, Guinness & Albon's (1982) work on their behaviour and ecology, which highlights the differences between the two sexes. There is a great deal of literature on feeding ecology of herbivores, particularly ungulates, and how this relates to various life histories both between species and between the sexes of a species.

8.1.1. Fitness in red deer

Females

In females, reproductive success is proximately determined by annual body condition (fat levels), though when body condition is accounted for, hinds that have borne a calf the previous year are most likely to conceive again, suggesting some other (perhaps genetic) influence on fertility. Small body size is also positively correlated with success in females, once condition and previous reproductive status are accounted for (Albon *et al.*, 1986).

Small body size may have a genetic component, but is also influenced by early nutrition. Annual body condition must be profoundly affected by diet, though digestive capabilities and other physiological processes will also be influential. Genetic control of enzyme systems and physiology may be as important as diet in annual body condition cycles.

As female fecundity is distributed throughout the lifetime, genes affecting annual condition (fecundity) will be selected for in all ages above first reproduction, which can be at 1yr. Genes that affect survival will also be under selection in all animals, but the intensity of selection will decline throughout the lifetime.

Males

In males success is correlated to absolute body size, antler size and configuration, and age. Body size is influenced by early growth and age, but not by annual condition. It is likely to have a large genetically determined component (Pemberton *et al.*, 1988). Antler growth and configuration are also likely to be largely

genetically determined (Williams, Krueger & Harmel, 1994), though nutrition will influence their growth both annually and over the lifetime. As male reproductive success is a not distributed throughout the lifetime, but peaks at middle age (around 9 years; Clutton-Brock & Albon, 1989), survival to this age will require maintenance of a minimum body condition in early life. Genes that affect fecundity will not be under selection until relatively late in life, whereas genes that affect survival will be intensely selected in juvenile males.

At a common age, genes affecting likelihood of survival and those affecting fecundity will be under different intensities of selection in the two sexes. However, they may also be different loci.

8.1.3. Fitness in sika deer

Sika deer have not been the subject of such an exhaustive study of lifetime success as red deer, but in the absence of evidence to the contrary it is assumed that they experience similar selection pressures. The most likely exception is in the strategy of the males. There is some data to suggest that sika males in woodland may use a more territorial breeding system to that of the Rum (open range) red deer described above (Miura, 1984; D. O'Gorman, pers. comm.). It is unclear how this may be influenced by size and age. The evolution of combative antlers and sexual dimorphism suggest that male body size is still likely to be an important determinant of success (Clutton-Brock, Albon & Harvey, 1980; Clutton-Brock, 1982, 1987), but the relative importance in territorial males compared to those using the red deer harem strategy is unquantified.

8.1.4. The interaction of sex, size and time of fecundity

As sika have smaller body size than red deer, it might be expected that sika stags would be strongly disadvantaged against red stags. However as the introgression of sika genes in to red deer populations, illustrated by the data, is due to success of small sika-like males, it suggests that a behavioural mechanism during the rut in mixed populations outweighs the importance of male body size in some circumstances or that the survival rate of sika or hybrid calves is extremely high relative to red deer.

When applied to females, the implications of body size are different. Small females are more successful than larger ones of equal body condition in red deer (Albon *et al.,* 1986). This would imply that the sika phenotype would be advantaged in females and that genes controlling body size may be selected for in females.

Clearly the selection pressure experienced by sika genes in a mixed population may differ between the sexes. Selection on body size, as an example, is likely to be further complicated by involving a large number of genes controlling skeletal structure, whose interactive (epistatic) effects may be more important than the sum of individual selective coefficients. Body size may no longer have a simple relation to fitness in a hybridising population.

The introgression of neutral markers may also be enhanced by 'hitch-hiking' on some other trait allowing introgression of some genetic material whilst other genes experience a barrier or partial barrier to flow. Genes influencing small body size may be selected against in males whilst other sika genes advance, but they are likely to be selected for in females. Given the relative dependence of fecundity on size in the sexes it seems reasonable that the intensity of selection against small size in males will outweigh the selection for it in females. On the other hand as female fecundity is selected from an early age, selection will be stronger in females as it acts on a larger proportion of the population than it will in males, whose ability to survive to breeding age has already reduced their number before selection on fecundity acts.

After an introduction, genes that are universally favoured will fix in the population, those that are universally deleterious will be lost and those that persist at intermediate frequencies will be those that are neutral or that have an advantage that is not consistent across all members of the population. Genes that have different values in different sexes, ages or morphs could have pleiotropic effects or experience epistasis or dominance effects thus having different effects in different genetic backgrounds, or could influence traits equally, that are of varying importance in the life histories of different animals.

8.2. Diet choice as a function of genotype and sex in the Argyll population

In mixed populations competition for access to plants is between phenotypes: either by superiority in direct aggression or by ability to exploit plants inaccessible to the competitor (Jarman, 1974). This may be reflected in botanical composition of the

diet or in spatial dispersion of the competitors (Roughgarden, 1974; Dunbar, 1978; Gautier-Hion, 1980; Schroeder & Schroeder, 1984; Putman, 1986). Phenotype traits affecting diet selection and physiological processes are likely to be under the control of (many) different genes and may be selected separately in a population. They may also be selected separately in the sexes; though nuclear loci can spread through both males and females, the selection pressure they experience in each sex may differ (see above, 8.1). This anomaly between population mean data and the inference of behaviour of genotypes at the individual level is one of the most difficult problems at the interface between population genetics and ecology.

8.2.1. How do genotypes and sexes differ in diet selection?

If, in female deer, annual body condition is the most important factor in reproductive success, this suggests that autumn diet may be important. Farmed sika and red deer females can calve each year from their first year if diet quality is good (Hamilton & Blaxter, 1980; Blaxter *et al.*, 1974, Suzuki & Ohtaishi, 1994), but wild populations rarely achieve this level of fertility, suggesting that either nutrition is inadequate or that other factors reduce fertility.

In the population studied here, the interactions of sex and genotype with diet are best summarised in Figure 7.1.3e above, which shows differences in plant community use by different male and female genotypes. No difference in diet quality was found between sexes or genotypes after selection of a botanical community. As genotype is classified on an index of only 5 loci, a tiny fraction of the entire genome, the purity of animals at the extreme ends of the scale is far from certain. What is important is what can be inferred from the trends across the genotype scale.

The greatest overlap in diet selection is between sika females and hybrid males, though how far overlap extends across the genotype index is difficult to test. Although errors can be calculated on the estimate of each component in the diet, changes in proportions must covary and the distribution of the error is complex. Measures of competitive overlap which aim to calculate the relative depression in growth rate of a population if a member is replaced by a competitor (MacArthur & Levins, 1967), show that sika females have greatest effect on other genotypes and there is least effect of red females on other genotypes.

The segregation of sexes in red deer has been well documented and is largely explained in terms of body size differences (Clutton-Brock, Guinness and Albon, 1982; Gordon & Illius, 1989). When considering the hybridising population, it is

clear that equivalent genotypes, in terms of proportion of red genes, respond functionally differently in the different sexes, a possibility raised in the last section. Sika males and females seem to select a botanically more similar diet than red deer. This may be because their requirements are more similar for annual condition, or because resources are sufficiently abundant that they are not competing. The lack of evidence for selection of different diet qualities suggests that winter requirements of all animals are similar, though achieved through different feeding strategies.

If diet selection is not the most important influence on reproductive success, the genes that control it may be selected differently to the selected genes linked to the documented marker loci. In this case we may expect some discrepancy between the genotype designated and the response of the animal in diet choice. It is likely that diet choice is important to success in herbivores. It is less certain that it is being selected the same way as the marker loci in this hybrid zone.

8.2.2. Explanations of the patterns

The theory of how body size affects feeding ecology would predict that (small) sika females would be the best competitors on depleted swards and largest red stags the would be competitively excluded first. Although sika females are shown by the asymmetrical niche overlap measure (MacArthur & Levins, 1967) to be the best potential competitors, red females are shown to be the most inferior competitor, not red males (see Table 7.1.4a&b). The apparent overlap between sika female and hybrid male diets may not result in actual competition because densities of hybrid males are likely to be low (see Chapters 3 & 5). Competition requires that the density of competitors reduces the available resource for each one. Although niche overlap would usually predict that the less abundant competitor would be ousted (MacArthur, 1968), in this case hybrids will be continually created from parental populations occupying different feeding niches and not in intense competition with sika females.

Although there is no data available on skeletal size differences in hybrids, it is likely that they will be intermediate between red and sika. In this case the notions of the body size concept would predict that greatest competition would be between red females and hybrid males where the range of body sizes is likely to overlap. This appears not to be the case and is evidence that other mechanisms are likely to be involved in diet choice (Weckerly, 1993). Possible other factors are the proximity of shelter to feeding grounds, group sizes or competition with herbivores other than deer.

Body size arguments would also predict that the smallest animals (sika females) would choose the highest quality diets and the large red males would choose

the lowest quality. The data here do not show highly significant differences in diet quality between groups the trends are consistent with these predictions. However, Hofmann (1982) classes sika as grazers on the basis of rumen characteristics, including a relatively larger rumen than red deer, putting them in the bracket of bulk selectors whilst red deer are concentrate selectors. This greater gut capacity would allow sika to chose a slightly lower quality diet in greater bulk than their body size alone would predict, perhaps confusing comparisons over the small range of body sizes considered in red-sika populations. However, in Scotland, winter grass quality has been shown to be higher than heather quality and red deer herds forced to utilise heather perform worse than those able to graze (Ratcliffe, 1988). Sika, as grazers and with higher relative gut capacity than red deer should compete effectively against red deer. The amount of grass in the diet is highest in sika males, followed by all females and least is found in the diet of red males. The relatively large amount in the diet of sika males is unexpected, compared to that of females, yet does not translate to a better quality diet, as measured by log (L/ADF) or lignification alone, which is perhaps why it features less in the diet of females.

The diet choice of red and sika deer probably cannot be explained by size alone and is likely to be influenced by relative gut size, habitat use patterns in response to shelter requirements, group sizes and predation pressure, and possibly other social factors. Significant differences do exist between the genotypes and sexes and in competition sika females seem likely to be the superior competitor in Argyll.

8.2.3. Comparison to the predictions made in Chapter 6 for the behaviour of mixed populations

In Chapter 6 I stated three potential fates of a mixed population. They were that either a) a physiological or behavioural trait which improved foraging efficiency by lowering costs or improving gathering rate would be selected, allowing animals to choose a botanically similar diet regardless of genotype,

b) that competition between animals choosing an overlapping diet would lead to divergence of diets, with correlation between phenotype and diet probably equating to correlation between genotype and diet,

c) that diets would not overlap initially, and that selection would act to preserve this difference, assuming intraspecific competition remained lower than interspecific competition would be through expansion of the niche. In newly-invaded populations

where sika are relatively infrequent, this is a valid assumption, but may lose validity in populations where sika densities are higher.

The data show that diets do overlap considerably and so the last scenario can be discarded. It would be possible for elements of both the other situations to occur. The fact that diets do not overlap fully could be, though is not necessarily, the result of some divergence through competition. Hybrid genotypes appear to fall between pure types suggesting that variation in diet choice is related to genotype rather than a learnt behaviour and that hybrids may be overlapping both parental niches. As discussed above, competition between hybrids and parental types will not be able to lead to the eradication of hybrids, even though they are less frequent (MacArthur, 1968), as they are produced from genotypes experiencing different competitive pressures in a different niche. In fact the difficulty of niche definition in a hybrid zone, genotype by genotype, simply crystallises the problem of all species niche definition, which is always defied by anomalies in the behaviour of the individuals (Hutchinson, 1968; Emlen, 1975; Diamond, 1978). The data are unable to show competition between genotypes or sexes, but suggest that diet overlap means that competitive interactions could potentially occur if resources are depleted and that this would probably result in sika females exerting a density dependent effect on other genotypes.

There is still also the possibility that the first scenario, of selection on a physiological trait, is occurring despite other mechanisms of resource partitioning or competitive interaction. If such a locus were selected, we may expect to see patterns of hitch-hiking in the marker loci, and may expect different intensities of selection in the sexes, dependent on whether the locus affected early fecundity (females) or survival (males). As shown in Chapter 5, the clines in allele frequencies do indicate that the markers may be hitch-hiking on a strongly selected locus, or multi-locus trait, which would be consistent with this pattern. The possible fates of a population and of neutral markers under this type of selection could be modelled using computer simulation and the strategy is outlined in section 8.3..

8.2.4. The potential for a mosaic type structure in the hybrid zone

As there is evidence for plant community use differences between genotypes and sexes, there is perhaps potential for a mosaic structure to develop in the *Cervus* hybrid zone (Rand & Harrison, 1989). The current distribution of sika phenotype seems correlated closely to woodland distribution (Horwood & Masters, 1970; Ratcliffe, 1987a) and the large use of woodland type plants by sika females compared

to red females may reflect this. Deer use habitat for shelter as well as feeding and the importance of this may also differ between genotypes, though to date there is little data on this (Staines, 1974, 1976; Grace & Easterbee, 1979). Chadwick, Ratcliffe & Abernethy (in press) show a significant difference between red and sika females in their proximity to cover during feeding, with sika females remaining within 30m of forest edge, whilst red females readily move >100m into open habitat. The dispersion of a hybridising population in relation to habitat is mapped by Harrington (1979) and shows an association between the sika phenotype and woodland. This is perhaps evidence for the potential for development of habitat dependence in a mosaic zone, however in this population introgression is much more extensive than in Scotland and habitat genotype associations may not be strong enough to limit gene flow (Harrington, 1979, 1982).

As deer are known to exhibit very different ranging patterns and social groupings during the rut than at other times of the year (Clutton-Brock, Guinness & Albon, 1982; Davidson, 1979), there is potential for gene flow across the hybrid zone to be influenced differently to patterns which are discernible in the genetic composition of resident populations at other times. Many hybrid zones are now found to have a habitat-dependent component to their internal structure (MacCallum, 1994) and it is likely that this is the case in *Cervus*. Understanding the influence of habitat is likely to require sampling the distribution of genes both in winter populations where mortality and therefore selection is intense, and in breeding populations where distribution may change the potential barrier to gene flow.

8.3. Predicting the further spread of sika genes

The data presented in this thesis have raised many theoretical questions as to how genes behave after introduction of a founding population, and what parameters are crucial in determining their spread. A significant gap in our knowledge is an expectation of patterns in gene frequency typical of various selection regimes in expanding populations, to which we could compare empirical data. This section outlines a first strategy for simulating possible outcomes of introduction and gives preliminary results of the application of these ideas to the sika introduction.

8.3.1. Simulating the travelling wave

Exotic organisms are often introduced to new environments where they may hybridise with native species. Severe consequences have been documented for indigenous populations from the introduction of new predators, competitors or disease, (Atkinson, 1989) but less research has focused on the introduction of hybridising fauna (Conway, 1988). Introductions of exotic fauna are becoming increasingly frequent and the need to assess and understand their genetic impact is correspondingly important. Genetic consequences of introductions depend largely on the number of loci and fitness differences involved in selected traits. Computer simulations of the spread of neutral and selected loci under various conditions could be used to generate expected clines in gene frequency under various models of selection and dispersal, and including details of the mating system and population density. These would then be a useful tool in interpretation of observed clines in post-introduction gene frequency.

Directly selected advantageous alleles spread as Fisherian waves (Fisher, 1937), initially held in association by linkage disequilibrium with neutral loci dragged (hitch-hiking) behind them (Maynard Smith & Haigh, 1974; Hedrick, 1979; Baird, 1994; Figure 8.3.1). As recombination dissociates neutral loci from the selected traits, they cease to increase, but merge into the population by diffusion. We assume that some sika alleles may have been deleterious in the new environment or genetic background, but that these would have been rapidly lost from the population.

Post-introduction allele frequencies and the behaviour of waves of advance could be simulated initially for nuclear and mtDNA at the extremes of a single selected locus or an infinite number of selected loci. and resultant waves compared to empirical data by maximum likelihood. Bounds of generation time, dispersal distance and reproductive capacity applicable to sika deer in Scotland would be applicable to the analysis, though these could in theory be tailored to meet the requirements of modelling other populations.

In deer, as males disperse further (Davidson, 1973, Abernethy, 1994), neutral nDNA will diffuse more rapidly through the population than mtDNA. This effect could be countered by differing selection pressures on the nDNA and mtDNA, or between the sexes. If sika females have relatively greater fecundity than males, mtDNA frequency will increase faster than nDNA. If however they are less fit than males, mtDNA will increase more slowly.

So far I have attempted to explain the advance of nuclear genes, but simulations of the fate of the mitochondrial genome could also be a useful tool in

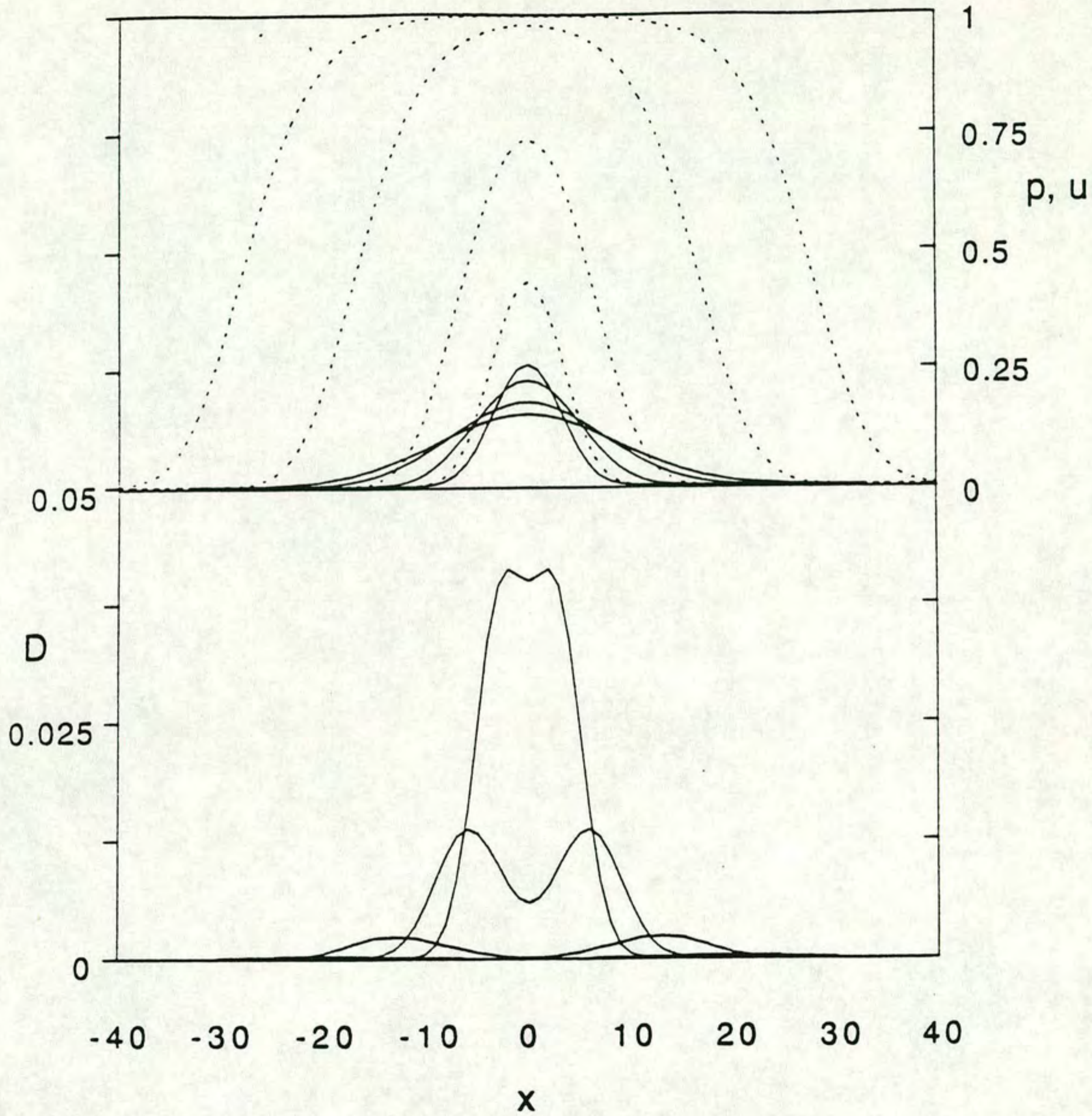


Figure 8.3.1. The wave of advance of a selected allele (upper figure; broken lines), a neutral marker hitch-hiking with this (upper figure; solid lines), and the linkage disequilibrium between the pair of loci (lower figure). Curves are given for t = 20, 40, 80, and 120 generations after introduction. The initial frequency of the introduced genes was 1% in the central deme, and selection and recombination rates are set at 10%. Figure redrawn from Baird (1994).

understanding the dynamics of the zone, especially in conjunction with models of nuclear spread (Baird, 1994). At the time of introduction, sika mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) must both have been of equal frequency and distribution. Two forces influence their spread; dispersal and selection. As mtDNA is carried only in the matriline, but nDNA is biparentally inherited, the effective dispersal distances and selection strengths on them may differ. Any differences in the wave forms for mtDNA and nDNA give us information on selection and dispersal differences between the sexes. Genes could spread by asymmetric sexual selection favouring sika (Lande, 1981). Assortative mating is acknowledged as a possible contributory factor to the rate of sika spread and to the linkage disequilibria generated in the hybridising populations.

8.3.2. Initial comparisons of *Cervus* data to the models.

The observed cline in sika gene frequencies does not fit the Fisherian form (Baird & Abernethy, in prep.). The strength of linkage disequilibria and heterozygote deficit in the hybridising populations can be used to infer that overall selection must be quite high (Mallet *et al.*, 1990), sufficient to form the wave rather than being swamped when initially at low frequency. If the markers were experiencing direct, strong selection we would expect them to conform to a Fisher wave, so we reject the hypothesis that the marker loci are under significant direct selection, which would have been highly unlikely anyway. We propose that the markers may be selectively neutral, or under negligible direct selection, but are hitch-hiking on a selected attribute.

The behaviour of hitch-hiking markers is currently being simulated, again for the two extremes of a single strongly selected locus or an infinite number of selected loci (Baird & Abernethy, in prep.).

In these early simulations, a mean dispersal distance is used. However we know that there is differential dispersal between the sexes and it is possible that there is also differential selection on them. A problem is to separate the effects of these two seemingly confounding variables which influence wave dynamics. We could simulate the wave that we might expect if the only difference between sexes was dispersal distance, using empirical dispersal estimates and equalising selection. Given the noise in dispersal we may not be able to say much, but perhaps we may say that it cannot alone account for the patterns and that some different selection pressure on males and females may be required to explain the data.

8.3.3. Discussion

The hypothesis that the marker alleles are selectively neutral and hitch-hiking on a polygenic selected attribute is supported by these early models and appears so far to be the best explanation of the both the genetic and quantitative data, though it is not exclusive of other selection regimes.

Increasing numbers of selected genes in the complex will decrease the selection coefficient of each of them but increase the chance of linkage to neutral loci. In the sika case, the neutral marker loci may currently appear concordant because there has been insufficient time to separate them, even if they are linked in differing degrees to the selected attribute. Clearly if linkage differences are insufficient to separate marker clines before they are dissociated from the selected attribute itself, then no difference in their behaviour will be perceived under the two scenarios.

The spread of "sika" oversimplifies the impact of this introduction as both phenotype and genotype need to be considered. Firstly, sika have one or more genetic attribute that is highly selected in Scotland and likely to sweep quickly through the cervid population. If a large number of genes are involved, clines are unlikely to have advanced much beyond the neutral marker loci as individual selection coefficients will be weak. In this case the apparent neutrality of phenotype may be misleading, as sampling error in what is inherently a noisy measure may mask its association with the selected attribute.

If a small number of genes are involved, therefore with higher individual selection coefficients, it is likely that they have advanced considerably further, yet no change in phenotype has been perceived beyond the cline in neutral markers. This would make it more credible that the components of phenotype assessed in the field are indeed unselected. It becomes also increasingly likely that recombination will dissociate them from the hitch-hikers and the sika phenotype will be broken down slowly by a process of diffusion and recombination. In Wicklow, Ireland a population of red and sika which is thought to have been hybridising for around 10 generations longer than the Argyll population shows much more phenotypic variation (C. McLean & E. Tappin, pers. comm.), which is consistent with this proposed pattern of introgression.

Researchers in New Zealand have mapped 200 DNA loci in cervids (M. Tate, pers. comm.) which will allow us to test these predictions in the field. By choosing molecular markers to locate one (or more) per chromosome we can examine the advances of loci of known linkage and infer their selection coefficients. Our

expectation is that loci in or closely linked to the selected trait will advance as a Fisher wave, that most loci will behave in a similar fashion to the four neutral markers already investigated and that loci weakly linked to the selected trait will travel at an intermediate rate. We expect the overall increase in relative fitness to be approximately twofold, corresponding to our selection coefficient of 0.9.

Laboratory selection of divergent lines has been used to map quantitative trait loci (QTL) by their linkage to marker loci in additive models (Nuzhdin, Keightley & Pasyukova, 1993; Keightley & Bulfield, 1993). Changes in specific phenotypic traits, in relation to frequency changes in a dense array of mapped marker loci in this system could provide insight into the position of and selective pressure experienced by various QTL in nature. This will go a long way to strengthening predictions of how the red deer phenotype may be affected in the future.

8.4. Conclusions

Clearly the simplest results of this introduction have been the increase of sika deer numbers in Scotland and the introgression of sika genes to the red deer genome. It seems highly unlikely that any mainland red deer population may remain free of sika genes in the future, however the extent of their effect on the red deer *phenotype* remains to be seen.

The present genetical structure of the Argyll and Great Glen populations, together with computer simulations of selection on introduced genes, suggest that some trait of sika is highly advantageous in Scotland and is spreading fast, but that it is not affecting a prominent phenotypic trait. The behaviour of marker loci and the linkage disequilibrium in populations just behind the wave of advance of sika genes is consistent with predictions from simulations of hitch-hiking on a polygenic selected trait and it is likely that many sika genes are now disassociated from that trait and will spread by diffusion into the population. Irish sika-red populations, which have been hybridising for longer than the Scottish ones show wider phenotypic introgression, which is consistent with expectations and seems the likely fate of Scottish populations.

The possibility that genotype-habitat associations will limit gene flow between red and sika seems unlikley. The evidence from Wicklow suggests that habitat associations may slow down hybridisation and may even maintain genetically distinct taxa as in other older hybrid zones (MacCallum, 1994; Gollman, in press, Nurnberger et al., in press) but do not indicate that associations are strong enough to allow the taxa to retain their current genetic identities.

The feeding ecology of the Argyll population shows that there is clear potential for competition between the genotypes, most especially between sika females and hybrid males and suggests that sika females would be the superior competitor. Relative sizes of the taxa and sexes are unable to fully explain diet selection and it is likely that other factors, such as shelter requirements may affect use of habitat and perhaps also affect relative nutritional requirements. Hybrids appear to fall between the parental taxa

8.5. Future management of mixed sika-red populations

8.5.1. Management for damage control

Sika deer, like red deer are a threat to commercial and natural forestry through overbrowsing, though deer in low numbers are an integral part of the highland woodland ecosystem. Management of sika for control of damage follows essentially the same lines as control of red deer, through culling and fencing. Culling regimes have been widely researched in recent times and the population demography of red deer under various culling pressures has been well documented (Ratcliffe, 1987b, 1988; Clutton-Brock, 1994). The response of sika to selective culling pressures is as yet unresearched, but likely to be similar to that of red deer, and so management plans for mixed populations can be formaulated as those for red deer. Goals being set as target population densities to allow tree growth. The biggest problem in defining management goals is likely to be in assessing the acceptable density of sika, which may differ from that of red deer if damage patterns differ. For instance, there is some evidence that sika may strip bark from trees at a higher rate than red deer (Bennetsen, 1982), but this has not been related to densities of sika and may not be controllable simply by lowering numbers.

8.5.2. Conservation genetics ?

Concern has been voiced in many places over the implications of sika hybridisation for the conservation of red deer (Ratcliffe, 1987a; Callander & MacKenzie, 1991; Red Deer Commission, 1993; Scottish Natural Heritage, 1994). Indeed such concern was a motivating force for this study. Defining the management goals for populations to be 'conserved' is far more dificult.

The Scottish deer herd is genetically different from other European populations (Gyllensten et al., 1983; Linnell & Cross, 1991), but not more so than other local populations differ from each other across Europe. It has been subject to many restocking events, the origins of which are often unknown (Whitehead, 1950, 1964), and is likely to contain at least some genes that originated outside Scotland fairly recently (more recently than the separating of Britain from the European land mass). It has also been subject to huge selection pressures to adapt to loss of woodland habitat over the last three centuries. Thus it is not the same genetic stock as it was when red deer arrived in Scotland, in fact, then it was probably closer to sika than the present day descendants (see Chapter 2).

However, despite its history, the Scottish deer herd has now a recognisable identity, different from the Japanese sika, and its appearance as 'the Monarch of the Glen' is an important part of the Scottish heritage, as well as a marketable asset to Highland estates.

The fact that sika are performing well in Scottish woodlands is the result of natural selection on some trait they possess and this is likely to benefit the red deer population through hybridisation, as a universally favourable trait will spread to fixation in a population even with low levels of gene flow (Fisher, 1937). An introduction is equivalent to the sudden arisal of many mutations in a population and increases genetic diversity in the local population, both through increased numbers of polymorphic loci and increased variability within loci. This is likely eventually to lead to increased phenotypic variation in the population, as has been seen in the Wicklow herds, and increased opportunity for adaptation. This may be seen as a good thing by some managers.

However, increased phenotypic variability may also equate to loss, or reduction to very low frequency, of the phenotypes we now see: the demise of the 'Monarch of the Glen' and the loss of the strains of sika now represented in Britain.

In managing mixed populations the first problem is to decide what the outcome of the management plan is, and is this is realistic given the present population. The genetic structure of the Scottish populations suggests at present that some sika genes are likely to sweep through the entire mainland deer population quite quickly, but that obvious phenotypic introgression is much slower. If in fact it is the phenotype, based on the majority of the genome remaining together, rather than the absolute genetic integrity, that is the goal, then management through increased selection against hybrids (culling) may acheieve this for several decades. If absolute genetic integrity of red deer is the goal, then only island populations of red deer are likely to survive, and even these will have to be protected against later sika introductions.

Acknowledgements

I am, of couse, in the debt of many people for their help, encouragement and advice during the last three years.

The work in this thesis could never have been done without the help of the Forestry Commission staff in Loch Awe, Kintyre and Knapdale and Fort Augustus districts and the Wildlife and Conservation Research Branch at Ardentinny. To them I extend special thanks for hours of hard work in sample collecting. I am particularly grateful to Andy Chadwick and Colin Lavin for helpful discussions and suggestions in designing the sampling programme.

Madan and Monica Oli, besides being my friends through the last three years gave me enormous help with the fibre analysis programme, for which I thank them.

My supervisors, Nick Barton, Andrew Illius and Phil Ratcliffe, have always been supportive, have given advice readily and have put up with my demands on their time with remarkably good humour. I am grateful for their interest and hope they have enjoyed being involved in this project.

I carried out most of the lab. work in Josephine Pemberton's lab in Cambridge and Mike Bruford's lab. in London. Both welcomed me, fitted me into busy schedules and were encouraging and interested throughout my stay. Thanks go especially to Josephine and Mike, but also to all the other members of those groups, who put up with my incessant questions and explained lab. techniques to me with infinite patience.

The work was originally inspired by visits to Taynish NNR in Argyll in 1990, where I saw and heard my first sika deer and became interested in their biology. Since then I have spent many happy hours studying them in more detail and my thanks go to John Halliday and Bettina Vettori for their constant hospitality on the west coast. Likewise I thank Dominic White for hospitality on visits to the Great Glen.

I have been very happy in Edinburgh, despite the rigours of thesis writing, and thank all of the people here who have helped make life fun. I also thank my family who have always been understanding of the pressures of work and have been encouraging throughout.

Thanks to Colleen Covey, Colin McLean and Lee White for use of their photographs in the thesis.

I thank the Natural Environment Research Council, the University of Edinburgh and both my parents for financial support.

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Appendices for Chapter 4.

Appendix 4.2a

DNA Extraction

To extract genomic and motochondrial DNA from animal tissue

Day 1. (or morning)

Preprepare; Incubator @ 37°C or 55° C — *pestle and mortar

Scalpel — *Flask of liquid N_2

Petri dish

1 x TNE buffer

00% ethanol and tissues

25% SDS (sodium dodecyl sulphate) solution

15 ml tubes (1 per sample) containing:-

3ml 1 x TNE

690µl dH_2O

300µl Tris HCl pH 7.5

10µl proteinase K @ 10mg/ml

a) Take a 500µm sample of tissue.

b) Chop finely to a jelly with scalpel on petri dish.

OR

b) Cover in liquid nitrogen in pestle and mortar. } better for

c) Grind to powder. } tough tissue.

N.B. if the spatula is also frozen in liquid N_2. it helps prevent powder sticking.

d) Add to preprepared TNE/proteinase K tube.

e) Wash utensils and wipe with alcohol before next sample.

f) Prepare all samples to be extracted as above.

g) Add 100µm 25% SDS to each tube.

h) Incubate at 37°C overnight (or 55°C for 2 hours).

Day 2. (or afternoon)

Preprepare

Equilibrated phenol : chloroform: isoamyl alcohol (25:24:1)

Chloroform : isoamyl alcohol (24:1)

100% ethanolß

70% ethanol

4 x15ml tubes per sample

1 eppendorf per sample

1 x TE buffer or dH_2O

Wide bore pipette tips

If there is a lot of Hb present, do a PHENOL only extraction prior to phenol chloroform, use same method i) - l)

i) Add 4ml phenol : chloroform to each tube that has digested to a liquid with no tissue lumps. Leave others to digest to this stage before adding p : c, then continue.

N.B. there may be white lumps of fat visible - these are lost at the first phenol clean.

j) Mix tubes gently.

k) Spin at 3700rpm. for 10 mins.

l) Pipette off top layer **avoiding interface**.

m) Repeat i) -l) .

n) Repeat i) - l) using 3ml chloroform instead of phenol: chloroform.

o) Add 6ml 100% ethanol to each tube and swirl to precipitate DNA. It should appear as a white, woolly pellet.

N.B. if pellet is invisible, spin at 3700rpm for 5 mins then gently decant off ethanol. Add 1 ml 70% alcohol to wash, resuspend and spin down as above, decant off alcohol and air dry. Add 0.5ml, 1 x TE buffer to tube to store.

p) Remove DNA pellet on a fine glass rod and wash gently in 70% ethanol.

q) Transfer pellet to eppendorf containing 1ml 1 x TE buffer, or dH_2O for short term storage.

r) Label and store @ 4°C.

Appendix 4.2b

Agarose DNA Checking Gels

To check quality, size and presence of DNA after a procedure like extraction, PCR, digestion etc..

Preprepare 0.5 x TBE buffer
Gel mould with suitable well-forming combs
Agarose
5 x TBE Bromophenol-blue loading buffer
dH_2O
Lamda Hindi III DNA standards for checking extraction quality and conc.
or 123bp DNA ladder for checking PCR or digestion fragment sizes
10mM Ethidium Bromide bath (in fume cupboard)
UV camera and transluminator

a) Measure out enough 0.5 x TBE buffer for gel.to fill mould.
b) Add agarose powder for correct percentage (0.7-1.0% w:v).
c) Microwave (or heat over a bunsen) to boil and clear (~3 mins).
d) Cool to hand hot and pour.
e) **When gel is set** remove combs and place gel in tank with enough 0.5 x TBE to cover it.
f) Take 1-3µm sample of DNA suspension (PCR or digestion product ,etc.).
g) Add 4-7µm loading buffer to each sample on a microtitre plate.
h) Add dH_2O to make up to well volume (check combs used).
i) Pipette entire content carefully into well, **noting sample loading positions,** and mixing by drawing through pipette tip.
k) Load 10um of each lamda standard in order, with Hindi III first, or a single well of 123bp ladder.
l) Connect to power @ 40mA, 80V and run toward **positive**.
m) Check dye to see length of run (dye runs beyond DNA). Usually 1-2 hours is enough.
n) Switch off and remove gel carefully.
o) Place in Ethidium bromide bath (0.5µm/litre) for 15-20 mins.
p) Remove WITH GLOVES and place on UV transluminator.
q) Focus and photograph. **Do not use UV for more than a few seconds**.

Appendix 4.2c

Buffers

'AC' Electrophoresis running buffer

Dilute this buffer to 1 in 20 to make the homogenisation buffer. i.e. 200mls buffer + 3800mls dH_20.

Citric acid (0.04M)	8.4 grams
N-(3-aminopropyl)morpholine	10 ml
dH_2O	1.0 litres

a) Add citric acid to water.

b) Wait until pH stabilizes.

c) Add N-(3-aminopropyl) morpholine with dropper until pH. 6.1 is achieved.

TE

1M Tris-HCl pH 7.5

0.5M EDTA (ethylene diamine tetra acetate)

TNE

50mM Tris-HCl pH 8.0

10mM NaCl

5mM EDTA

TBE

1M Tris-HCL pH 8.0

5M Boric acid

0.5M EDTA

Loading buffer

PARR-excellence™

Supplied by Advanced Biotechnology, 9, Hilltop Road, West Hampstead, London. Tel 071 625 4440.

Appendices for Chapter 6.

Appendix 6

Protocol 6A: Sieving and subsampling

Preprepare — Preserved rumen samples in 1% Formalin

Sieve stack of mesh apertures 4.75mm
2mm
1mm
0.5mm
0.25mm
0.15mm

Wet sieve shaking apparatus, i.e. *Endecotts* 'Octagon 800'

Drying ovens at 60°C

a) Thoroughly mix contents of preserved rumen sample.

b) Take 3 x 300ml subsamples of whole contents and place in aluminium trays.

c) Dry at 60°C to constant weight (for fibre content analysis) and record dry weight.

d) Wet sieve each of another 3 x 300ml subsamples through a stack of 6 sieves at 3mm shake amplitude for 5mins, 2mm for 5mins and 1mm for 5mins with the water at constant flow.

e) Carefully transfer the contents of each sieve to an aluminium tray. If need be wash material held in the mesh into the tray with a little water. Label and dry at 60°C to constant weight (around 48hrs drying).

f) Sieve a 300ml subsample of mixed whole rumen contents through a 1mm mesh sieve only. Use the same 15 min. shaking regime above.

g) Label and freeze for later botanical analyses.

h) When all samples are dried to constant weight, weigh dried material and store in sealed polythene bags.

Protocol 6B: Volumetric dry weight separation

a) For each sample sieve a 300ml subsample of mixed rumen contents through a 1mm mesh sieve as Protocol 6A.

b) Take 4 x10ml volume (blotted dry) subsamples for analysis.

c) For each subsample, tip into a standard grid tray (10 x 10, 20mm squares) and separate evenly across tray. Float the sample in 100ml water.

d) Record identity of each particle crossing or touching grid lines.

N.B. For particles crossing more than once, record *each* 'hit'; this compensates for large particles.

e) After three replicates, check consistency of data. If categories vary greatly (>10% difference in hits) do a fourth replicate.

f) For categories such as fungi where particle size is very large, remove *all* particles from tray and take a direct volume using displacement in a measuring cylinder.

N.B. to be accurate blot off all surface water before measuring.

g) Calculate volumes from regression curves and check it adds up to about 10ml.

Protocol 6C: Calculation of regression lines

a) Score subsamples of the rumen to be analysed and identify the species in it.

b) Collect fragments of each plant species found. Fragments should be collated from as many different animals as possible to a total volume of 10ml in each bulked category (see step 3).

c) Bulk together collections from as many animals as possible, whilst keeping different animals separate i.e. males, females, adult, juvenile, different species or populations.

d) Blot the fragments dry and then measure out 1ml volume by displacement.

e) Spread these in the SAME standard tray as was used for the scoring of the unknown composition sample.

f) Score as a sample with 3 replicates.

g) Repeat with 2ml, 3ml, 4ml volumes etc.... until a 10ml volume is tested.

h) Plot the three scores against each trial volume and calculate the regression coefficient for each species/animal type.

Protocol 6D: Dry weight botanical separation

a) For each sample sieve a 300ml subsample of mixed rumen contents through a 1mm mesh sieve.

b) For each sieved sample, take a 10g subsample (wet weight).

c) Spread the subsample over a tray in 100ml water and separate into its constituent parts, using forceps and a 12x lens. Plant fragments should be identified to species level if possible, or to categories such as 'grasses'.

N.B. This takes a lot of practice. At least 10 samples should be thoroughly sorted before any reliable data are taken and a test run on a sample of known composition should be tried to test observer limits.

d) Store fractions in paper and dry to constant weight (100°C for 8 hrs) before weighing.

e) Calculate percentage of the total sample represented by each component by totalling component dry weights to get the 100% sample weight.

Protocol 6E: Sequential Fibre Analysis Protocol

Day 1.(NDF)

a) Make up ND and AD reagents (as Appendices 6.4a&b).

b) Put two crucibles for each sample to be analysed in a 100°C oven for two hours.

c) Place (previously 100% dried) samples to be analysed separately in drying oven at 40°C for two hours

d) After two hours cool the crucibles in a dessicator for 20-30 minutes, then record their exact weights.

- This weight is 'CRUCIBLE 1'.

e) Place ~1g (record exact weight) of ground, dried sample into a 200ml refluxing (round-bottomed) flask . Each sample should be tested in duplicate.

- This weight is 'SAMPLE I'.

N.B. Record engraved label on flasks with sample no.(a&b), as ink labelling may be burnt off later.

f) Add 100ml of ND reagent to each flask.

g) Turn on the water to the condensers on the refluxing unit.

h) Place flasks in the refluxing unit.

i) Record the time each flask boils, reduce heat and let simmer for 1 timed hour.

j) Start 2-3 litres of distilled water boiling near the end of the hour.

k) Place the weighed crucibles on the filter manifold and turn on vacuum.

l) As each flask completes one hour boiling, pour as much of the contents into a crucible as possible.

N.B. some of the sample may have splashed into the condenser during refluxing. If so, wash it through into the flask with a little hot water and transfer to the crucible.

RECORD CRUCIBLE LABEL WITH SAMPLE NUMBER.

13. Open the vacuum to the crucible, increasing vacuum strength as required.

14. Repeat until the flask is empty.

16. Rinse the flask into the crucible with hot, distilled water until clean (Use a squeeze bottle).

14. Rinse the residue in the crucible with acetone twice. Vacuum off acetone.

15. Place the crucibles in 100°C oven overnight.

16. Place 2 extra crucibles for each sample in the oven for the ADF test .

Protocol 6E (continued)

Day 2.(ADF)

a) Cool ALL crucibles in a dessicator for 20-30 mins. and weigh.

b) Record exact weights of crucibles plus NDF residue from Day 1.

- This weight is 'SAMPLE II'.

c) Record weights and numbers of new crucibles.

- This weight is 'CRUCIBLE II'.

d) Working with one sample at a time out of the dessicator, scrape as much of the residue from the NDF crucible as possible into a scoop and weigh.

N.B. Be careful not to damage sinter base of the crucible as this will falsely inflate the weight of the subsample taken.

e) Record exact weight and transfer this subsample to a refluxing flask.

- This weight is 'SAMPLE III'

f) Record flask label and sample number

g) Add 100ml AD reagent to each flask.

h) Turn on the water to the refluxing unit.

i) Place the flasks in the refluxing unit.

j) Record the time each flask boils and boil for one timed hour

k) Near the end of the hour put on 2-3 litres of distilled water to boil and turn on the vacuum to the filter manifold. Place clean, weighed crucibles on the filter manifold.

l) As each hour is completed pour and rinse the contents of the flask into a crucible as for the NDF assay.

RECORD CRUCIBLE LABEL WITH SAMPLE NUMBER

m) Rinse the residue with acetone twice and vacuum off.

n) Place the crucibles in 100°C oven overnight.

o) Make up 1 litre 72% sulphuric acid for Day 3.

Protocol 6E (continued)

Day 3.

a) Cool crucibles and ADF residues in a dessicator for 20-30 minutes and weigh when cool. Record exact weights.

- This weight is 'SAMPLE IV'.

b) Place each crucible in a glass crystallizing dish in safe place, like a fume cupboard.

c) Place a glass stirring rod in each crucible and add and mix in about 15ml of 72% sulphuric acid

d) Add more sulphuric acid until the crucible is half full.

e) Leave at room temperature for 3 hours stirring every half hour and topping up if the acid drains out.

f) Near the end of 3 hours start 6 litres of distilled water boiling.

g) After 3 hours put crucibles ONE BY ONE on a 1 litre Buchner flask with a rubber cup and vacuum off remaining acid. This should be very little.

MAKE SURE ALL ACID IS DRAINED OFF BEFORE YOU ADD ANY WATER TO A CRUCIBLE OR FLASK

h) Fill crucible with COLD distilled water and vacuum off, then wash residue 5 or 6 times with hot distilled water.

N. B. Do not rinse with acetone.

i) Place crucibles in 100°C oven overnight.

Protocol 6E (continued)

Day 4.

a) Cool crucibles in a dessicator for 20-30 mins. and weigh. Record exact weights.

- This weight is 'SAMPLE V'.

b) Place crucibles in furnace and set at 500°C.

c) Leave to burn to ash overnight or during 8 hour day if possible.

Day 5.(or later on day 4. if possible)

a) Switch off muffle furnace and leave to cool an hour.

b) Take out crucibles after an hour and cool 30-45 mins in a dessicator. Weigh. Record exact weights.

- This weight is 'SAMPLE VI'.

N.B. Ash is very light and care should be taken not to expose crucibles to draughts, including opening the furnace too soon or too quickly.

c) Clean crucibles for next set of tests.

d) Make the following calculations of fibre contents for each replicate of each sample.

CALCULATIONS (If samples are not weighed fully dry a correction for dry matter content should be made)

$$\% \text{ ASH} = \frac{(\text{SAMPLE VI - CRUCIBLE II})}{\text{SAMPLE III}}$$

$$\% \text{ NDF residue} = \frac{(\text{SAMPLE II - CRUCIBLE I})}{\text{SAMPLE I}}$$

$$\% \text{ ADF residue} = \frac{(\text{SAMPLE IV - CRUCIBLE II})}{\text{SAMPLE III}}$$

$$\% \text{ LIGNIN} = \frac{(\text{SAMPLE V - CRUCIBLE II})}{\text{SAMPLE III}}$$

Appendix for Chapter 7.

***** Principal components analysis *****

*** Latent Roots ***

1	2	3	4	5
40023	29326	9504	3907	2371

*** Percentage variation ***

1	2	3	4	5
43.86	32.14	10.42	4.28	2.60

*** Latent Vectors (Loadings) ***

	1	2	3	4	5
Cvul	0.86610	0.14632	0.25788	-0.25203	0.01338
Ecin	-0.00967	0.00501	-0.00861	0.03655	0.00221
Vmyrt	-0.01915	0.03076	-0.05131	0.43647	0.68885
Conif	0.00167	0.01091	0.00220	0.04443	-0.10248
Birch	-0.00716	-0.01411	-0.07063	0.42822	-0.60583
Mgale	-0.05899	-0.02730	**-0.75137**	-0.53185	0.04051
Wood	-0.00729	-0.00705	-0.00595	0.04228	-0.06535
bark	-0.00366	0.00239	0.00127	0.00198	-0.00543
grass	**-0.44966**	0.61342	0.42714	-0.35035	0.00809
Nsedg	0.02173	-0.00286	-0.07322	0.00014	0.03020
Bsedg	-0.01333	0.00246	-0.01299	0.03210	-0.01072
Juncus	0.00790	0.01068	-0.00423	-0.00115	-0.04813
Luzula	-0.01852	0.00220	-0.01838	0.14957	0.25436
Lichens	-0.03077	0.03187	-0.02983	0.05749	-0.18047
Moss	0.00046	-0.00344	0.00606	-0.00230	0.00570
ungi	-0.00025	-0.00031	0.00057	-0.00057	-0.00068
Tbryoph	-0.03059	0.02813	-0.02317	0.05456	-0.17550
Gsax	-0.00014	0.00028	-0.00111	0.00132	-0.00110
otherher	-0.02357	0.01085	-0.04185	0.00961	0.05284
Fernroot	**-0.19188**	**-0.77248**	0.41174	-0.30073	0.03565
Fernleaf	-0.05016	-0.03777	-0.00911	0.17670	-0.07535
other	-0.02793	-0.00002	0.00622	0.02106	-0.02390

Publications arising from the thesis

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551–562

(145)

Molecular Ecology (1994) 3, ~~000–000~~

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 ?? ???? 1993; revision received ?? ???? 1994; accepted ?? ???? 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 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).

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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, cytonuclear 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

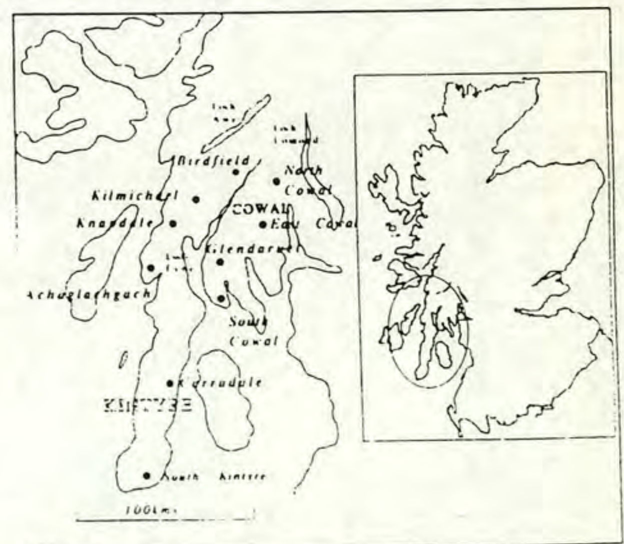

Fig. 1 The study area in Argyll, Scotland, showing the nine forests sampled (named sites) on the Kintyre and Cowal peninsulas.

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_2O) 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 *Msp*I, *Hin*fI and *Hae*III. 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 reduced 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

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

of polymorphism in the sika population could be a result of the small number of founder animals. Animals were 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 [illegible],

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 loci, measured by maximum likelihood (Barton & Gale

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	8	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

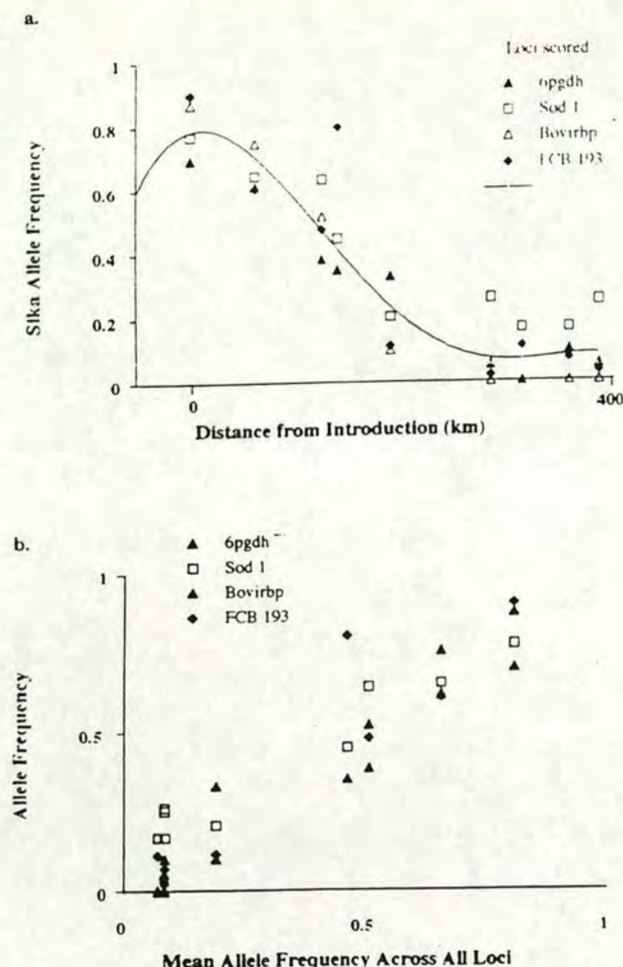

Fig. 2 Sika nuclear allele frequencies at each locus. (a) plotted against overland distance along the transect. The curve is fitted to the mean frequency. (b) plotted against mean sika allele frequency across all loci. The cline for each locus is concordant with all others. Heterogeneity of slope, $a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

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 ($\chi^2 = 1.89$, 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 estimates are too large to draw robust conclusions from

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 ($\Delta \log L_5 = 4.11$) 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

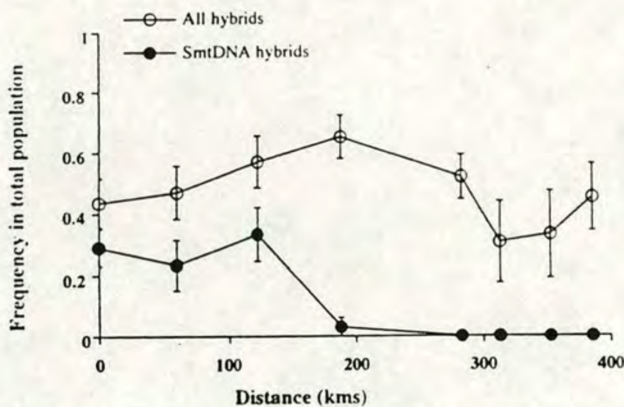

Fig. 3 Hybrids were assessed as those animals possessing alleles from both taxa or a mtDNA haplotype differing from their nuclear genotype. The proportion of hybrid animals in each population is shown by the open circles. Hybrids possessing sika mtDNA are shown in closed circles, also as a fraction of 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.

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

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

	Locus			
Forest	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*

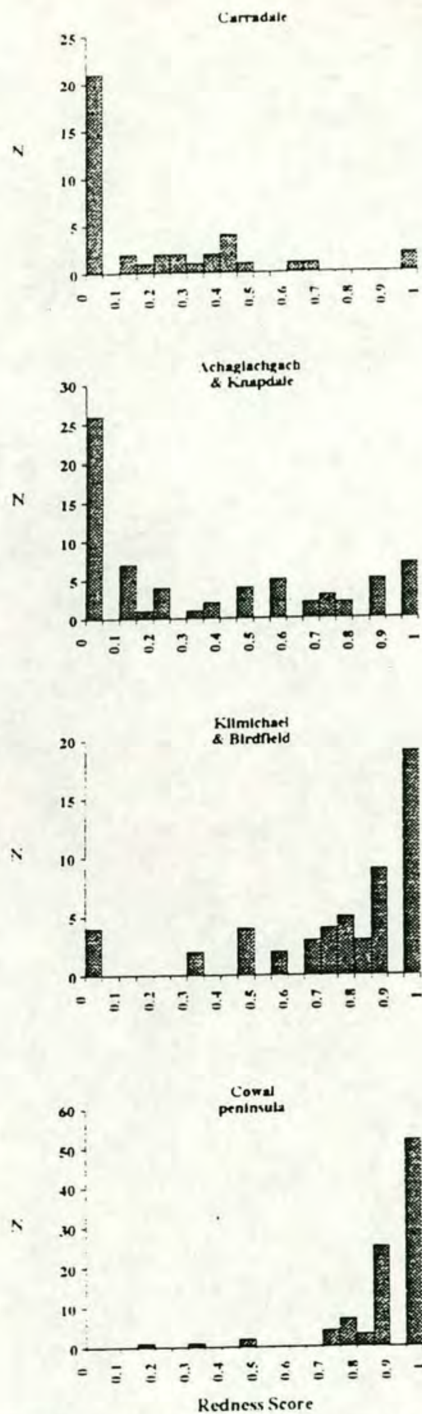

Fig. 4 The distribution of individuals across a hybrid index, described above. 'Pure' red individuals score 1, 'pure' sika individuals score 0. Scores are pooled across populations dependent on mean sika alleles frequency. From top; $P = 1.0–0.75$, $P = 0.749–0.5$, $P = 0.499–0.15$, $P = 0.149–0.0$. 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.

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 unidirectional (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 (cyto-

plasmic 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

I thank the Forestry Commission staff who carried out the sampling and gave advice: Alistair Strang, Stuart Downie, Derek Farrar, Alisdair Cameron, Bob Blackwell, John Sweeney, Alistair Clarke, Davey Sutherland, Hugh Gibb, Stan Strange and especially Andy Chadwick and Phil Ratcliffe. I also wish to thank Drs. Josephine Pemberton, Mike Bruford and Heather Hall for help with laboratory techniques and accommodation in their busy laboratories. Drs Nick Barton, Josephine Pemberton, and three anonymous reviewers gave helpful comments which much improved the manuscript. The work was funded by the Natural Environment Research Council.

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