The Genetic and Phenotypic consequences of translocations of deer (Genus *Cervus*) in Scotland

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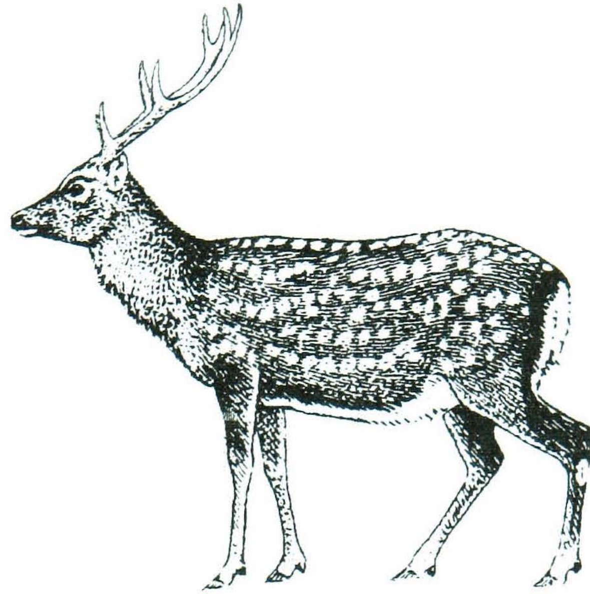
To
Lisbeth and Rory



"The Japanese are a most satisfactory little deer.."

Viscount Powerscourt (1884)

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Abstract

Asian sika deer (*Cervus nippon* Temminick, 1836) were introduced across Scotland at the turn of the last century and have since established several large feral populations. Their potential to cause economic damage to forestry and hybridise with native red deer (*C. elaphus* L. 1758) has provoked widespread concern and highlighted a lack of basic ecological and genetic data relevant to their management. A preliminary genetic investigation of two Scottish populations (Great Glen & Argyll) revealed introgression at both sites. The aim of this thesis is to expand and develop this work by investigating the genetic and phenotypic causes and consequences of hybridisation across Scotland.

Tissue samples and cull data were collected from 670 red deer, 266 sika and 8 phenotypic hybrids originating from 9 areas around Scotland with documented sika introductions. The samples were genotyped for 10 microsatellite and one mitochondrial DNA (mtDNA) marker loci. Skeletal material and genetic samples were collected from a further 339 red and 307 sika from Argyll in an intensive study involving an additional 15 microsatellite markers. All markers used were putatively diagnostic, though the interpretation of some rare alleles was problematic.

Samples with several introgressed alleles at different loci provided strong evidence for hybridisation since introduction in Argyll, Galloway and Sutherland and Ross-shire, but different patterns of introgression emerged. In Argyll, hybridisation is rare but followed by repeated backcrossing to give substantial introgression, with 62% of sika-like deer and 33% of red-like deer containing opposite-taxon alleles. Evidence of recent hybridisation was restricted to sample sites at the leading edge of sika colonisation. In Galloway a number of early-generation hybrids were identified. In Sutherland and Ross-shire, introgression was common (>75% of individuals) in sika-like deer at 5 of the 10 microsatellite loci but almost absent at the other loci, suggesting a hybridisation event(s) several generations ago followed by backcrossing and strong assortative mating. Introgression was uncommon in the remaining sika-like populations (<20% of individuals) and rare in red-like populations (<5%), but most red-like populations contained rare alleles whose origins were difficult to interpret. These alleles may represent polymorphism, introgression from unreported sika introductions or introgression from introduced wapiti (*C. canadensis* L. 1758). Across all samples, mtDNA introgression was rare and restricted to Argyll sika-like deer having red mtDNA. Furthermore, all recently generated hybrids contained red mtDNA, suggesting hybridisation events usually involve sika males covering red females.

Genetic distance measures were calculated for populations following the removal of introgressed alleles. Neighbour-joining trees revealed no consistent pattern between hybridising and non-hybridising populations. The Borders sika were confirmed as the most genetically differentiated sika population in Scotland.

The identification of hybrids by stalkers was generally poor and restricted to the most recent backcross offspring. Only 2% of the genetically identified hybrids in Argyll showed any obvious visual sign of introgression and around the rest of Scotland, only one sample was correctly identified by the stalker as a hybrid. Detailed measurements of several phenotypic traits confirmed this pattern and after several generations of backcrossing, hybrids and non-hybrids could not be differentiated reliably, even though there were significant increases in some sika-like hybrid measures.

Hybridisation has occurred, and continues to occur, between red and sika across Scotland. The existence of hybrids in an area did not correlate well with the physical size of local red-like and sika-like deer. Similarly, there was no evidence that particular genetic strains of red or sika were more likely to be involved in hybridisation. However, there does seem to be an association with the colonisation process of sika stags. Why this has happened in some cases but not all remains unclear and may be associated with forest design. Nevertheless, there is good evidence that strong assortative mating preferences exist where mate choice is available and that backcrossed offspring quickly attain the parent taxa phenotypes. Under these circumstances it will be difficult to prevent the introgression of opposite taxa alleles by selective shooting.

1 Introduction

1.1 General introduction

1.1.1 Hybridisation

Hybridisation is a common natural process which occurs when individuals from genetically distinct populations interbreed to produce fertile offspring (Barton & Hewitt, 1989). The issue of hybridisation is of great importance in two areas: in the identification, classification, and conservation of particular species (Porter, 1990; O'Brien & Mayr, 1991; Grant & Grant, 1992; Hodder & Bullock, 1997), and in understanding the fundamental evolutionary processes involved in speciation (Hewitt, 1988; Barton & Hewitt, 1985, 1989; Johnson & Wade, 1996; Arnold, 1997).

Under the strictest definition of what differentiates species from populations [reproductive isolation; i.e. the Biological Species Concept (Mayr, 1942)], hybridising populations cannot be described as separate species. However, a more flexible approach to the species concept is often adopted (see Avise, 1994 & Arnold, 1997) and "hybridisation" is most commonly used to describe inter-breeding between species. In this thesis, I shall adopt the second convention, the implications of which shall be discussed in the final chapter (Chapter 8).

Where two distinct taxa meet and interbreed along a narrow geographical region, they can form what is known as a hybrid zone (Barton, 1979). Studying these populations can be of wider scientific value as they provide a rare opportunity to investigate the key evolutionary processes involved in divergence and speciation, and how alternative adaptations spread in populations (Hewitt, 1988; Barton & Hewitt, 1989; Johnson & Wade, 1996). The analysis of these zones can also give reliable estimates of the selection pressure acting on different traits, the

rates of gene flow between the taxa and the number of loci involved: parameters which are otherwise difficult to measure (Barton & Hewitt, 1985).

1.1.2 Conservation implications

There are two principle reasons why hybridisation has been seen as a negative phenomenon in the context of establishing and achieving conservation objectives. The first is that, following the translocation of species, native populations can be swamped with exotic genetic material that is poorly adapted to the local environment, resulting in a lowered fitness of the native species, i.e. outbreeding depression (Templeton, 1986; Ellstrand, 1992; Levin *et al.*, 1996). This is of particular importance in the context of small, already threatened, species where any adverse affect on average fitness could have a significant effect on their viability (Grant & Grant, 1992; Levin *et al.*, 1996). Although the documentation of such extinction events is rare, there are several examples of where significant decreases in fitness have been described in both plant (see Levin *et al.*, 1994) and animal [e.g. mammals (Davidson *et al.*, 1999); fish (Dowling & Childs, 1992 and bird (Deon *et al.*, 1994)] species.

The second primary concern is that the transfer of genetic material from one taxa into an other (introgression) is undesirable because it destroys "genetic integrity" (e.g. Abernethy, 1994b, Lorenzini & Fico, 1995), the assumption being that there is some intrinsic conservation value in preserving the current genetic differences between taxa (Balharry, et al., 1994). In this case the actual consequences (e.g. changes in average fitness) of introgression are less important than the principle that genetic "purity" (i.e. phylogenetic history) should be conserved. It is however, rarely possible to quantify "genetic purity" as there are no established criteria for differentiating species on basis of relative genetic distance. This in turn creates practical problems regarding the formulation and enforcement of conservation legislation, as has happened, for example, with the red wolf (which appears to be a recent coyote-grey wolf hybrid;

Wayne & Jenkins, 1991) under the U.S. Endangered Species Act 1973 (O'Brien & Mayr, 1991; Brownlow, 1996).

Particular difficulties can arise when the "pure" parental reference populations are not available (e.g. where one population is already introgressed) and in this situation it may only be possible to identify recent hybridisation within the last few generations (Goodman *et al.*, 1999). This has proved a particular problem in the formulation of conservation strategies of two of Britain's native carnivores, the polecat (*Mustela puorius*) (Davidson, *et al.*, 1999) and Scottish wild cat (*Felis silvestris*) (Hubbard *et al.*, 1992). This issues will be discussed in more detail in Chapter 4.

In Scotland, frequent translocations of deer (Genus *Cervus*) have affected the ecological and genetic status of the indigenous red deer (*Cervus elaphus* L. 1758) by hybridisation (Goodman *et al.*, 1999). Of primary concern have been the several introductions of Asian sika deer (*Cervus nippon*, Temminick 1836) (Ratcliffe 1987a; Balharry *et al*, 1994; Scottish Natural Heritage 1994; DCS, 1997), but other translocations of Canadian wapiti (*C. canadensis* L. 1758) and various red deer subspecies and their hybrids have also occurred (Ritchie, 1920; Whitehead, 1964). This situation presents a challenge to those involved in the stewardship of Scotland's natural heritage and highlights the need for basic ecological and genetic data (Balharry *et al.*, 1994), so that informed, practical and effective deer management practices can be established across Scotland (Ratcliffe, 1987a; Abernethy, 1994a).

An initial genetic and ecological investigation of a hybrid zone between red and sika deer in Argyll, Scotland, highlighted the potential benefits of just such an investigation (Abernethy, 1994a, b; Goodman *et al.*, 1999) and provided a platform for the work described in this thesis.

1.2 The Genus *Cervus*

This section aims to give a brief description of the appearance, classification and genetic background of the three *species* relevant to this study (red, sika and wapiti) and does not deal with the other species within the genus. More detailed and extensive background information is available elsewhere and was reviewed by Abernethy (1994a).

1.2.1 Phenotype

Detailed descriptions of red, sika and wapiti can be found in Whitehead (1972, 1993), however this section aims to summarise the main features of the three species.

The most obvious differences between Scottish red, sika and wapiti are body size and coat colour. Although there is a wide geographic and temporal variation in body size within taxa, sika deer are generally much smaller and stockier than red in all habitats and wapiti are much larger. The winter carcass weights (head, lower legs and internal organs removed) of British male sika are approximately half those of red (32 kg and 56 kg respectively; see figure 7.1) and their shoulder height is around 40cm lower (65-85 cm in sika and 120-150 cm in red) (Abernethy, 1994a; Ratcliffe, 1991). However, some Japanese sika populations may be much larger, reaching more red-like proportions (Whitehead, 1993). Wapiti male carcass weights are about double, or more, those of red deer males and they stand around 150cm at the shoulder (Whitehead, 1993).

In the summer months sika develop a reddish brown coat scattered with distinct yellowish-white spots running parallel to a dark dorsal stripe, whereas red and wapiti rarely show any spotting as adults. The winter coat of sika is also generally much darker than that of red and wapiti, and can appear almost black. Sika also have a distinctive rump (caudal) patch which is bright white with a characteristic black margin along the top and sides, whereas red and

wapiti rump patches are generally creamy, extending above a short beige tail (both sp.). Although red and wapiti have similar coats, wapiti have a shorter tail, a more defined rump patch and a lighter, greyer coat colour (Whitehead, 1972).

The metatarsal glands of the sika have elongated white hairs and are usually very distinct, whereas the red and wapiti's gland hairs are normally far less prominent. Facially, sika have a shorter snout, more rounded ears than red and wapiti and often have a distinctive dark line of hair above their eyes, especially noticeable in stags. The males of all three species have deciduous antlers which they lose in the spring. However, sika antlers are simple and rarely have more than eight points (four each side), whereas red and wapiti antlers are more elaborate with more points (Whitehead, 1964; Ratcliffe, 1991; Polziehn & Strobeck, 1998).

All three species are sexually dimorphic in size, though wapiti are less dimorphic than red or sika (see Polziehn & Strobeck, 1998).

Throughout this thesis I have used the terms "red-" and "sika-like", and "phenotypically red/sika" interchangeably to be less monotonous, both indicating that the individual looked like either red or sika deer. Clearly, "phenotypically hybrid" and "hybrid-like" refer to hybrid looking deer.

1.2.2 Taxonomy

Red, sika and wapiti are members of the order Artiodactyla (even-toed ungulates), family Cervidae and are placed in the subfamily Cervinae along with other members of the genus *Cervus*. The status of these taxa as separate species has been controversial and remains the source of much debate (Harrington, 1985; Groves & Grubb, 1987; Lowe & Gardiner, 1989; Cook 1993; Emerson & Tate, 1993; Cronin *et al.*, 1996; Polziehn & Strobeck, 1998). The primary reason against awarding wapiti individual species status has been their phenotypic similarity to red deer and ease with which they hybridise to produce fertile offspring (Dratch &

Gyllensten, 1985; Lowe & Gardiner, 1989). Despite hybridisation, there has never been any real suggestion that red and Japanese sika deer should not be considered as separate species (Cook, 1993), only that the status of some Asiatic mainland sika subspecies be reconsidered (Lowe & Gardiner, 1975; Harrington, 1979). This confusion exemplifies the more general problems associated with the application of Mayr's (1942) biological species concept based on reproductive isolation to hybridising species (Balharry *et al.*, 1994).

The subspecific systematics of the genus *Cervus* has been described as "superficially chaotic" (Caughley, 1971) with as many as eighteen subspecies of red (including wapiti) (Ellerman & Morrison-Scott, 1951) and thirteen subspecies of sika deer (Whitehead, 1993) being described. This confusion has largely arisen due to the broad cline of morphological characters displayed across the genus' distribution (Geist, 1987), the translocation of individuals for zoological collections and game hunting (Lister, 1984), the ability to hybridise (Harrington, 1979; Lowe & Gardiner, 1975; 1989) and the over-zealous attention of taxonomists (Abernethy, 1994a). Three general systematic groups of red deer subspecies have been recognised, the European group (including North Africa and southwesternsouth-western Asia), the central Asia group, and the east Asian and North America group, which includes wapiti (Groves & Grubb, 1987).

The red deer in Scotland are thought to be the direct descendants of the red deer which colonised Britain after the last Ice age around 11,000 years ago (Lowe & Gardiner, 1974; Gyllensten *et al.*, 1983). As such, they are generally recognised as belonging to the subspecies *C. elaphus scoticus* Lonnberg, 1906 (Scottish Natural Heritage 1994; Whitehead 1993) and distinct from the continental populations (Lowe & Gardiner, 1974; Gyllensten *et al.*, 1983). There have, however, been several intentional introductions to Scottish populations from England, continental Europe and America (Whitehead, 1964) and many escapes from deer parks around the country (Callaender & Mackenzie, 1991). Included in these translocations were

several recorded cases of wapiti, or their hybrids, having been introduced to increase the body size of the native stock (Whitehead, 1964; see section 1.3).

Despite the problems surrounding sika systematics, an overall pattern emerges from the literature: one subspecies is described for each of the main Japanese islands, one each in Taiwan, Vietnam and Sichuan and various contiguous subspecies in the east Asian mainland (Cook *et al.*, 1999) (Figure 1.1). The main criticism of this scheme is the suggestion that the mainland and Taiwanese sika are in fact stabilised hybrid species of Japanese sika (*C. n. nippon*) and the Chinese red deer (*C. elaphus xanthopygus*) (Lowe & Gardiner, 1975; Harrington, 1982). However, recent mitochondrial DNA evidence (see section 1.2.2), supports the monophyletic status of mainland and insular sika deer and found genetic differences between five sika subspecies [*aplodontus* (*centralis*), *hortulorum, nippon, pseudaxis*, and *taiouanus*], suggesting evolutionary divergence times of between 0.1 and 1.9 million years ago (Cook, 1993; Cook *et al.*, 1999). Nagata *et al.* (1999), extended their sample range to include sika samples from Hokkaido (*C.n. yesoensis*) and found them to be similar to the other northern sika populations, and no more different than those samples within northern Honshu. They concluded that the current subspecies classification does not accurately reflect the mtDNA differences between populations and that some adjustment should be made, probably to reduce

In this thesis red deer, sika deer and wapiti are treated as separate species due to their overall phenotypic and genetic differences (Polziehn & Strobeck, 1998; Cook *et al.*, 1999). Interbreeding of red deer and wapiti is therefore considered hybridisation in the same way as interbreeding between red and sika deer.

1.2.3 Genetics

Several studies have compared red, sika and wapiti genetically, primarily to establish the systematic relationship between them (Johnson, 1968; McDougall & Lowe, 1968; Baccus *et al.*,

1983; Dratch & Gyllensten, 1985; Dratch, 1986; Linnell & Cross, 1991; Cook, 1993; Emerson & Tate, 1993; Polziehn & Strobeck, 1998), or in the identification of hybrids (Harrington, 1979; Abernethy, 1994a, b; Tate *et al.*, 1998).

Johnson (1968) used serum proteins and haemoglobin to compare red and wapiti and was unable differentiate between them, while Linnell & Cross (1991) found little difference between red deer and wapiti using enzyme loci. However, using a variety of molecular techniques, including further protein electrophoresis, Dratch (1986), Baccus *et al.* (1983), Dratch & Gyllensten, (1985), Emerson & Tate (1993), Polziehn & Strobeck (1998) and Tate *et al.* (1998) have all found either fixed allelic differences or significant differences in allele frequencies between the species. The exact genetic distances (D, Nei, 1972) estimated vary widely between enzyme studies (red-sika, 0.13-0.5; red-wapiti, 0.02-0.32; and sika-wapiti, 0.06-0.39), indicating that the specific loci and perhaps populations used has had a significant effect on the outcome of these studies.

Within red deer, a number of authors have compared populations within Europe using protein electrophoresis [e.g. Gyllensten *et al.* (1983); Herzog *et al.* (1991)] and mitochondrial DNA (mtDNA) (Hartl *et al.* 1995). Within continental European red deer populations there is a consensus across studies that significant genetic subdivision can be found between regions that broadly correspond with national boundaries (Herzog *et al.* (1991); Herzog & Krabel (1993); Hartl *et al.* (1995). In the most recent comprehensive comparison of European populations, Hartl *et al.* (1995) used mtDNA to compare 15 populations scattered across Western, Central South-eastern Europe. They identified nine haplotypes that were almost fixed within each of the populations studied.

However, there are no comprehensive comparisons of Scottish and European red deer populations. The only work to include a small number of Scottish samples was carried out by

Gyllensten *et al*., (1983) and found as much differentiation between Scottish populations as between Scottish and continental populations.

Within Asian sika populations, relatively little genetic work was published until recently. Cook (1993) compared the mitochondrial DNA (mtDNA) of five Asian subspecies [*aplodontus* (*centralis*), *hortulorum, nippon, pseudaxis*, and *taiouanus*] and found clear differences between the northern sika populations (Korea, *hortulorum*; Japan, *centralis*) and those from Vietnam (*pseudaxis*) and Taiwan (*taiouanus*). There was, however, some ambiguity regarding the phylogenetic position of the southern Japanese samples from Kyushu Island, as some analyses grouped them with the southern sika, and others with the northern samples.

In a subsequent comparison of three sika populations in Japan, Nagata *et al.* (1995) found a clear difference in mtDNA between the northern (Hokkaido and N. Honshu) and southern (Nagasaki) samples. This finding was latter repeated by Nagata *et al.* (1999), again using mtDNA, who compared 13 populations from across the Japanese Islands. Two distinct phylogenetic lineages were evident between the northern and southern populations, dividing not between islands as was expected, but somewhere in southern Honshu. No previous studies that have compared Scottish sika populations.

The only paper to compare wapiti populations genetically found little genetic differences between North American, New World, populations using enzyme electrophoresis (D ~0.005; Dratch & Gyllensten, 1985). Little is known about the genetic background of the wapiti introduced to Scotland and the previous genetic studies comparing Scottish red deer populations have tended to ignore their introduction to Scotland (Dratch, 1983; Gyllensten *et al*., 1985; Abernethy, 1994a, b).

1.2.4 Phylogeny

The exact evolutionary history of the genus *Cervus* has been the subject of some debate (see Lister, 1984; Geist 1987; Groves & Grubb, 1987), especially that surrounding the true phylogeny of red deer, sika deer and wapiti (Ellerman & Morrison-Scott, 1951; Flerov, 1952, Gyllensten *et al.*, 1983; Harrington, 1985; Lowe & Gardiner, 1989; Whitehead, 1993, Cook *et al.*, 1999). Although Harrington (1985) argued that a wapiti-like deer was the ancestral form of the extant species, the most widely excepted history is that red, sika and wapiti evolved from a common sika-like ancestor in Asia during the late Pliocene around two million years ago (Lister, 1984; Cook, 1993; Polziehn & Strobeck, 1998). The most recent estimates of genetic divergence (see above, section 1.2.2) between red, sika and wapiti indicate a split between all three around two hundred thousand years ago in eastern Asia (Polziehn & Strobeck, 1998).

Red-like deer are first recognised in the fossil record in Britain around the Middle Pleistocene (c. 400,000 BP) and have been continuously present since the beginning of the last postglacial around 10,000 BP (Lister, 1984). The red-like characteristics of larger body size, more elaborate antlers and exaggerated sexual dimorphism are thought to have evolved as red deer migrated north and east from Asia towards Europe and Siberia. In more open habitats ungulates tend to form larger groups in response to more homogenous food distribution and as a predator vigilance strategy (Jarman, 1974). The mating success of males then tends to become more skewed as increasingly polygynous mating systems evolve in response to larger female group size (Clutton-Brock, 1984). In response larger, more aggressive, males are selected for increasing overall male size and secondary sexual characteristics such as antlers (Lister, 1984; Geist, 1987).

Following comparisons of sika mtDNA, Cook (1993) suggested that sika spread south along the coast of east Asia, colonising Taiwan within the last 200,000 years, and Japan twice,

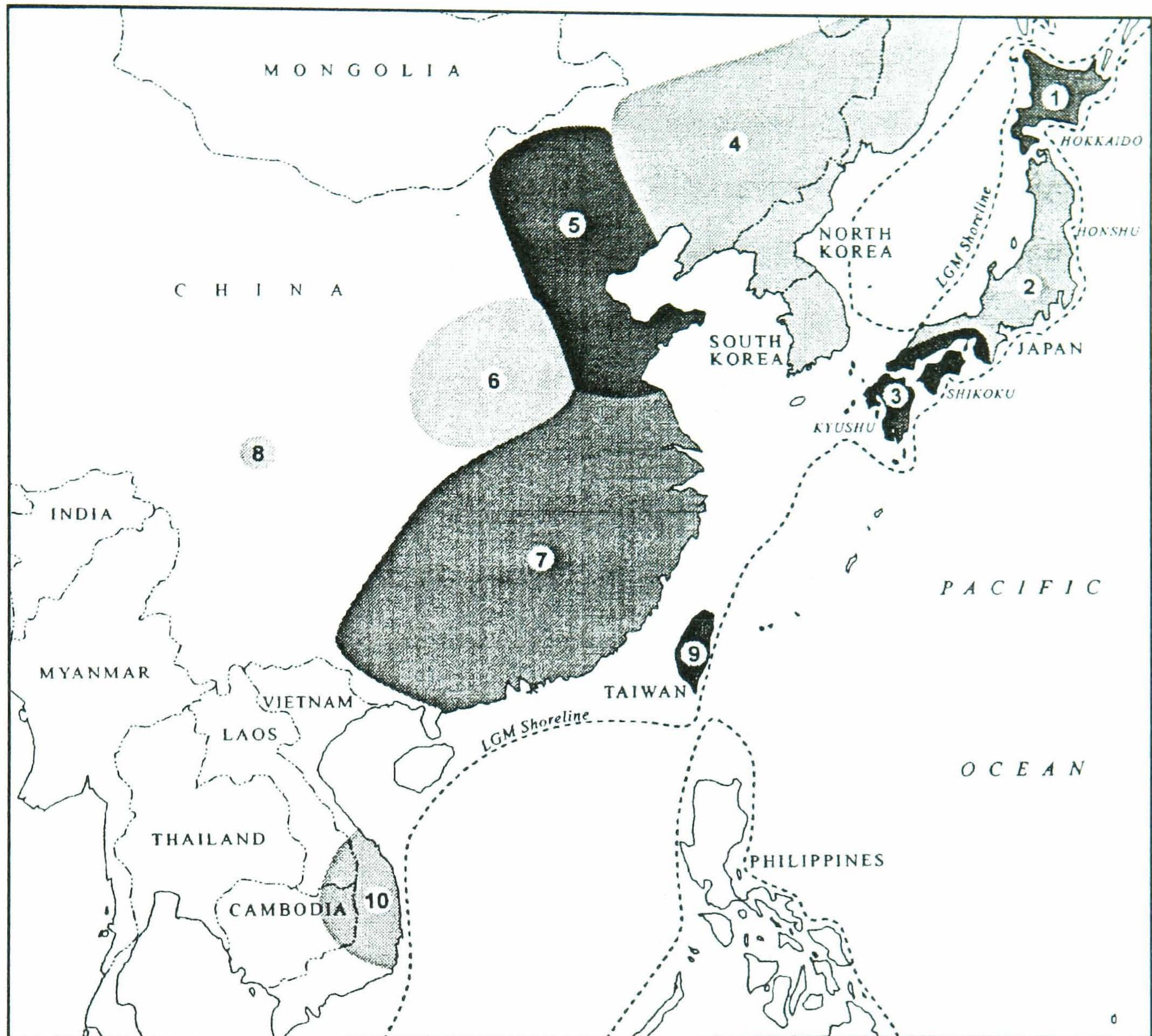

Figure1.1 Sika deer (*Cervus nippon*) subspecies and their distributions, from Cook (1993).

Key: 1. *C.n. yesoensis*, 2. *C.n. aplodontus*, 3. *C.n. nippon*, 4. *C.n. hortulorum*, 5. *C.n. mandarinus* 6. *C.n. grassianus*, 7. *C.n. kopschi*, 8. *C.n. sichuanicus*, 9. *C.n. taiouanus*, 10. *C.n.pseudaxis*

initially around one million years ago and then again during the penultimate glacial period, one to two hundred thousand years ago. More recently Nagata *et al.* (1999) confirmed this result in a more extensive mtDNA investigation of Japanese sika (see section 1.2.2).

The North American and Siberian wapiti appear to have differentiated around the time of the last land bridge between Alaska and Russia, some fifty thousand years ago (Polziehn & Strobeck, 1998). It is not clear why wapiti, which are as polygynous as red deer, are less dimorphic (Polziehn & Strobeck, 1998); perhaps males have reached some upper limit to size set by energetic constraints.
the number of subspecies in Japan to two, one representing the northern populations and one the southern populations.

In this thesis red deer, sika deer and wapiti are treated as separate species due to their overall phenotypic and genetic differences (see Polziehn & Strobeck, 1998; Cook *et al.*, 1999). However this definition is not critical to the results of this thesis, what is important, is that interbreeding of red deer and wapiti is considered in the same way as that between red and sika deer, i.e. as *hybridisation*.

1.3 Translocations into Scotland

1.3.1 Red deer and wapiti

The re-stocking of red deer populations, depleted by 18th century hunting, was carried out in many areas of Scotland with English and Irish deer park stock. Whitehead (1964) lists more than 100 occasions when recorded introductions of park red deer and states that "there must have been numerous other park introductions of which no record has been kept."

From one red deer herd alone (Warnham Court, Sussex) around 115 stags and 147 hinds were introduced to various parts of Scotland between 1894 and 1962. There were also at least

three separate translocations of continental red deer to Scottish estates: Caucasian red deer to Ardnamurchan, Argyll (late nineteenth century), Carpathian red deer to Attadale, Ross-shire (1885) and Hungarian red deer to Letterewe, Wester Ross (1910) (Whitehead, 1964). Across Scotland at least seven different introductions of wapiti occurred prior to 1964 and in most cases hybridisation with the local wild red deer was thought to have taken place (Ritchie, 1920; Whitehead, 1964): Monymusk, Aberdeenshire; Glenmuick, Sutherland; Glenfinnan, Inverness-shire; Killiechonate, Inverness-shire; Mamore, Inverness-shire; Meoble, Inverness-shire; and Arran, Bute.

1.3.2 Sika

Sika were introduced to deer parks and private collections across Scotland between 1870 and 1930 (Figure 1.2), and subsequently escaped or were released into the surrounding habitat (Whitehead, 1964; Ratcliffe, 1987a). The majority of these populations (Dawyck in the Borders; Rosehall and Ledgowan in Ross-shire; Carradale in Argyll; and Aldourie and Glenmazeran in the Great Glen) have grown rapidly and expanded their range to cover approximately one third of Scotland (Rose, 1994). More recently Chadwick *et al.* (1996) reported that young sika stags have been recorded in Glen Orchy and Glen Duror in the west, Skye, north of Loch Rannoch and near the English border, well outside their previously recognised range (Figure 1.2).

The origin and subspecific status of the sika in Scotland has generated much interest because of the possible role it may have in the hybridisation of red and sika (Ratcliffe, 1987a; Lowe & Gardiner, 1975; Putman & Hunt, 1993; Harrington, 1982; Abernethy, 1994a). All of the sika populations in Scotland are thought to have originated from the Japanese archipelago and belong to the subspecies *C. n. nippon* (Ratcliffe, 1987a; Abernethy, 1994a). Although the records are far from complete (see Ratcliffe, 1987), it is likely that all, except one, were brought

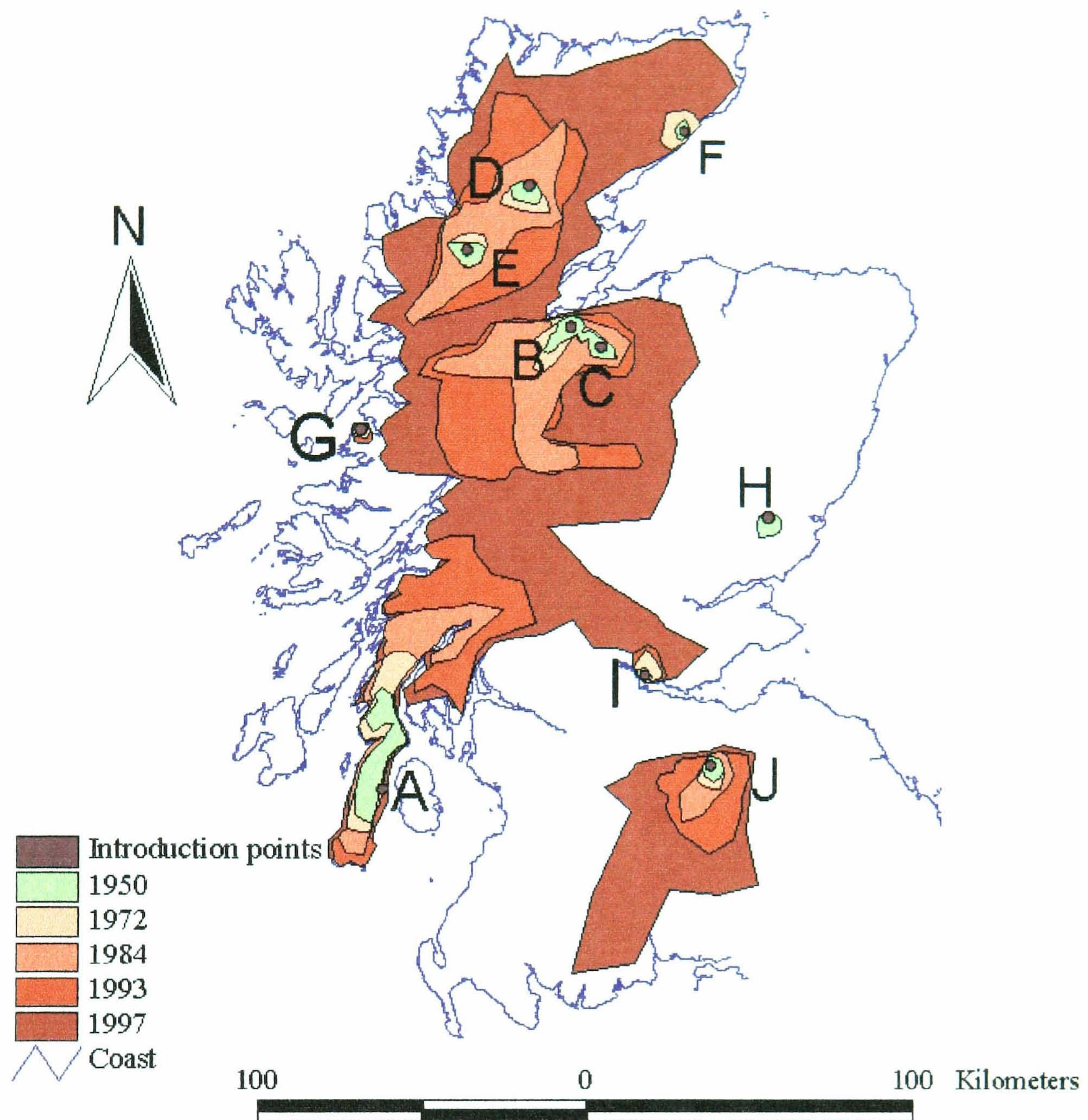

Figure 1.2 Sika introduction points and range expansion in Scotland, from Abernethy (1994a). A) Carradale, B) Aldourie, C) Glenmazeran, D) Achanalt, E) Rosehall, F) Berriedale, G) Loch Morar, H) Kinnaird, I) Tulliallen, J) Dawyck. The plots represent maximum range and not resident breeding population.

into Scotland via Powerscourt, Ireland, the original introduction point of Japanese sika to the British Isles in 1860 (Powerscourt, 1884).

However, Asian mainland Formosan (*C. n. hortulorum*) and Taiwanese (*C. n. taiouanus*) sika were known to have been kept at the same time in the deer parks that may have contributed to the Scottish populations (Ratcliffe, 1987a; Ratcliffe *et al.*, 1992). Only the sika introduced to Dawyck, Peebleshire are thought to have come directly from Japan and on this basis are likely to be pure Japanese sika (Whitehead, 1964). However, this population may have been introgressed by an stag of unknown provenance introduced from Whipsnade Zoo in 1939 (Whitehead, 1950). The Fife sika were also re-stocked with unknown provenance park stock sometime after the First World War (Whitehead, 1964).

Skull morphometric work has shown that Scottish sika have overlapping distributions, except for the Dawyck sika which resemble examples of "*C. n. nippon*" from Honshu, Japan (Ratcliffe *et al.*, 1992). Although practical, Ratcliffe's (1987a; 1992) use of *C. n. nippon* to include all Japanese island subspecies, may be confusing as the origin of the Honshu skulls was within the historical range of the subspecies *C. n. centralis*, now recognised by Cook (1993) as genetically distinct from the *C. n. nippon* described by Temminick (1836). An added confusion surrounds the sighting of several melanistic sika at Dawyck (A. Chadwick pers. comm.), a trait which is a associated with an endangered subspecies *C. n. keramae* from the Ryuku Islands, Japan (Whitehead, 1972).

1.4 Hybridisation within the Cervinae

1.4.1 Background

Hybridisation has been noted between several species within the subfamily Cervinae, both within genera and between, although many of these events have been between captive deer

(Whitehead, 1993). In the wild red and sika have been reported to hybridise along their natural contact zone in East Asia (Bartos, *et al.*, 1981), and following sika introduction in Czechoslovakia (Bartos *et al.*, 1981; Bartos & Zirovnicky, 1981 and 1982), England (Lowe & Gardiner, 1975), Ireland (Harrington, 1979; Harrington, 1982), New Zealand (Davidson, 1973) and Scotland (Ratcliffe, 1987a; Abernethy, 1994a).

The frequency with which red and sika hybridise in the wild is thought to vary according to the particular circumstances of each population. Harrington (1979) carried out a number of crossing experiments between various combinations of age, sex, and species (including hybrids). He reported that matings were only successful between young red males and adult sika females, and between hybrids and either parental taxon. It therefore appeared that the first generation hybrids were most likely to result from matings between red males and sika females in the wild. However, the specific sizes of the deer used in these crosses was not reported by Harrington (1979), but involved relatively small sika from Killarney and relatively large local park red deer. The generality of these experiments to Scottish wild populations is therefore unclear. Harrington (1979, 1982) also used an immunoelectrophoretic technique an phenotypic measures to study the feral deer of Wicklow and Killarney in Ireland. He found that both phenotypic and genetic introgression was considerable in the Wicklow population and that no "pure" red or sika remained. The Killarney populations, however, appeared to have avoided hybridisation. Harrington (1979, 1982) concluded that initial hybridisation events were rare, depending on the relative size and genetic origin of the populations involved [Harrington (1979) assumed that the Killarney sika were pure Japanese, whereas that Wicklow sika were possible of hybrid origin].

Lowe and Gardiner (1975) compared skull measurements of red-, sika- and hybrid-like deer from populations in the north of England and Asia. There appeared to be differences between the rates of introgression between two English populations, one considered "pure" sika and the other not so. The English "hybrid" samples appeared to resemble skulls from

Manchurian sika, suggesting to Lowe & Gardiner (1975) that mainland Chinese sika are of hybrid origin. They again concluded that genetic origin and size was an important factor in promoting hybridisation between red and sika deer in the wild.

Bartos (1981) and Bartos & Zirovnicky (1981, 1982) studied various feral red and sika populations in the former Czechoslovakia, comparing skull morphology measurements, behaviour and external phenotypic characteristics. They reported no evidence of any ethological barriers between red and sika deer, and suggested that sika deer males were more likely to show an interest in red deer females than *vice versa*. They also found sika males to be more aggressive in general than red deer males (including to other species) and that they were able to fight competitively with red males, despite their smaller body size and antlers. Furthermore, the most likely context for hybridisation events was suggested to be when young male sika were colonising new territories which contained red deer populations (Bartos & Zirovnicky, 1982).

Phenotypic analysis revealed widespread introgression across populations, and although individual characters proved unreliable, at least one populations (Janovice) was considered entirely hybrid (Bartos & Zirovnicky, 1981). Skull morphometrics revealed some evidence of intermediate forms representing recent hybrids, but no clear cline in skull dimensions was found (Bartos, 1981).

Overall it appears that initial hybridisation events between feral sika and wild red deer are rare, but that introgression can spread throughout the population relatively quickly, i.e. rapidly enough to result in completely introgressed populations. Bartos & Zirovnicky (1981) differentiated between the initial hybridisation events and the subsequent backcrossing, suggesting that populations (especially sika) have an affinity to their original phenotype and that in the absence of continued hybridisation, will retain their overall appearance. The only phenotypic evidence of hybridisation may then be restricted to a small number of traits including some skull dimensions, antler form and size and possible slight increase in body size.

On the open hill, the fitness of red deer males is indirectly related to body size and age and directly related to harem holding and fighting success (Clutton-Brock & Albon, 1985). The smaller size of sika males should therefore prevent them from accessing females in oestrus. The mating success of the males is also highly skewed (Pemberton *et al.*, 1992), which suggests that there is strong male-male competition and few successful sneak matings. However, sika males may be more aggressive than red deer and some behavioural differences could override the effect of size alone (Bartos & Zirovnicky, 1982).

Are there further ecological and behavioural factors are potentially involved in promoting or preventing hybridisation? First, in commercial forests, the modification of the red deer harem system towards smaller groups (Staines, 1991) and possibly territorial behaviour (Putman & Mann, 1990) is likely to reduce the amount of skew in the success of males mating with red females (Clutton-Brock, 1989). Smaller groups of hinds may be more difficult to guard, increasing the opportunities for hybrid matings. Bartos & Zirovnicky (1981), further suggested that red males are more tolerant of sika males than vice versa (even at rutting time), perhaps providing opportunities for sika males whilst the red males are distracted by conspecifics. It is also possible that the better body condition generally found in sika deer compared with sympatric red (Chadwick *et al.*, 1996) allows them to rut longer and harder, and thereby more successfully.

Second, as the rutting period of sika and red deer greatly overlap in Britain (Ratcliffe, 1991), there is unlikely to be a temporal barrier to hybridisation. There may, however, be more chance of hybridisation by sika males at the end of the red rut when, recycling, poorer condition and younger females come into oestrus (Mitchell & Lincoln, 1973; Mitchell *et al.*, 1981), as by that stage the most competitive red males have stopped rutting (Clutton-Brock *et al.*, 1982).

The speculation above may give some ideas as to the possible ecological and behavioural factors important in preventing or promoting hybridisation. However, the apparent rarity of

hybridisation events, as indicated by a general lack of F_1 phenotypes, and context in which they occur (dense commercial forestry) makes any quantitative investigation of their relative importance difficult.

Finally, the issue of red and wapiti hybridisation has been ignored (e.g. Dratch, 1983; Abernethy, 1994a), largely because of the confusion over the taxonomic status of wapiti and the absence of extant feral wapiti populations. Also, since wapiti have a larger body size and antlers than red deer, which are considered positive traits by sporting estates, there has been less concern about the possibility of introgression.

1.4.2 Scottish studies

In Scotland red and sika were initially not considered at risk from hybridisation because of their genetic "purity" and origin: the Scottish sika were thought to be pure Japanese sika (*C. n. nippon*) and thereby less likely to hybridisate under natural conditions (Harrington, 1979, 1982; Lowe & Gardiner, 1975). However, phenotypic (Ratcliffe, 1987a; Ratcliffe *et al.*, 1992) and genetic (Abernethy, 1994a,b; Goodman *et al.*, 1999) evidence has recently indicated that hybridisation may occur wherever red and sika deer are sympatric, and that the size and genetic origin barriers suggested by Harrington (1973, 1979) and Lowe & Gardiner (1975) (see above) are not evident.

Ratcliffe (1987a) carried out the first comprehensive review of sika deer and hybridisation in Scotland, highlighting the potential ecological impact of an increasing sika population and the potential threat to red deer from hybridisation. The lack of any baseline data on the geographical extent and nature of hybridisation was emphasised by Ratcliffe (1987a) and acted as catalyst to the first genetic and ecological investigation of hybridisation between Scottish populations (Abernethy, 1994a,b).

Two areas in Scotland (Argyll and Great Glen, Invernesshire) containing red and sika deer were sampled for DNA to investigate the frequency of hybridisation and pattern of introgression. In addition, a number of the deer shot in Argyll were sampled for gut rumen samples so that winter diet choice could be compared with genotype. A set of two isozyme [superoxide dismutase (Sod-1) and 6-phosphogluconate dehydrogenase (6Pgdh)] and two microsatellite (BOVIRPB and OarFCB193) nuclear markers and one mtDNA marker (NADH complex) were used as putatively diagnostic markers between red and sika deer. A total of 235 deer from nine forests in Argyll and 136 deer from seven forests in the Great Glen area were genotyped for this panel of markers.

The results of the genetic study of the Argyll and Great Glen populations identified considerable introgression at both sites, significant heterozygote deficit (Fis ~ 0.60) and linkage disequilibria (34%). These results were interpreted as evidence of the rapid introgression of one or more traits by hybridisation and possible heterozygote disadvantage and/or assortative mating. Differences were also found between the steepness of the maternally inherited mtDNA clines and the nuclear clines which indicated that the sika or hybrid stags were leading the introgression.

Although the diet analysis data were highly variable within groups, an interaction of sex and genotype on the plant community used was identified, with sika-like deer using significantly less heath and more grassland than red-like deer. This in turn had an effect on the fibre composition and overall diet quality sampled at that time,

Following on from Abernethy's (1994a) work, Goodman *et al.* (1999) began to develop a larger number of diagnostic microsatellite markers that would be used in a larger study of hybridisation in Scotland (including the work described in this thesis). Candidate loci of bovine or ovine origin were first identified using a small test panel of four sika (two each from Kintyre and the Borders, Scotland) three red deer (one each from Galloway, Rum and the Great Glen,

Scotland) and one cow or sheep control. Any loci that appeared to have non-overlapping allele sizes in red and sika were tested on a larger test panel consisting of 44 sika (from five Scottish populations) and 44 red deer (from four Scottish populations). Of 271 ruminant microsatellite loci tested 30 (11%) appeared to have non-overlapping allele distributions in the Argyll samples (Slate *et al.*, 1998).

Nine of these initial putatively diagnostic microsatellite markers were then used to re-analyse Abernethy's (1994a) samples collected in 1991/92. Whilst two of the microsatellite markers used by Abernethy (1994, a, b) gave a similar pattern to the new microsatellite loci, two of the assumed diagnostic protein loci (superoxide dismutase, sod-1; and 6-phosphogluconate dehydrogenase, 6Pgdh) indicated introgression further north of the microsatellite cline, and were deemed undiagnostic due to a shared polymorphism with sika (Goodman, *et al.*, 1999).

Introgression was subsequently found to be rare (<7%) at individual loci, although where red and sika overlapped up to 40% of deer contained one or two apparently introgressed alleles. A new analysis technique was developed to allow for rare hybridisation (previous analysis of hybrid zones had relied on frequent hybridisation between the populations and considerable introgression; e.g. Barton & Gale, 1983), which treats the sika- and red-like populations separately and estimates taxon-specific rates of hybridisation and introgression (Goodman *et al.*, 1999). New hybridisation events were estimated as rare (1/500 matings in sika deer and 1/1000 in red deer), but three individuals were found to have significant linkage disequilibria, providing strong evidence of recent hybridisation. The predicted rates of introgression were less than those expected from the current rates of hybridisation and this was interpreted as possible evidence of selection against hybrids.

1.4.3 Consequences of hybridisation

Red and sika deer clearly differ genetically and phenotypically and these differences will become less distinct through hybridisation. However, the extent to which these differences affect the relative fitness of the various genotypes across environments will determine the dynamic interaction of the two species (Barton & Hewitt, 1989; Grant & Grant, 1992; Johnson & Wade, 1996). The differences which will have most effect are those which determine the amount of gene flow between the taxa and the age specific fecundity and mortality of the different genotypes.

Gene flow will be affected by the incidence of hybridisation and the extent to which the individuals disperse and backcross (Barton & Hewitt, 1989). The extensive ranging behaviour of red and especially sika (Davidson, 1973; Mitchell *et al.*, 1977; Ratcliffe, 1987a) and their significant overlap in diet (Abernethy, 1994a) suggests that the two species will not remain isolated through association with different habitats, as has happened in other older hybrid zones (e.g. fire-bellied toads, MacCallum 1994). However, there is some evidence to suggest that strong assortative mating is occurring, thereby reducing the amount of gene flow between the taxa (Goodman *et al.*, 1999). At present, there is little evidence of wide scale phenotypic changes associated with genetic introgression (Abernethy, 1994a).

In female red and sika deer fertility is directly related to body condition (Albon *et al.*, 1986; O'Donohue, 1991). As sika deer maintain better body condition than red deer over the winter period, it is likely that the higher reproductive performance sika achieved across Scotland compared with red deer (Chadwick *et al.*, 1996) is a reflection of this. When sympatric we would therefore expect red and sika females to compete for preferred habitats so that they can achieve maximum body condition. In times of resource depletion, smaller sika females may be able to displace larger red females to poorer habitats because of their smaller bite size and lower

absolute energy requirements (see Clutton-Brock *et al.*, 1987b; Gordon & Illius, 1989). The larger component of heath communities in the female red deer diet found by Abernethy (1994a) may be evidence of this.

Hybridisation presents several practical problems to those responsible for the conservation of Scotland's natural heritage. The government has recently encouraged land managers to apply rigorous control to sika populations to protect red deer from hybridisation and to control the spread of sika (DCS, 1997). If mainland red deer populations are likely to be further introgressed by sika, then the establishment of island refugia may represent the only practical way of protecting current red deer populations from further introgression (Ratcliffe, 1987a), assuming that these populations are free from introgression presently.

There is no recorded information available about the phenotypic consequences of local hybridisation with wapiti and it would be impossible now to identify any genetic effects from local differences in environmental conditions. Furthermore, no genetic data exists about the genetic background of the wapiti introduced to Scotland (see section 1.2.2), nor has there been any previous attempt to investigate the rate of introgression within any of the populations involved. Nevertheless, the small number of crosses documented may have resulted in significant introgression through drift or the selection of advantageous alleles (Barton & Hewitt, 1989). In the absence of further hybridisation the fate of any introgressed material will now be determined by these two evolutionary forces.

1.5 Objectives of study

The translocation and subsequent hybridisation of *Cervus* deer has clearly raised many ecological and genetic issues for the conservation of Scotland's natural heritage. For example, what will the fitness effects of hybridisation with sika deer be for Scottish red deer? Will the

phenotype of Scottish red deer be broken down through continued hybridisation with sika deer? Will the loss of "genetic integrity" of Scottish red deer affect their conservation status?

Phenotypic hybrids have been reported in several areas in Scotland (Ratcliffe, 1987a) and genetic hybrids have been found in the Argyll (Abernethy, 1994a,b; Goodman *et al.*, 1999) and the Great Glen (Abernethy, 1994a, b). However, red and sika are sympatric over much of Scotland without apparent hybridisation, and where it has occurred, there is little evidence of phenotypic disruption. Why then has hybridisation occurred in some populations and not others? Are there ecological, genetic or phenotypic factors that predispose populations to hybridisation? Where it has happened, what are the genetic and phenotypic consequences of hybridisation, and are they always the same?

Previous studies have begun to address some of these questions, but have concentrated mostly on the genetic processes occurring in one population, Argyll (Abernethy, 1994a,b; Goodman *et al.*, 1999). Ratcliffe (1987a) recognised the importance of establishing the national extent of hybridisation so that areas of conservation priority could be identified, and of collecting the basic demographic and ecological data necessary to manage any species effectively. The aims of this thesis are to build on this work and to address some of the main outstanding questions outlined above. The primary aims are to establish how extensive hybridisation between red and sika deer is in Scotland and to balance the previous bias towards purely genetic investigations by collecting detailed phenotypic information that could be compared simultaneously. This will allow a phenotypic comparison between hybridising and non-hybridising populations and also a detailed investigation of the phenotypic consequences of introgression.

1.6 Specific Aims

- Establish the extent of hybridisation and introgression in the various introduced sika populations and sympatric or local red deer populations.

- Identify any phenotypic and/or genetic correlates of the incidence and extent of hybridisation in the study populations, e.g.:
    - (i) Does the body size difference between sexes and species affect their likelihood of hybridisation?
    - (ii) Are populations that hybridise more similar genetically compared with those that have not?

- Describe the phenotype of the parental red, sika and hybrid deer of the sampled Scottish populations and investigate the following questions:
    - (i) Can hybrids be identified by stalkers?
    - (ii) Do hybrids differ in body size, skull shape, condition or fecundity from non hybrids?
    - (iii) Are F_1 foetuses produced early, or late in the rut relative to the other matings?
    - (iv) What is the species, body condition and size of any females with F_1 foetuses?

- Discuss the possible outcome of the introgression process and provide information relevant to the management of red and sika deer in Scotland.

2 Materials and Methods for Genetic Study

2.1 Sampling Regime

2.1.1 Overview

Tissue samples from phenotypically red, sika and hybrid deer were collected from sites in the UK and Ireland. The majority of these samples came from targeted populations on Forest Enterprise land associated with sika introduction sites. However, a number of other samples were provided by individual land owners or stalkers and other research projects. Two separate sampling strategies were employed across the Scottish sika introduction areas. In Argyll, an intensive study was carried out across most of the area, collecting samples from the majority of FE properties. This is the area where Abernethy (1994a) had previously described a moving hybrid zone and where several phenotypic hybrids had been reported previously (Ratcliffe 1987a; D. Hendry pers. comm.).

In the other areas, sampling was aimed at collecting representative samples from any surviving sika populations, as well as any neighbouring red deer populations which could potentially have hybridised. This more extensive survey was supported by a national publicity campaign requesting the collection of tissue samples from suspected hybrids, based on phenotype.

2.1.2 Intensive Argyll study

Following the screening of 246 individuals at four nuclear and one mitochondrial marker, Abernethy (1994) reported the presence of a moving hybrid zone between red and sika deer in Argyll. Although these samples were available for a more detailed genetic study using more

markers (Goodman *et al* 1999), the lack of detailed phenotypic data associated with these samples, combined with the relatively small sample sizes at some of the sites (e.g. n = 6 at Kilmichael was insufficient to estimate an inbreeding coefficient, F_{is}), suggested that a second more intensive re-sampling of the area should be carried out. Sightings of hybrid and sika deer further north of Abernethy's (1994) sample sites by local FE staff also indicated that an extension of the transect was required to fully capture the extent of hybridisation in the area.

Across the Argyllstudy area, 23 distinct Forest Enterprise owned areas were identified as sample sites which corresponded to general deer management areas, 12 in W. Argyll District, 7 in Lorne District and 4 in Cowal District (Figure 2.1). This mammoth task was only possible with the enthusiastic support and encouragement of the Chief Ranger, Donald Hendry. Tissue samples were collected by FE ranger staff from shot deer during normal culling operations over the winter cull season, 1996-97. A small additional set of sixteen samples was collected from the Loch Avich population at the start of the following winter cull due to the rare occurrence of several phenotypic hybrids in the area.

The samples were collected as the cull proceeded from December 1996 onwards as this coincided with the peak of the female cull. Females were chosen because of the larger potential sample [the cull is female biased and adult size is reached earlier in females (Ratcliffe, 1987b)] and the extra information potentially available from them through sampling foetuses. Genotyping foetuses allows investigation of assortative mating and potentially the identification of F1 parents, while morphometric measurements can provide information about the conception date (see chapter 6).

Although red and sika have separate culls targets in the FE Districts involved, this is related to their estimated density and not to any taxonomic preference (D. Hendry pers. comm.). Rangers culled animals as they were encountered and it is reasonable to assume that this was random with respect to phenotype. Sika have previously been reported as being significantly

more difficult to shoot than red deer due to their cryptic behaviour (Maclean 1996), but this is not thought to be the case in Argyll (D. Hendry pers. comm.).

A total of 1313 tissue samples were collected from Argyll during this study, with corresponding phenotypic material and larder data. A full list of sample areas and totals is shown in Table 2.1.

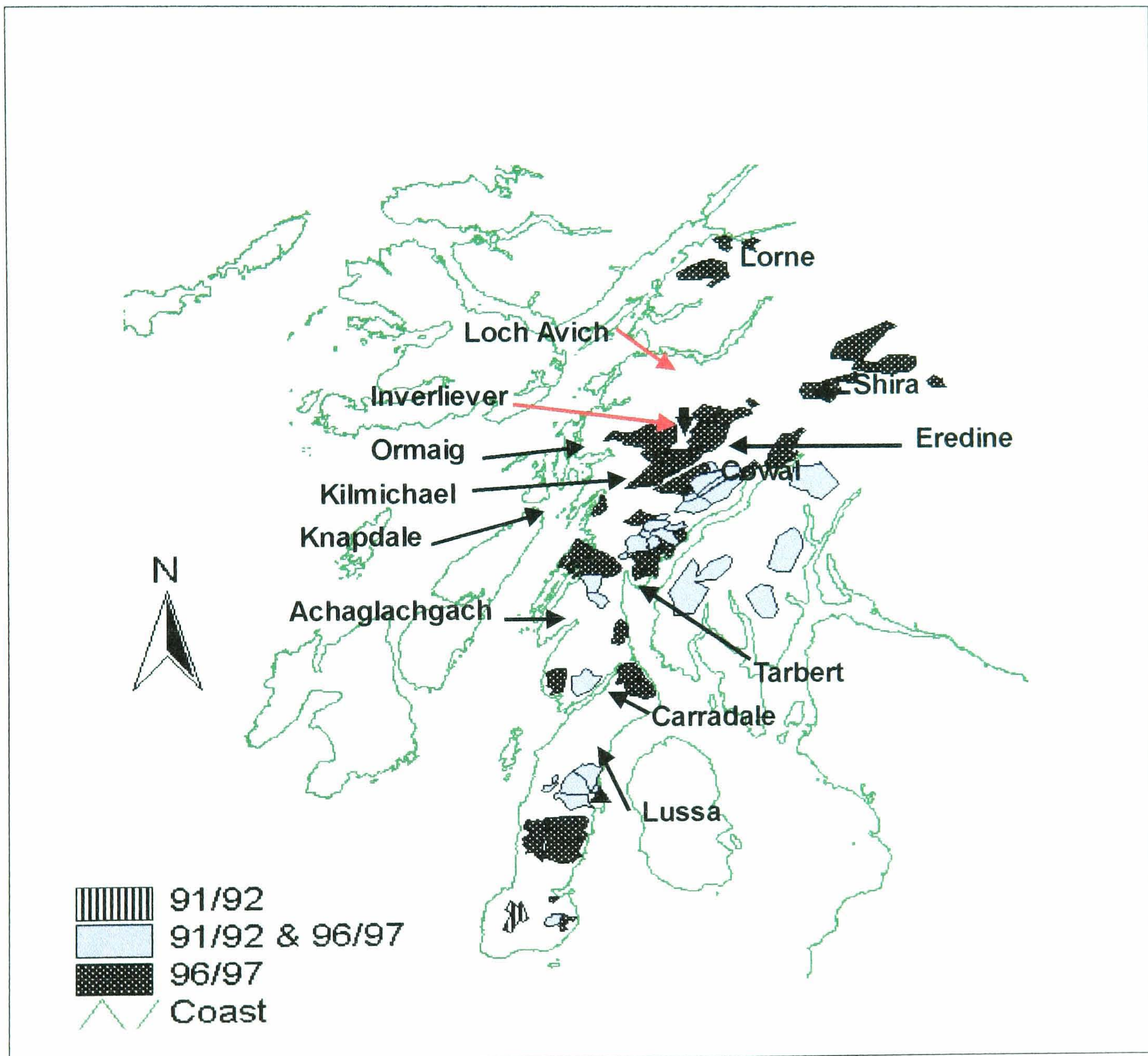

Figure. 2.1 Map of Argyll, Scotland showing sampling sites for Abernethy's (1994) 1991/92 transect and this project's 1996/97 survey starting in south Kintyre. In the text, sites are numbered from south to north.

Table 2.1. Argyll sample locations, phenotypic descriptions and sample sizes. Distances from Carradale (Kintyre) introduction site and from Loch Morar (*) for sites in Lorne farthest from Carradale.

District	Population	Pop. Code	Distance from Intro. (km)	Total Red	Male Red	Female Red	Red Foetus	Total Sika	Male Sika	Female Sika	Sika Foetus	Total Hyb.	Male Hyb	Female Hyb.	Hyb. Foetus
W. Argyll	Carradale	0	7					26		26	16				
	Lussa	1	11	2		2		133	19	62	52				
	Tarbert South	2	28					27		17	10				
	Tarbert North	3	39	10		8	2	3		2	1				
	Achaglachgach	4	46	30	1	22	7	35		25	10				
	Knapdale	5	51	38	1	25	12	48	2	33	13				
	Kilmichael	6	62	85	2	60	23	68	6	40	22				
	Ormaig	7	66					6		3	3				
	Inverliever	8	76	39	1	18	20								
	Eredine	9	78	99	12	70	17					2	1	1	
	Loch Avich	10	89	42	11	23	8	17	3	9	5	6	1	5	
	Shira	11	93	32		22	10								
	Total			**377**	**28**	**250**	**99**	**379**	**30**	**217**	**132**	**8**	**2**	**6**	
Lorne	Lochaline	21	220 (58*)	62	1	56	5								
	Mid South Mull	22	250 (66*)	31	7	6	18								
	North Mull	23	270 (93*)	64	8	48	8								
	Oban South	12	92	29	3	4	22								
	Oban North	13	106	4	2	1	1								
	Glen Lochy	14	107	74	9	37	28								
	Glencoe	15	121	26	3	23									
	Glen Orchy	16	123	36	13	22	1								
	Total			**326**	**46**	**197**	**83**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Table 2.1 continued. Cowal sample locations, phenotypic descriptions and sample sizes. Distances* from Carradale (Kintyre) introduction site.

District	Population	Pop. Code	Distance from Intro. (km)*	Total Red	Male Red	Female Red	Red Foetus	Total Sika	Male Sika	Female Sika	Sika Foetus	Total Hyb.	Male Hyb	Female Hyb.	Hyb. Foetus
Cowal	Glenbranter	17	126	93	15	59	19								
	Benmore	18	135	44	8	26	10								
	Ardgarten	19	108	86	2	67	17								
	Glendaruel	20	138	8	1	4	3	1		1		1	1		
	Total			**231**	**26**	**156**	**49**	**1**	**0**	**1**	**0**	**1**	**1**	**0**	**0**

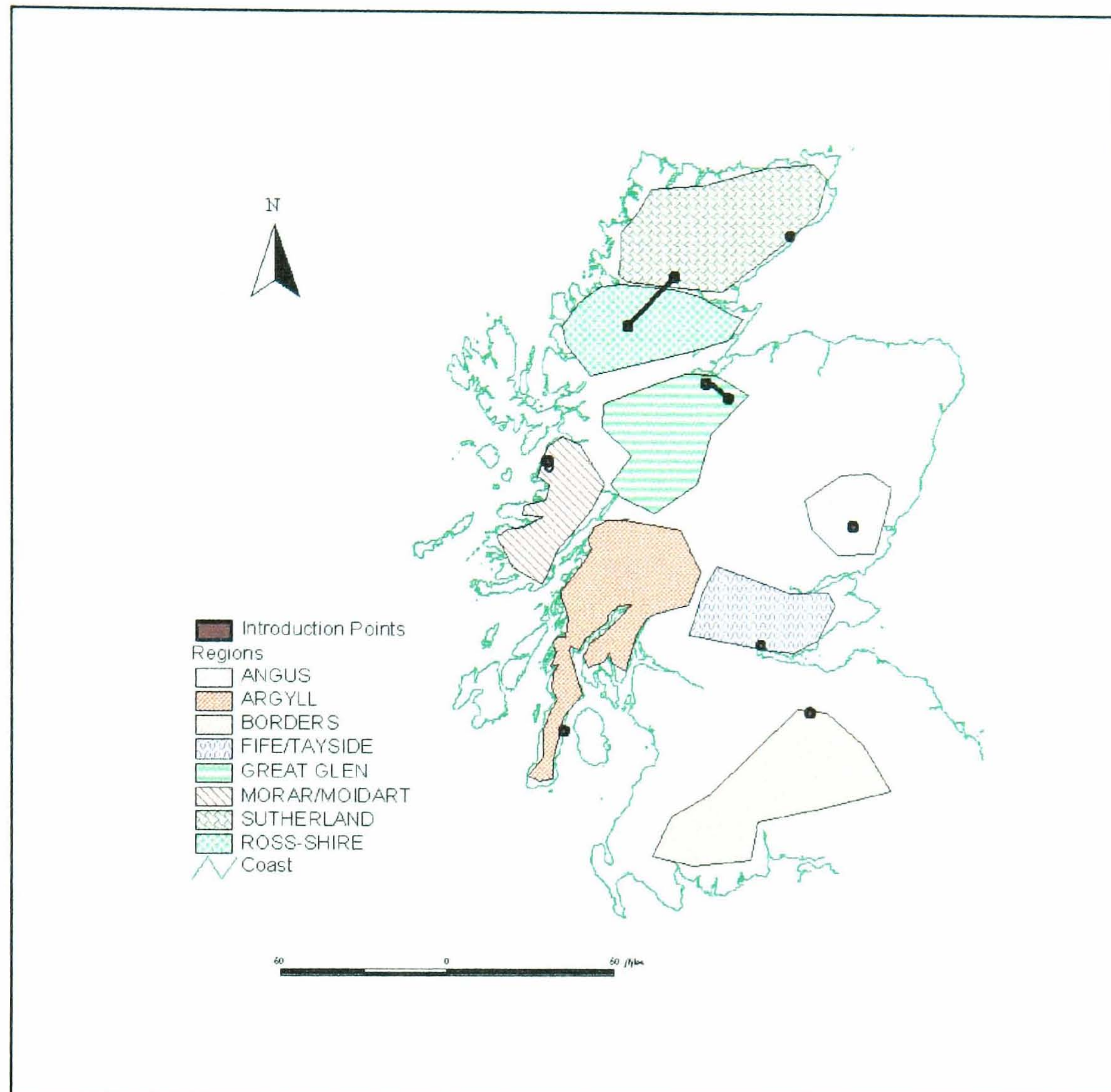

Figure 2.2 Map of Scotland showing sika introduction points (dots joined by lines where populations have merged) and corresponding red and sika deer sampling areas (shaded). Names of introduction points are given in Figure 1.2.

2.1.3 Extensive Scotland wide survey

As in Argyll, six of the other Scottish sika introductions have expanded and colonised much of the mainland red deer range (see Figure 1.2). Of these, two pairs (Aldourie and Glen Mazaren, Great Glen; Rosehall, Sutherland and Achanalt, Ross-shire) have combined to form single merged populations covering the Great Glen and North West Scotland respectively (Fig.

2.2). These populations fell within the range of two FE districts (Fort Augustus, Great Glen and Dornoch, N.W. Scotland) and were sampled in a similar, but less intensive, way to Argyll by FE staff (section 2.1.2). In the two other areas with large sika populations, Fife was sampled mainly by FE rangers (Aberfoyle District) and Borders was sampled by a combination of FE rangers (Galloway & Ae Districts) and private land owners or managers. At the remaining three introduction sites the sika population was either extinct (Kinnaird, Angus), or small and rarely shot (Berriedale, Sutherland and Loch Morar, Inverness-shire) and therefore not sampled. However, in two of these areas (Berriedale & L. Morar) local red deer samples were collected and in the third (Kinnaird) red deer samples were available from a previous study (see below).

Across the whole of Scotland, a number of other populations and individuals were sampled in response to a publicity campaign highlighting the issue of hybridisation. A leaflet (Appendix 1) was distributed to all deer controllers by the Deer Commission for Scotland, requesting the collection of samples from sympatric red and sika populations and from any phenotypically hybrid deer.

In total 670 deer (phenotypically 336 red, 326 sika & 8 hybrid; of both sexes as available) were sampled across Scotland as part of the extensive study (Table 2.2). In addition, 325 samples from phenotypically red deer collected from several parts of Scotland in 1991-1992 by the British Deer Society were made available by Dr Mike Bruford, Institute of Zoology, London.

Table 2.2. Details of Scotland wide sample collection by area (including Institute of Zoology, London samples). Introduction areas are shown in Figure 2.2. The sex of samples was not always recorded which accounts for the higher total adult values.

Area	Introduction point	Red adult	Red male	Red female	Red foetus	Sika adult	Sika male	Sika female	Sika foetus	Hybrid adult	Hyb. male	Hyb. female
Borders	Dawyck (Tweeddale)	156	52	47		68	30	25		2	1	
Great Glen	Glenmazeran/Aldourie	91	15	40	6	107	37	31	5	3	1	1
Easter Ross	Achanalt	135	88	51		69	8	3	3			
Sutherland	Rosehall	82	40	38		59	63	35	8	1	1	
Fife/Tayside	Tulliallen	82	37	25		17	1			2	1	
Angus	Kinnaird	68	25	42								
Morar/Moidart	Loch Morar	79	51	23								
Caithness	Berriedale	10										
Total		**703**	**308**	**266**	**6**	**320**	**139**	**94**	**16**	**8**	**4**	**1**

2.2 Molecular Markers

2.2.1 Diagnostic Markers

Detecting hybridisation and introgression genetically requires that genetic differences between the taxa involved can be identified. In principle this task is simple due to the fact that, by definition, hybridisation and introgression occur between two genetically distinct groups (Avise, 1994). Alien genetic material can therefore be detected following the identification of taxon-specific molecular markers, although this ideal of completely taxon-specific markers is desirable it is seldom possible to achieve, or prove, in practice (Tate *et al.*, 1998). This is especially true when there is no "pure" reference population available for the charactrerisation of the parental allele frequencies. Furthermore, where hybridisation is rare, it may be impossible to separate variation due to hybridisation from variation due to ancient shared polymorphism (Goodman *et al.*, 1999) or from convergent marker evolution (homoplasy) (Bruford & Wayne, 1993). The extent to which these factors are important will depend on the genetic history of the two taxa, the type of marker being used and the type of analysis employed (Tate *et al.*, 1998; Goodman *et al.*, 1999). One practical solution is to assign some probability of descent to the different allele frequencies found in each parental taxa (see Tate *et al*, 1998), but again this relies on having good reference populations and will also be affected by the marker choice. For example, if the parental populations used to choose the markers were introgressed to some small extent, loci that did not appear diagnostic because of the introgression would be rejected, and only loci that were not introgressed would be chosen. The exercise then becomes rather self-defeating, searching for the few loci not involved in introgression.

The resolving power of any 'hybrid test' will also be affected by the number of independent markers used as it is this that determines the precision of the estimate of introgression (Boecklen & Howard, 1997). These issues will now be discussed in the context of this study.

2.2.2 Number of markers

The number of markers to use will be crudely dictated by the level of precision required; detecting F1's from parental types will require far fewer markers than assigning individuals to backcross categories in a population. In the absence of selection the number of markers required to assign individual status accurately increases exponentially with the number of generations of backcrossing, because the proportion of introgressed genome halves with each generation (Boecklen & Howard 1997; Goodman *et al.*, 1999). If the aim of the study is to document recent hybridisation in a population, or crudely group individuals into parental, F1, or recent backcross categories, a small number of markers (5-10) may be sufficient (Boecklen & Howard 1997). However, differentiating accurately between more distant backcross categories and pure parental types may be impractical due to the large number of markers required (Boecklen & Howard 1997).

In this study there were different requirements for the two groups of samples. In Argyll, the planned intensive genetic and phenotypic study indicated that each individual should be screened for a large number of markers (>20). It was expected that this would allow the accurate estimation of the overall levels of selection, dispersal and non-random mating present in the hybrid zone. It would also allow the more precise allocation of individuals to hybrid categories (e.g inferred backcross generations or using hybrid indices) to investigate the phenotypic consequences of hybridisation. Similarly, individual samples that were sent as phenotypically suspected hybrids would require a large number of markers to unambiguously determine their hybrid status.

For the Scotland wide survey the main priority was to establish the location of recent hybridisation and therefore fewer markers were required (5-10).

2.2.3 Nuclear Markers

Several types of molecular marker have been used to investigate hybridisation and the phylogenetic relationships between *Cervus* deer populations (see chapter 1) ; immunoelectrophoresis (Harrington, 1979), protein electrophoresis (Linnell & Cross, 1991;

Tate *et al.*, 1992; Emerson & Tate, 1993, Abernethy 1994a,b), randomly amplified polymorphic DNA (RAPD) (Tamate *et al.*, 1995), restriction fragment length polymorphism (RFLP) (Tate, 1997), amplified fragment length polymorphisms (AFLP) (Tate *et al.*, 1998), and microsatellites (Abernethy 1994a,b; Goodman *et al*, 1999).

In the present study, it was desirable to choose a system that had the potential to provide a large number of unlinked locus-specific codominant genotypes. In addition, the system should be reasonably cost and time efficient due to the large number of genotypes planned. As reviewed in section 1.4., Abernethy (1994a) used allozymes with partial success in identifying hybrids, however, we planned to use methods based on DNA so that the storage conditions and type of material collected would be less critical (e.g. DNA methods no not require the material to be frozen continually).

Advances in deer molecular genetics had indicated that two of the above techniques had the potential to provide the large number of independent diagnostic markers required; RFLPs (Tate *et al.*, 1995) and microsatellites (Slate *et al.*, 1998; Tate *et al.*, 1998). Although RFLPs had shown considerable promise as diagnostic markers in other deer hybrid crosses (Tate *et al.*, 1998), in this case, microsatellites were chosen because: (a) there was a large amount of technical expertise and experience available in the laboratory; (b) a high proportion of ruminant microsatellites appeared to give polymorphic products in deer (Kuhn *et al.*, 1996; Slate *et al.*, 1998); (c) a deer mapping project was under way to map the location of more than 100 microsatellite markers (e.g. Tate *et al.*, 1995); (d) two of the first seven microsatellites tested had been shown to be diagnostic between red and sika (Abernethy, 1994a, b); and (e) large numbers of genotypes could be generated quickly at a reasonable cost. These benefits were thought to outweigh the potential disadvantages of microsatellites; null (non-amplifying) alleles (Callen *et al.*, 1993; Pemberton *et al.*, 1995) and high mutation rates resulting in homoplasy (Bruford & Wayne, 1993).

The process of identifying diagnostic microsatellite markers was undertaken by Dr Simon Goodman (ICAPB, University of Edinburgh) as part of the Red/Sika Hybridisation Project (funded by NERC). Candidate loci of bovine or ovine origin were first identified

using a small test panel of four sika (two each from Kintyre and the Borders, Scotland) three red deer (one each from Galloway, Rum and the Great Glen, Scotland) and one cow or sheep control. Any loci that appeared to have non-overlapping allele sizes in red and sika were then tested on a larger test panel consisting of 44 sika (from five Scottish populations) and 44 red deer (from four Scottish populations). Of the 271 ruminant microsatellite loci tested 30 (11%) appeared to have non-overlapping allele distributions in the Argyll samples. The size difference between red and sika allele distributions varied and sika generally had far fewer alleles, a probable consequence of their small founder populations (see chapter 3).

From the list of possible markers, two overlapping sets of markers were chosen; a 25 marker set to investigate the Argyll hybrid zone and a ten marker set to investigate the Scotland wide samples. The second set of markers consisted of the loci that appeared to be diagnostic for *most* populations on the second test panel. Overall, there appeared to be few markers that could be considered completely diagnostic for all populations; an observation that resulted in a new approach to the analysis of the Argyll hybrid zone (Goodman *et al.*, 1999). This issue will be dealt with in detail in chapter 4. A full list of primer sequences and PCR conditions used for each set of samples can be found in Appendix 2.

2.2.4 Mitochondrial Markers

Unlike diploid nuclear DNA, in most animals haploid mitochondrial DNA is inherited solely through the maternal line. By measuring the association between diagnostic nuclear and mitochondrial markers (cytonuclear disequilibria) in a hybrid zone it is sometimes possible to infer: (a) levels of gene flow between the taxa; (b) the gender direction of the first crosses; (c) the extent of any assortative mating; (d) the age of any reproductive barriers; (e) and possible mechanisms of selection on hybrids (Arnold, 1993). As the first three of these issues are central to the aims of the overall project [(a) and (b) specifically to this thesis], it was important to identify a reliable diagnostic mitochondrial marker: a single marker was sufficient due to the effectively asexual inheritance pattern of mitochondrial DNA (Asmussen *et al.*, 1987).

Abernethy (1994a,b) found four diagnostic RFLP markers from the ND genes of the NADH complex (see section 1.4.2). However, a technically simpler marker system was identified following phylogenetic studies of sika (Cook, 1993; Nagata, 1995; Cook *et al.*, 1999). A diagnostic polymorphism was identified in the mitochodrial control region. The number of tandem repeats of a 39 base pair sequence was shown to differ between red deer (one repeat) and sika deer (multiple repeats), and also to vary between some Asian sika geographical populations. This marker was tested on the second test panel described above and was considered fully diagnostic for all Scottish red and sika deer populations.

2.3 Laboratory Techniques

2.3.1 Overview

The routine laboratory techniques used in this study are now well established and described elsewhere (Sambrook *et al.*, 1989; Abernethy, 1994a; Slate *et al.*, 1998, Goodman *et al*, 1999). This section therefore summarises the methods used and refers to specific modifications of standard techniques where appropriate. A full list of laboratory protocols can be found in Appendix 3. The laboratory work was carried out by three different people: Simon Goodman genotyped the Argyll samples at 25 microsatellite loci, Antonis Rokas haplotyped the Scotland wide samples for the mtDNA marker and I carried out the Scotland wide microsatellite genotyping.

2.3.2 Sample Collection and Storage

Tissue sample and storage was arranged in two ways. Participants in the study were either supplied with, or had access to, local site freezers, or were sent 7ml sample bottles containing 5ml of 20% Dimethyl Sulphoxide (DMSO) solution saturated with NaCl. Samples were removed from culled deer within 48hrs of death and placed in labelled bags or tubes respectively. The frozen tissue was kept at -20°C until collection (one to twelve months) and then either transferred to a university freezer (-20°C), or left to defrost to allow subsampling

(e.g. foetuses) before being re-frozen for storage. Once retrieved the preservative bottles were again stored at -20°C for DNA extraction. The types and volume of tissue collected varied in relation to whether phenotypic material was supplied (see chapter 6). Heads, jaws, female reproductive organs, kidneys and tongues were all supplied in various combinations and a small piece of tissue (~2cm^3) was sub-sampled from the one of these (and from the foetus if available) for DNA extraction.

2.3.3 Extraction

Initially, DNA was extracted from the samples using a standard phenol-chloroform procedure described in Appendix 3a (Sambrook *et al.*, 1989). However, after problems with some sample DNA amplification, extractions were carried out using the chelex method (Walsh *et al.*, 1991). This method was, faster, used less toxic reagents and produced working template DNA solutions more consistently. For both methods, subsamples of tissue preserved in DMSO solution were initially soaked in 1ml distilled water overnight to leach salt (a polymerase chain reaction (PCR) inhibitor) from the sample. The end products of both extraction techniques (DNA and chelex solutions respectively) were stored at -20 °C until required.

Phenol-chloroform extracted DNA solutions were adjusted to working concentrations for PCR amplification (10x-1000x dilution) following inspection by standard agarose electrophoresis. Diluted aliquots (100μl) were stored in 96-well PCR plates under a mineral oil film at 4 °C prior to use. Chelex extractions were thawed, centrifuged (13000 rpm) for 2 minutes, and then 50μl diluted (2x-10x dilution by trial and error) supernatant was aliquoted directly into 96-well PCR trays and stored at -20 °C ready for use.

2.3.4 Microsatellite Markers

Microsatellite marker loci were amplified with direct incorporation of α-^{32}P. PCR products were separated by denaturing polyacrylamide gel electrophoresis (standard sequencing gels) and visualised by autoradiography (see Slate *et al.*, 1998). Individual

genotypes were assigned by comparison with a standard M13 sequence and a hybrid sample (GAL-027) heterozygous for "red" and "sika" alleles at most marker loci.

2.3.5 D-Loop Mitochondrial Marker

Mitochondrial haplotypes were assayed following (non-radioactive) PCR amplification of a fragment including a 39-bp tandem repeat in the mitochondrial d-loop (Cook, 1993; Goodman *et al.*, 1999). Amplified fragments were separated using 5% Nuseive (Flowgen) gel electrophoresis and were visualised using ethidium bromide staining viewed under UV light (Sambrook *et al.*, 1989; Goodman *et al.*, 1999). Genotypes were assigned in relation to two red and two sika standard individuals on each gel.

3 Genetic Data Set

3.1 Introduction

The previous chapter summarised the sample collection and genotyping methods used to investigate hybridisation between red and sika deer in this study. In this chapter, I describe, using conventional populations genetic statistics, the genetic data set which resulted. Analysis of hybridisation is described in the following chapter (4).

3.2 Overview

The around-Scotland samples were initially screened at twelve microsatellite markers and one mtDNA marker. However, two of the nuclear microsatellite markers were dropped after technical difficulties (FCB048) and concern over diagnostic resolving power (VH054). Samples were grouped by phenotype and location into "populations" representing their proximity to the nearest sika introduction area (Table. 3.1). Populations usually consisted of geographically distant individuals and may not have been true random-mating populations of deer.

As part of a more detailed study of an area with known hybrids, the Argyll samples were screened at 25 nuclear microsatellite markers and one mtDNA marker (Appendix 3). This data set is virtually complete with 90% of genotypes identified. In this chapter, for consistency, the summary statistics for Argyll are restricted to the ten markers used in the around-Scotland survey (the remaining allele frequency data is available in Appendix 4). Summary statistics are presented grouped by phenotype and the three FE districts within Argyll (Kintyre, KINT; Lorne, LOR and Cowal, COW).

Five wapiti (*Cervus canadensis*) samples originating from Canada were supplied by AgResearch, New Zealand and were genotyped at five markers. Wapiti allele frequencies for

four more of the loci (FCB193, BOVIRBP, BM6438 & RM188) derived from a larger set of samples were provided by Dr. M. Tate, AgResearch, N. Z.

All summary statistics presented below were calculated using the programme GENEPOP v3.1d (Raymond & Rousset, 1995).

3.3 Summary Statistics

3.3.1 Allele Frequencies

Population allele frequencies are presented graphically for each of the ten extensively-screened microsatellite markers (Figure 3.1). Overall, there is a general pattern for red to have more alleles than sika and most loci show clear separation of alleles in red and sika populations. However, there are several cases of shared alleles between phenotypic red and sika, which may be due to recent hybridisation, past hybridisation, ancient polymorphism (Goodman *et al.*, 1999), or recent convergent evolution (homoplasy) (Bruford & Wayne, 1993). The complex issues associated with differentiating between these possible sources of variation will be discussed in detail in the next chapter (4), as will the specific cases in each population.

In the Scotland wide survey all mitochondrial haplotypes were concordant with phenotype. MtDNA haplotypes of phenotypic hybrids will be discussed in chapter 4. In Argyll mtDNA introgression was restricted to phenotypic sika deer and occurred sporadically across the sample transect as shown in Table 3.1.

Figure 3. 1 (see over). Allelic distributions for ten presumed diagnostic microsatellite marker loci in the sampled populations. Each page shows a separate locus and contains ten allele frequency diagrams; nine representing the Scottish sika introduction areas and one (lower right) giving the mean allele frequencies of all populations. Each diagram shows the frequency of alleles found in phenotypically red and sika populations in each area, with the total number of alleles counted in each diagram. The Great Glen area contains two sika introduction sites and these are shown as GRG(A) and GRG(B) representing the Aldourie and Glenmazeran introduction areas respectively. On each page the lower right diagram also gives the allele frequency distribution for wapiti estimated from this study (n=5 individuals), or provided by Dr. M. Tate, Agresearch, NZ(indicated *).

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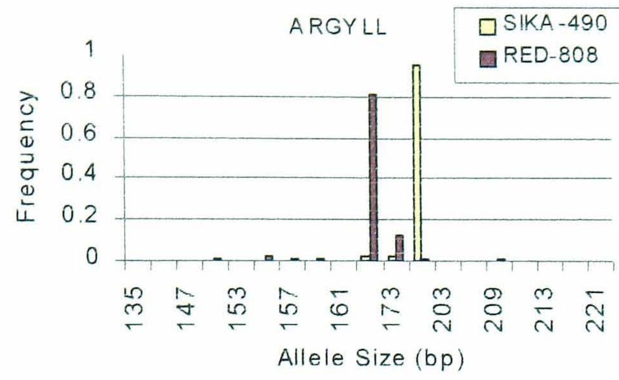

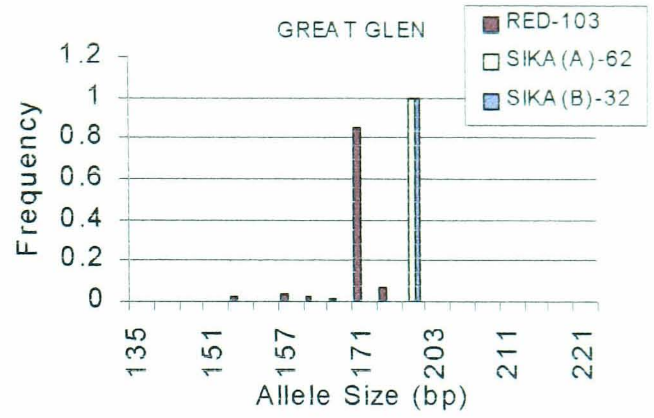

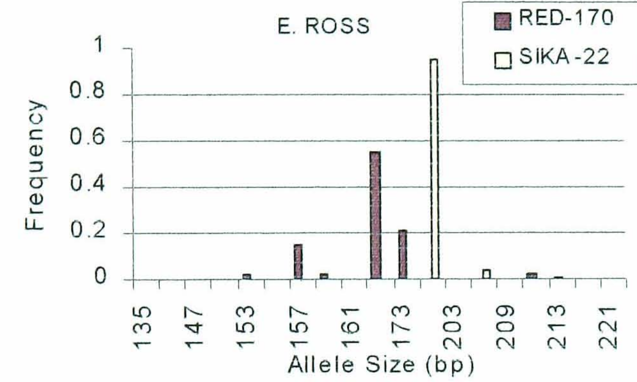

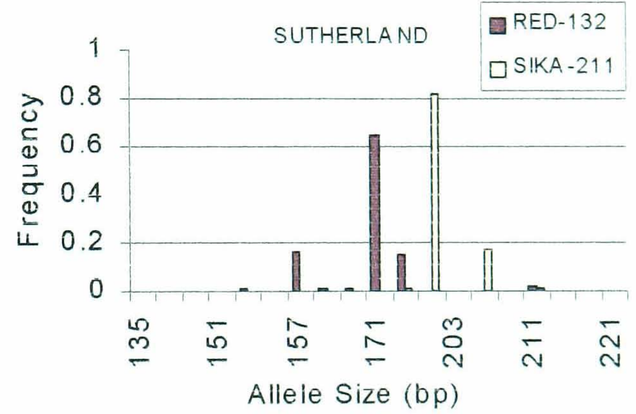

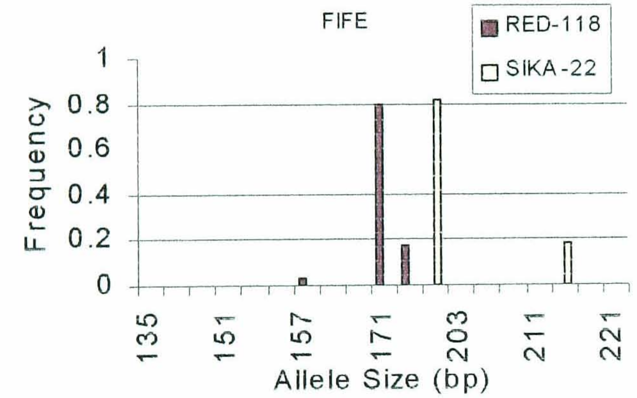

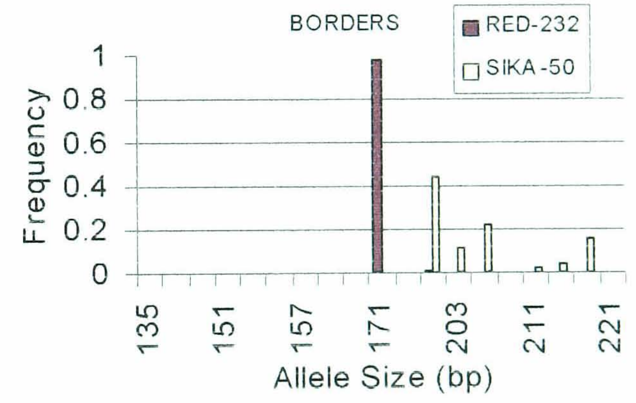

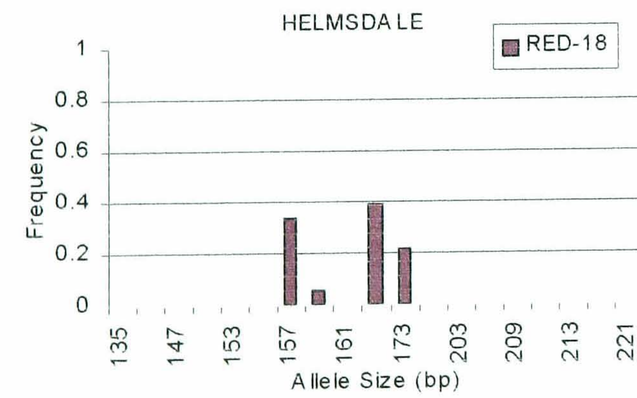

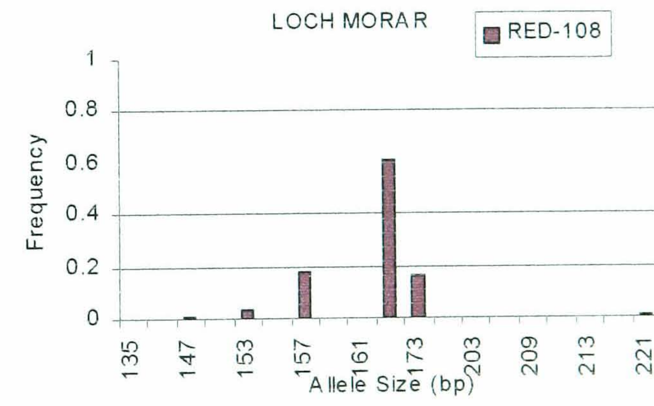

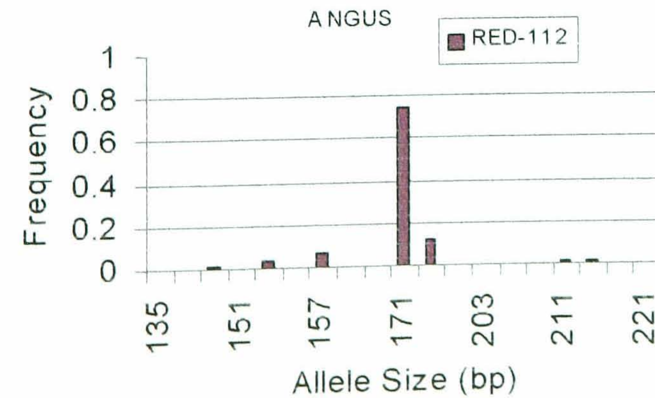

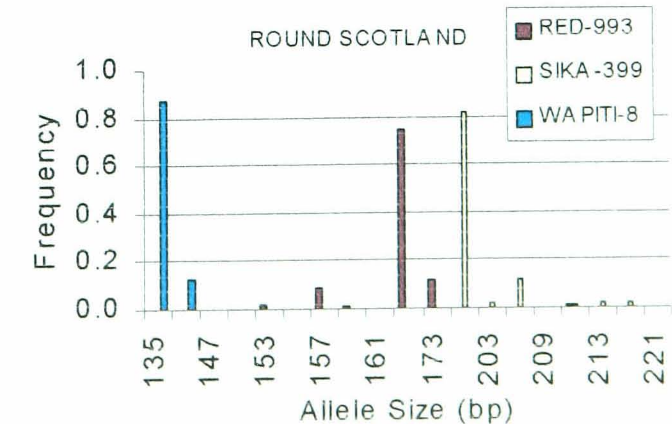

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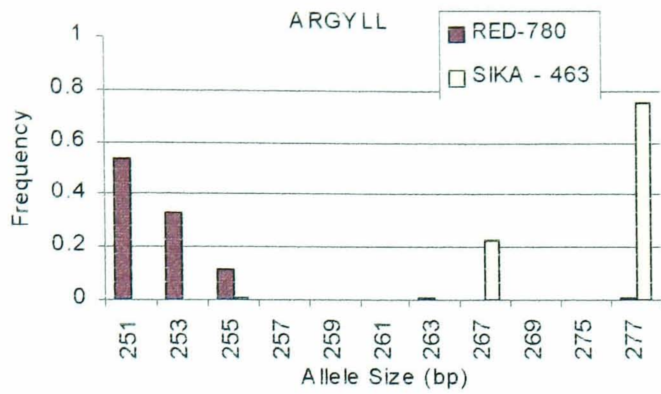

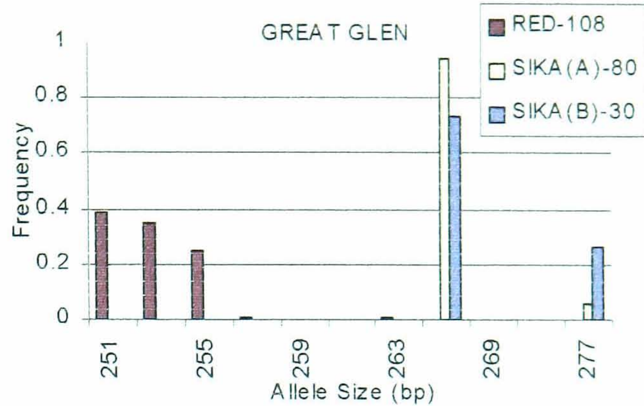

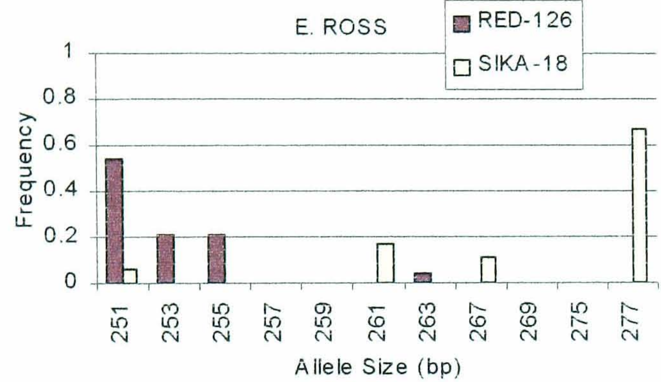

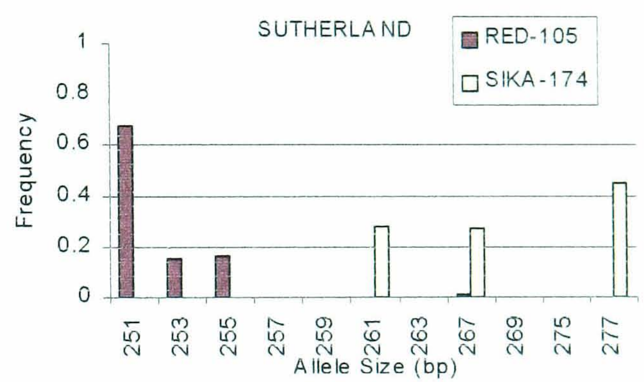

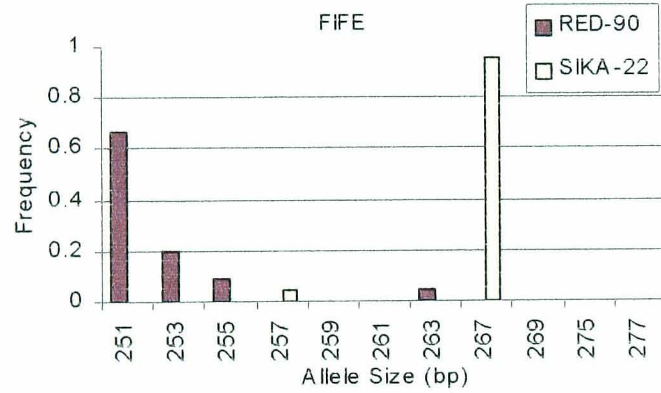

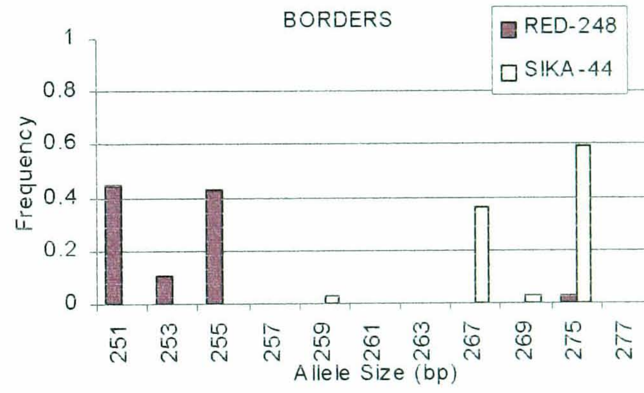

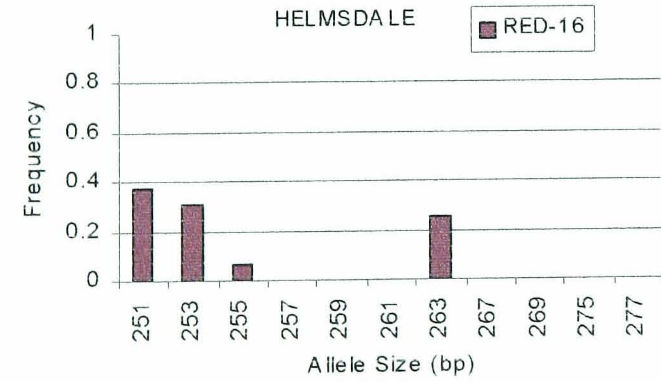

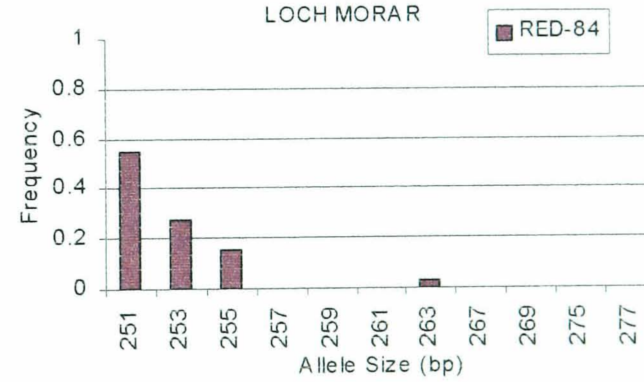

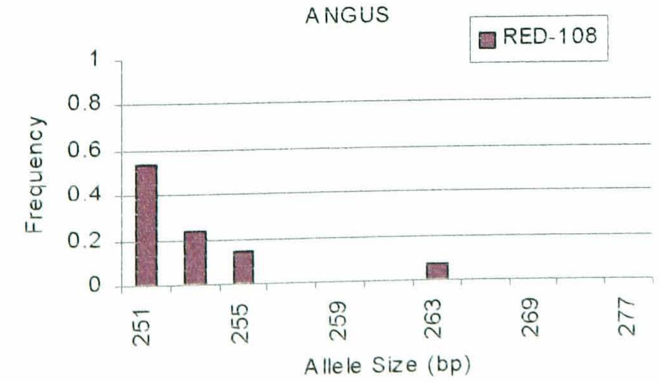

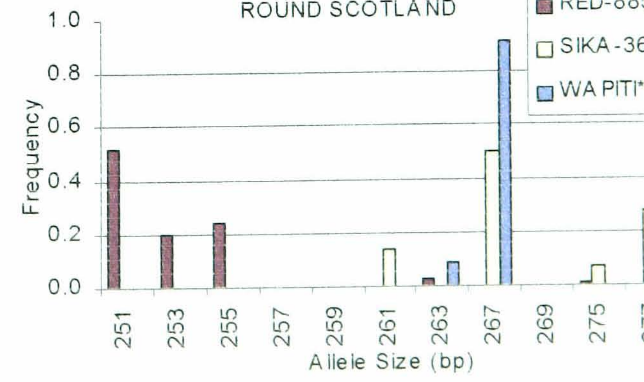

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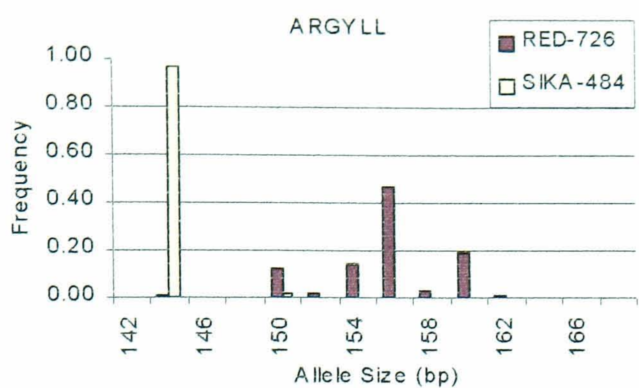

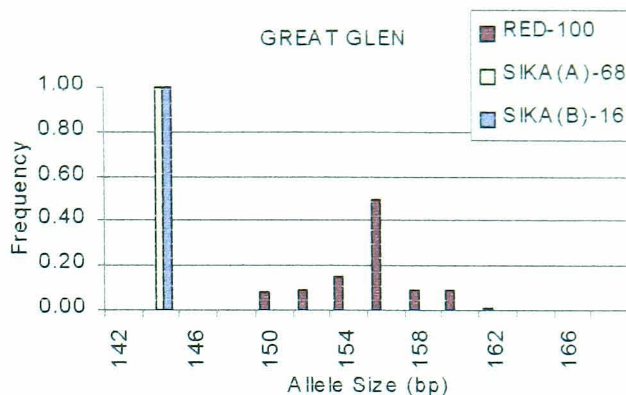

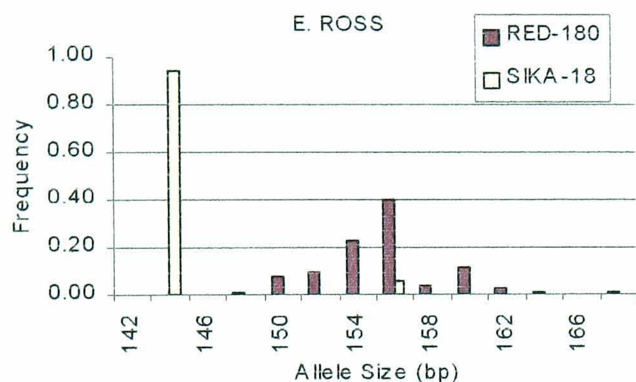

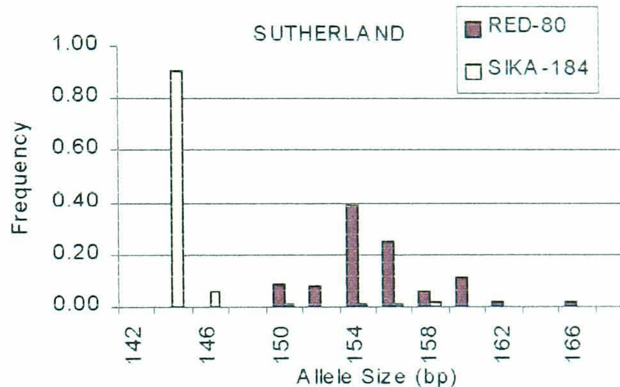

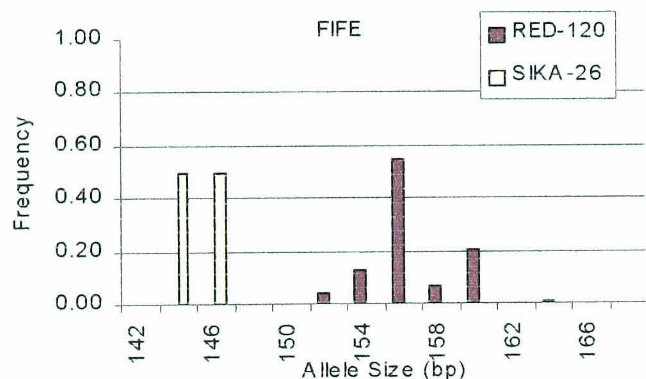

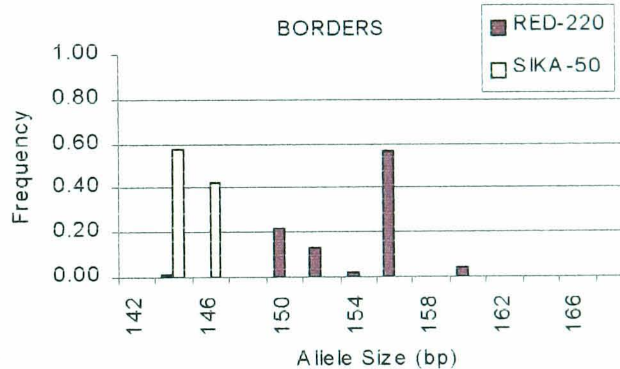

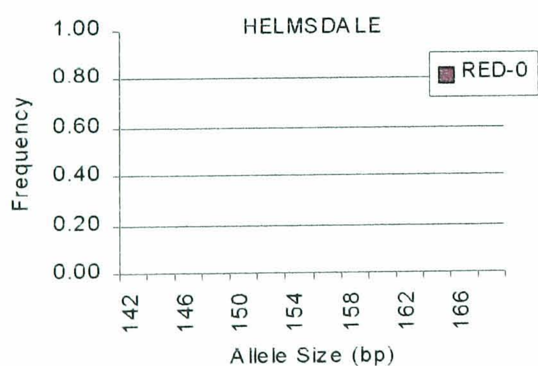

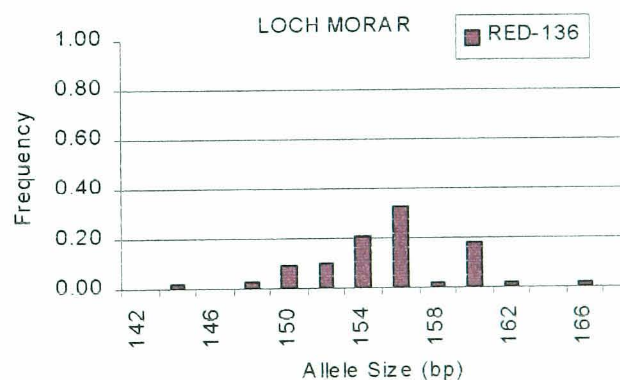

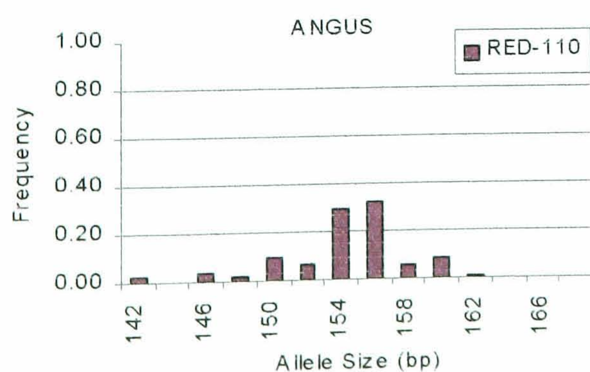

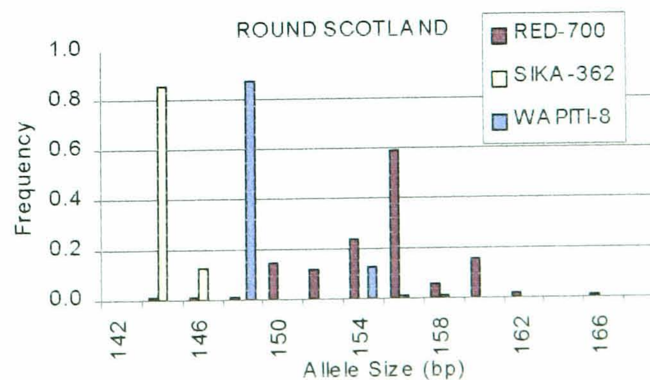

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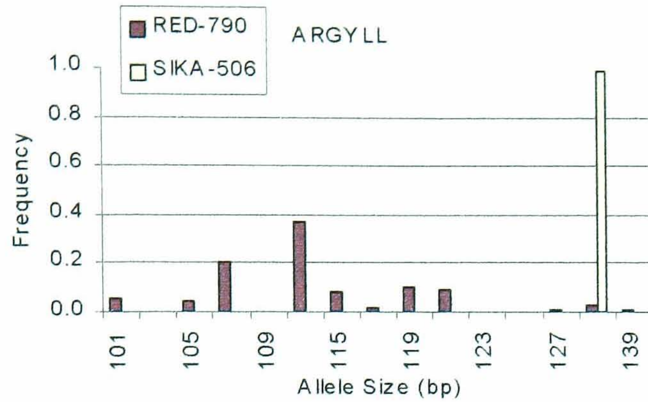

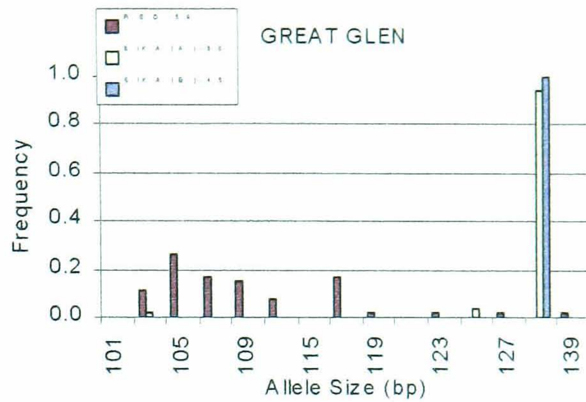

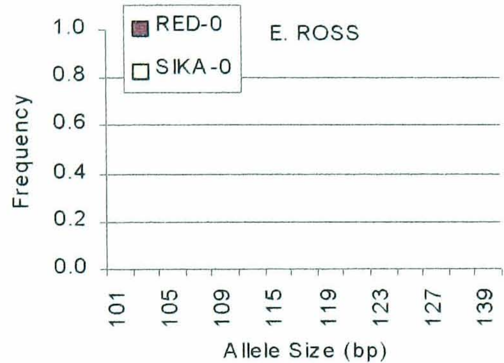

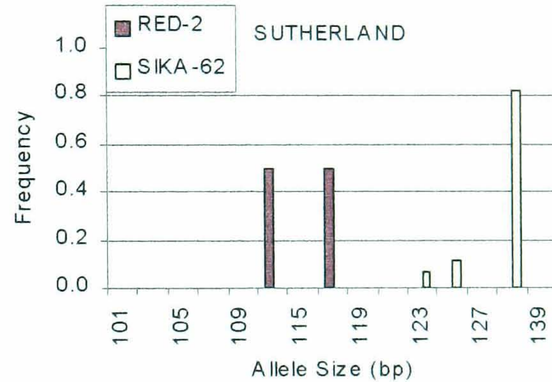

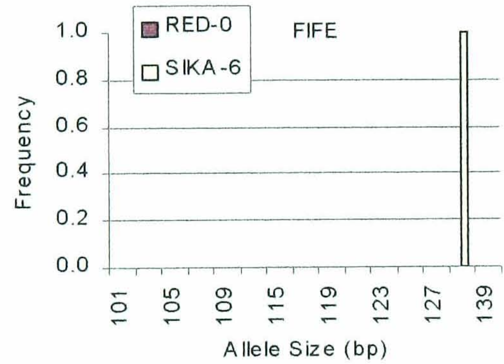

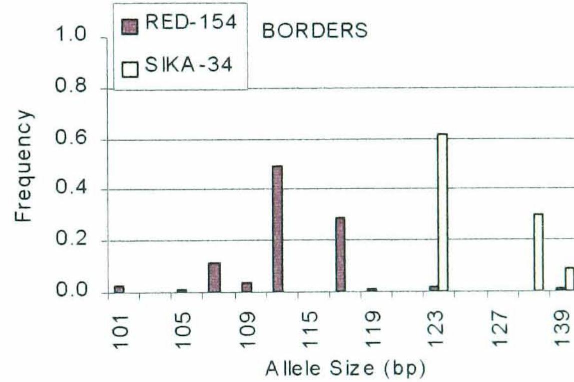

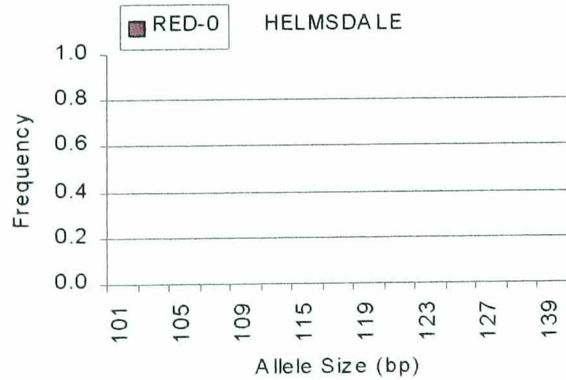

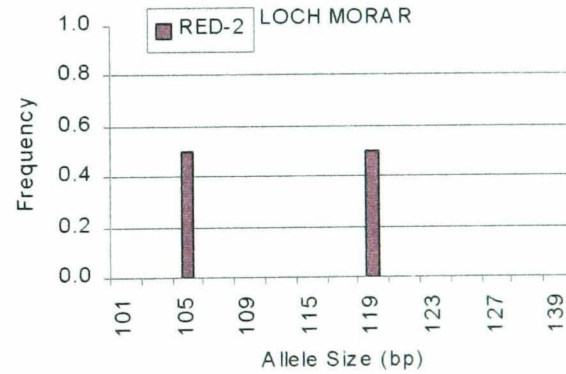

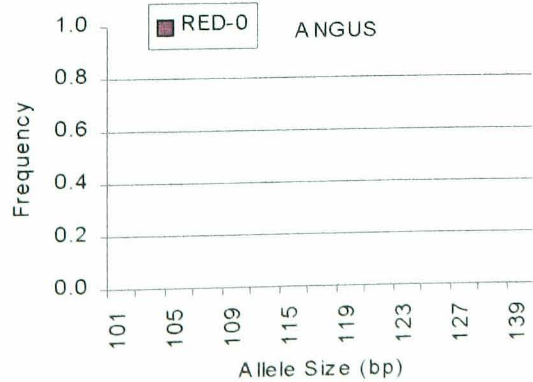

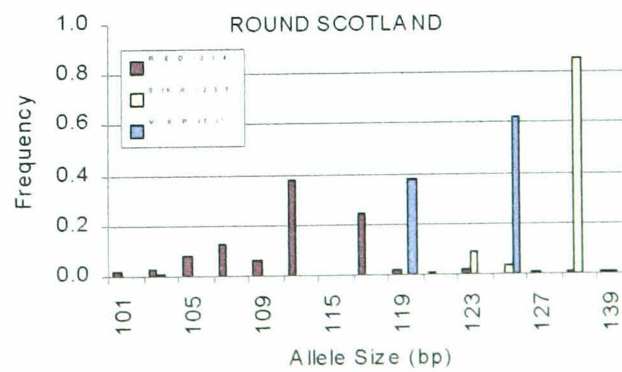

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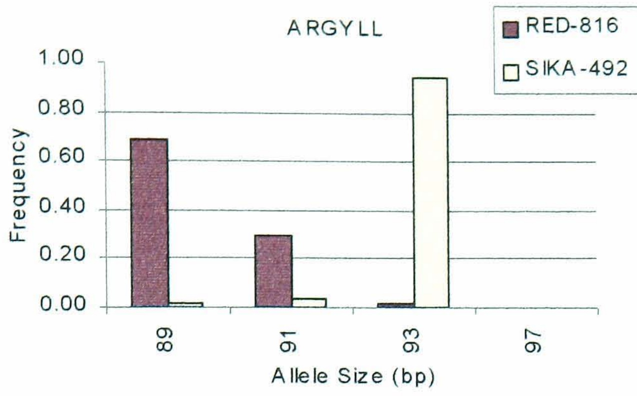

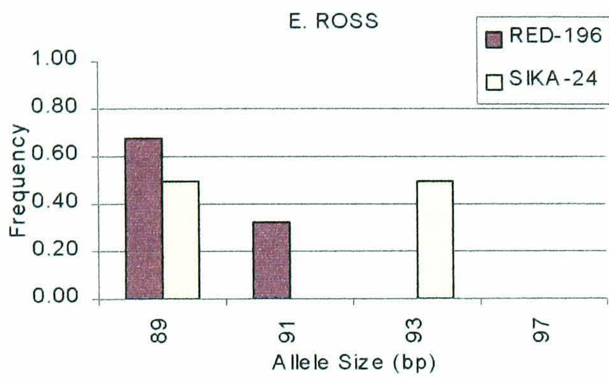

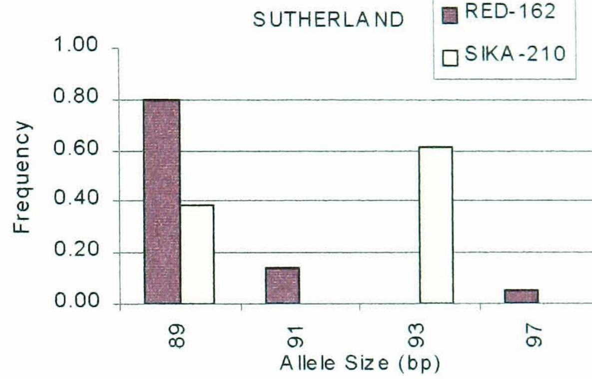

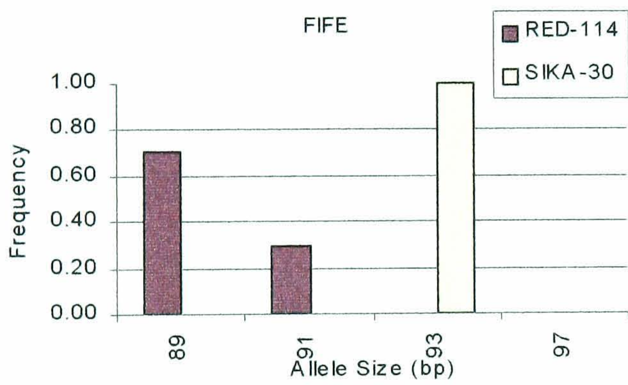

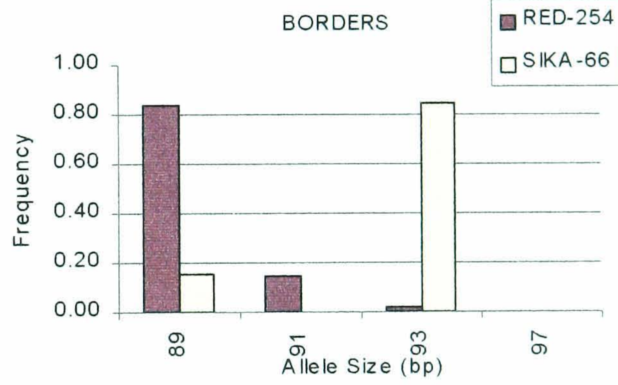

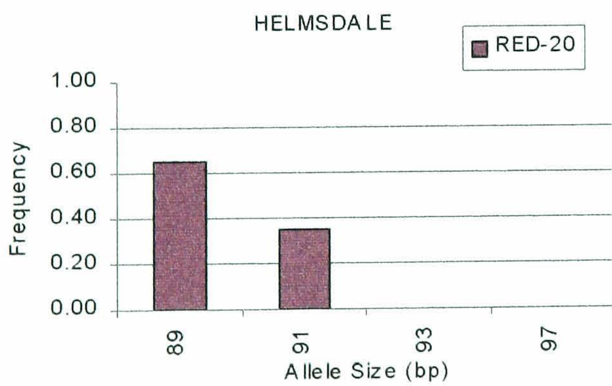

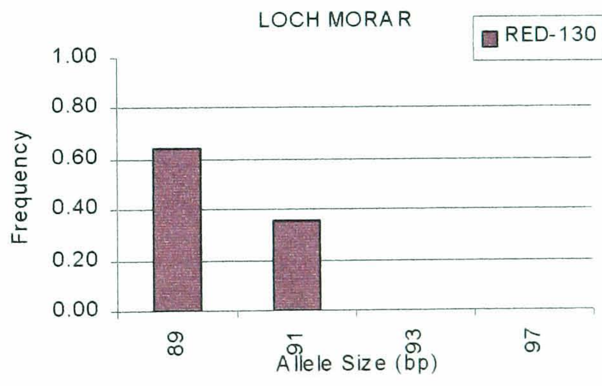

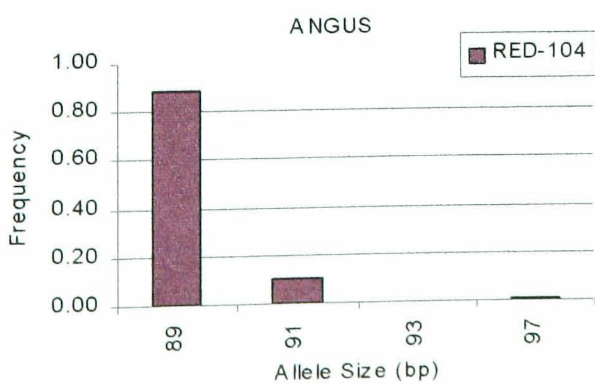

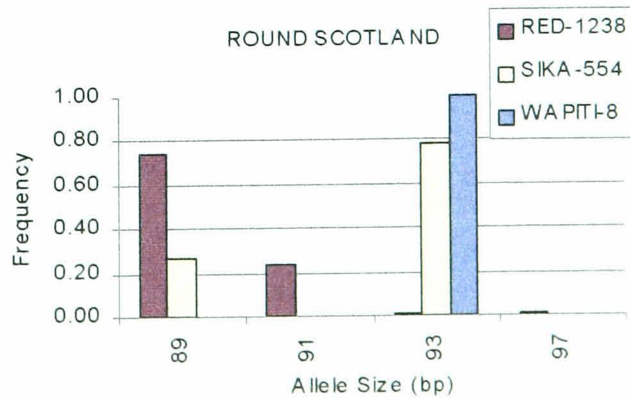

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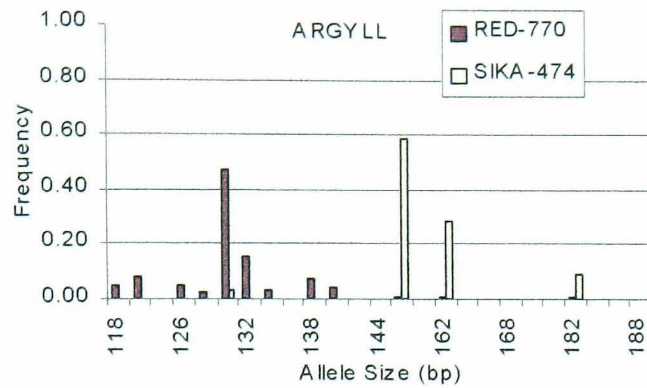

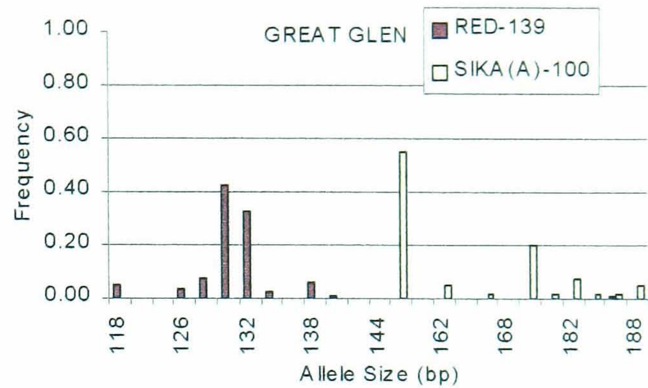

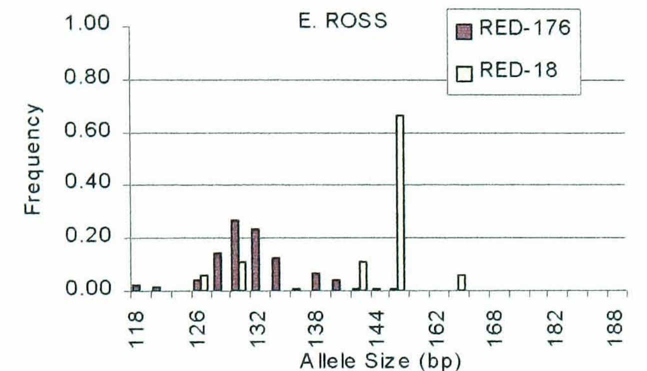

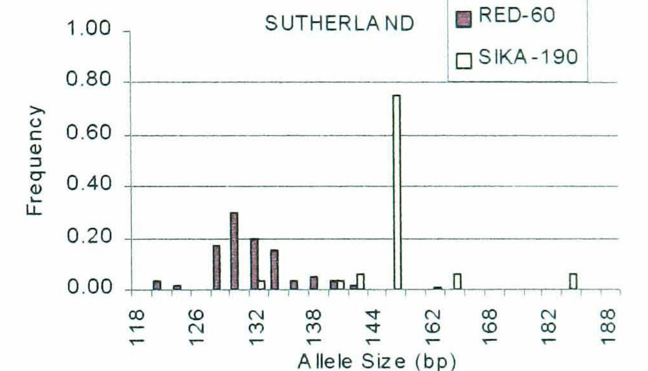

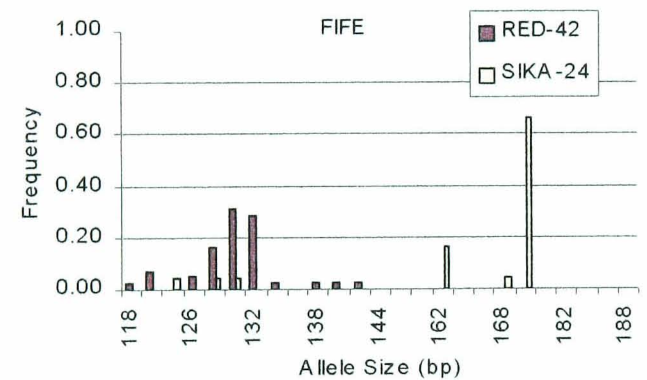

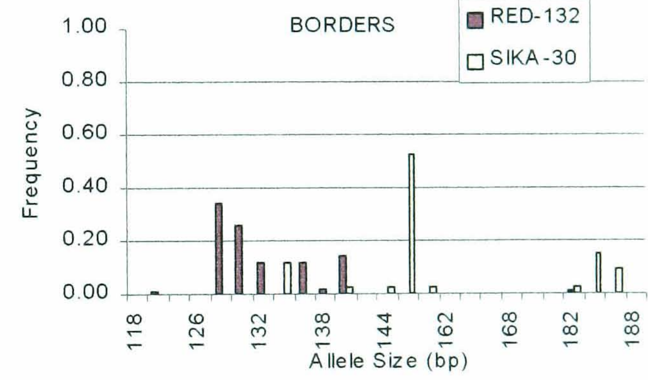

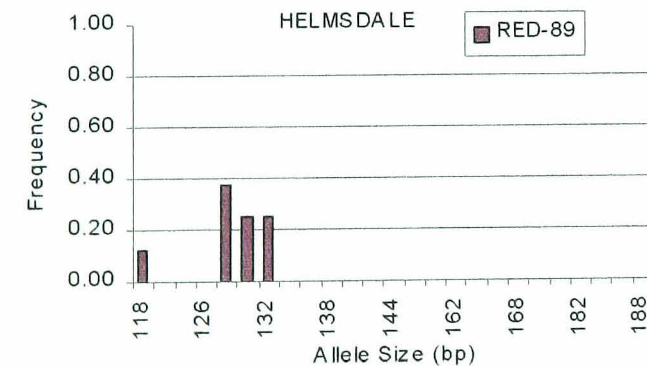

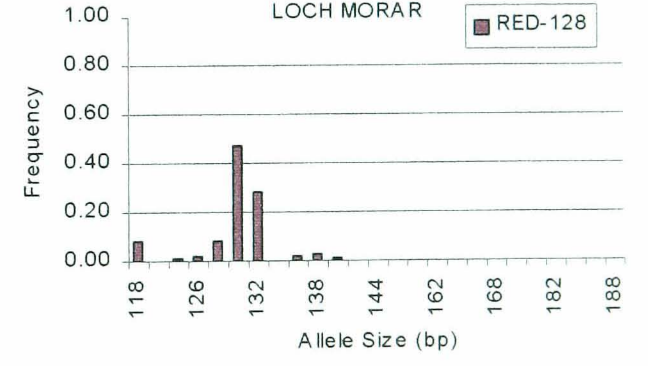

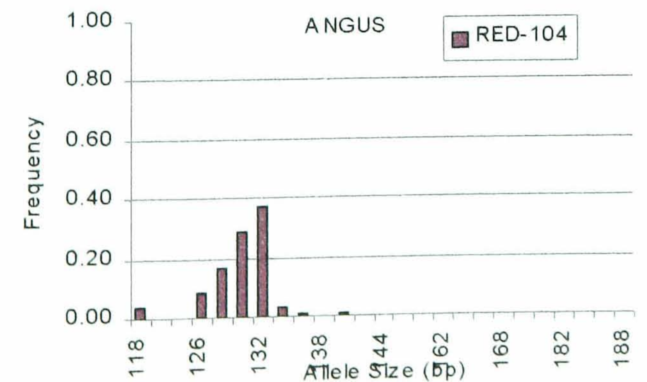

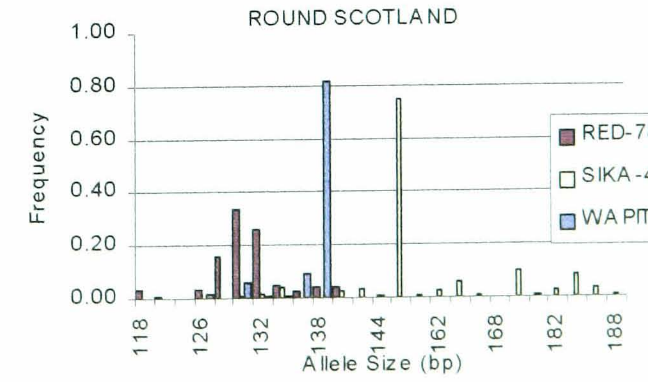

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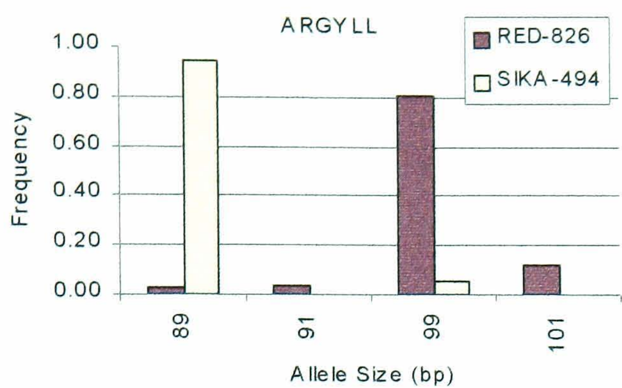

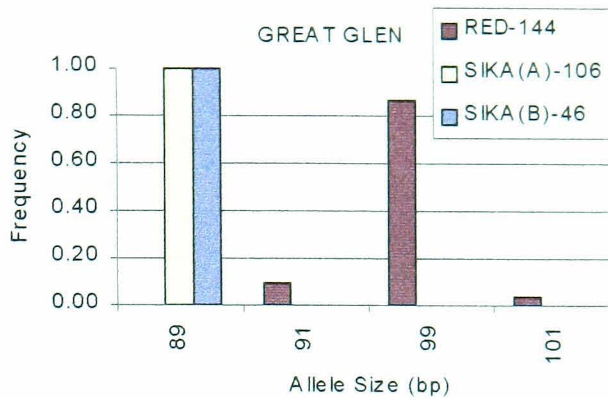

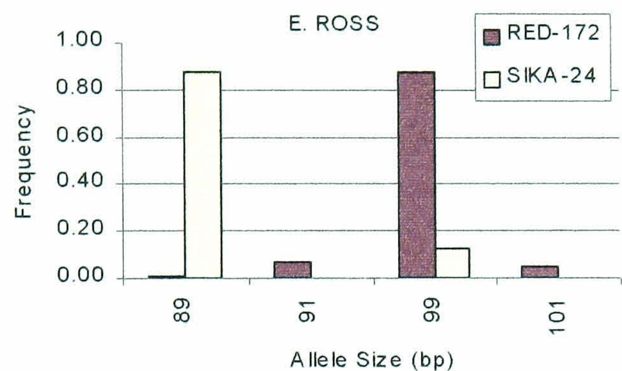

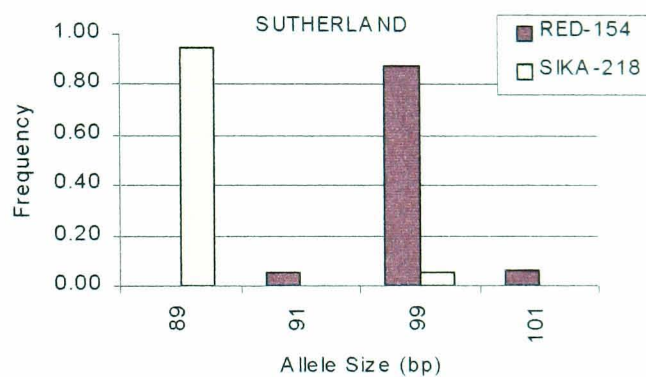

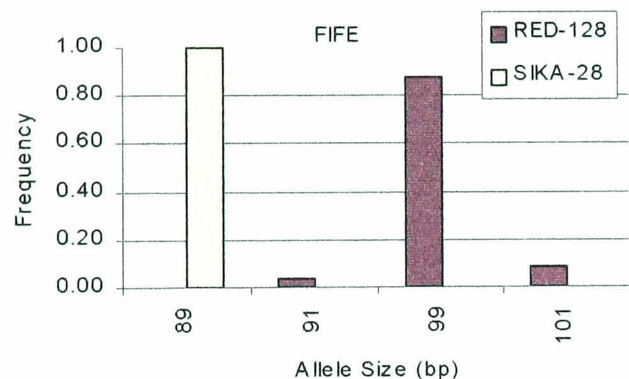

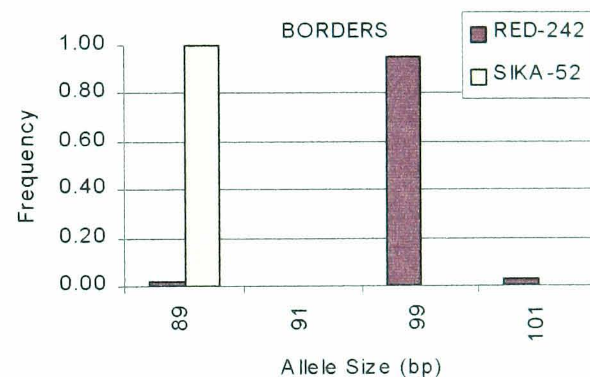

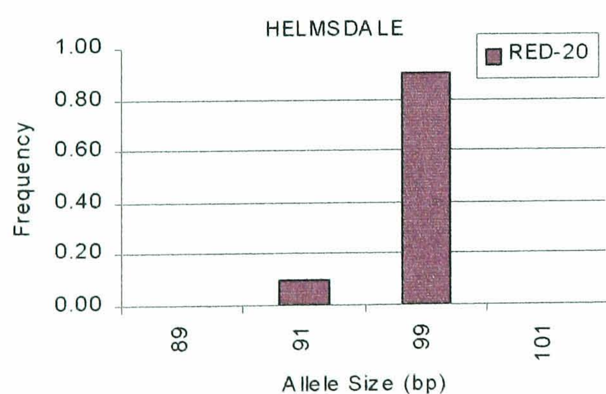

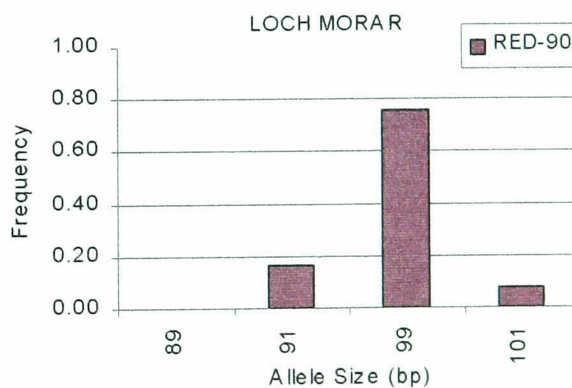

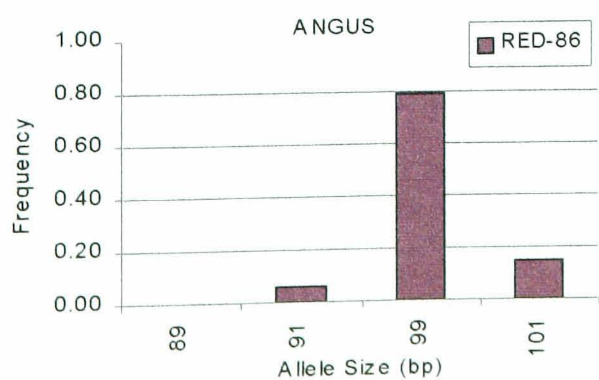

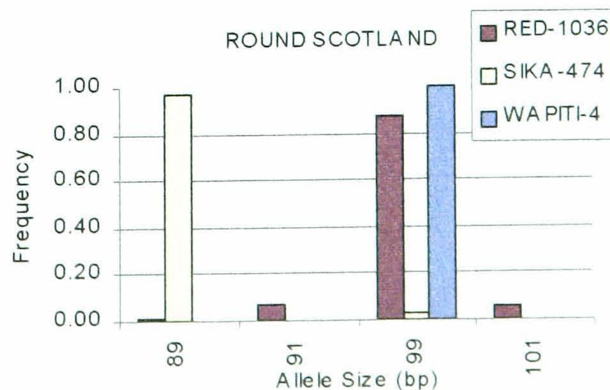

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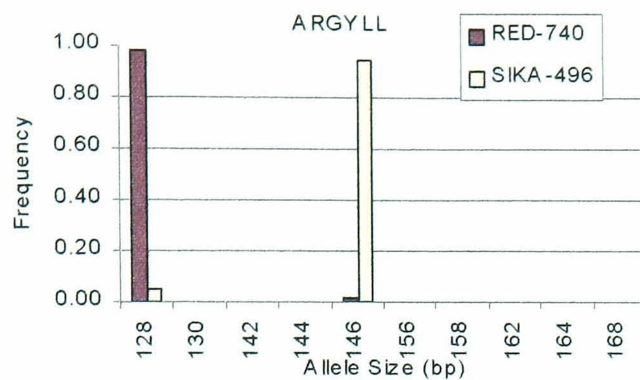

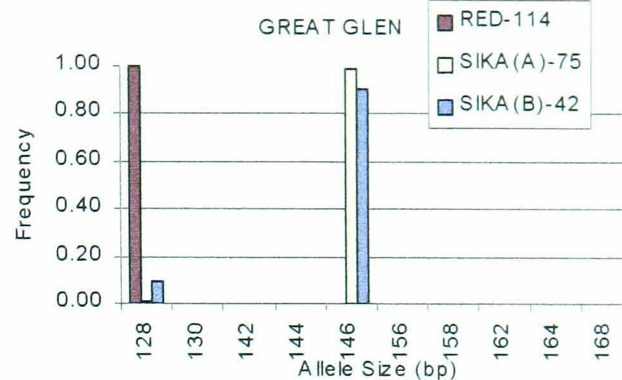

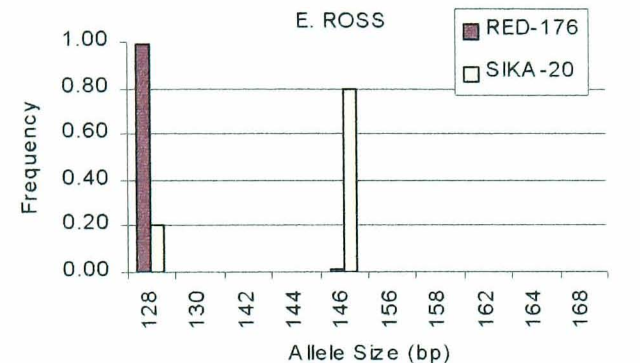

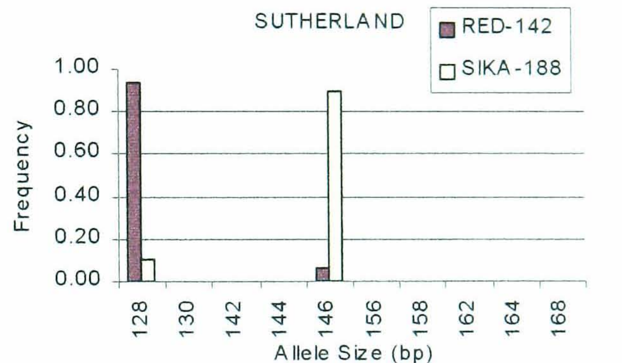

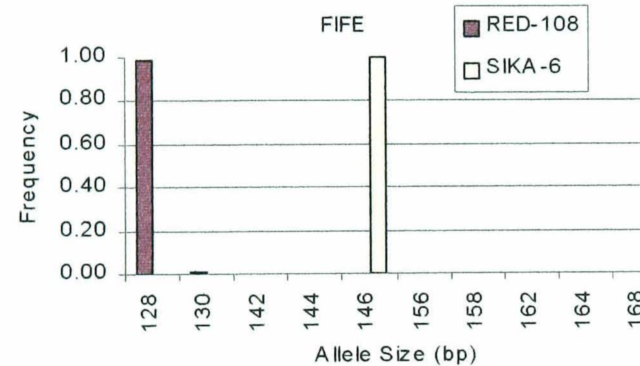

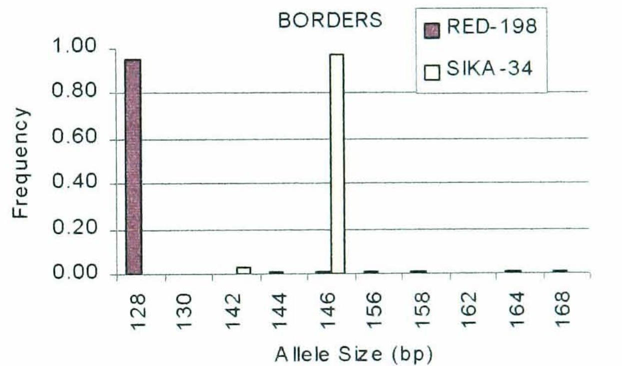

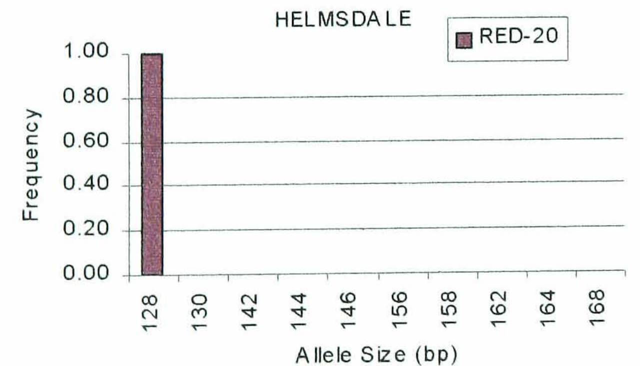

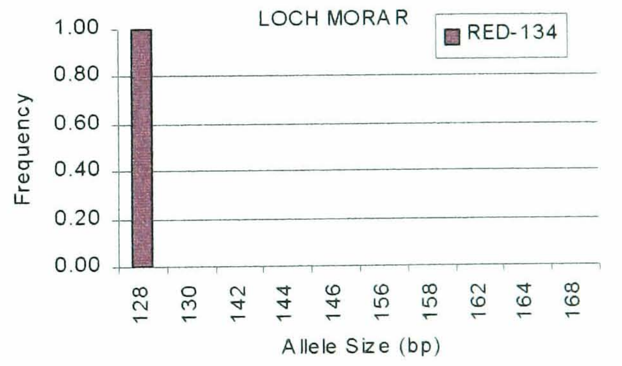

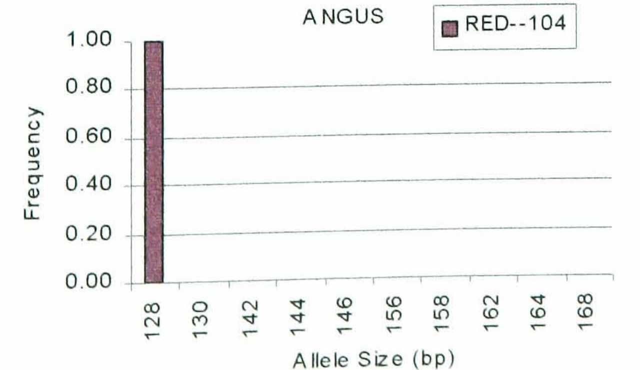

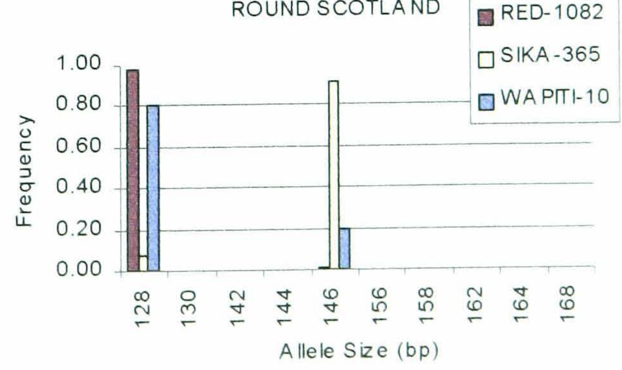

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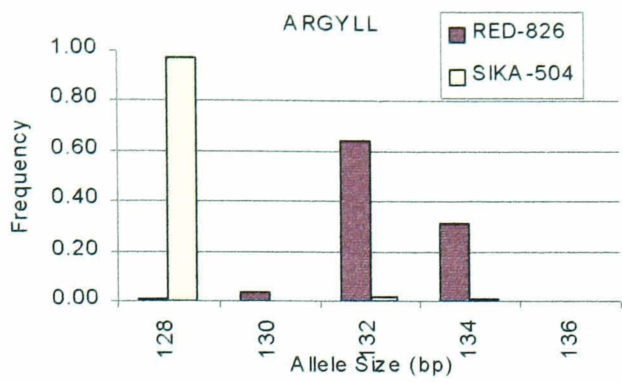

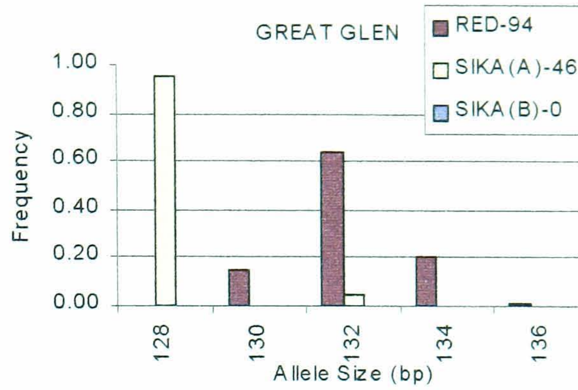

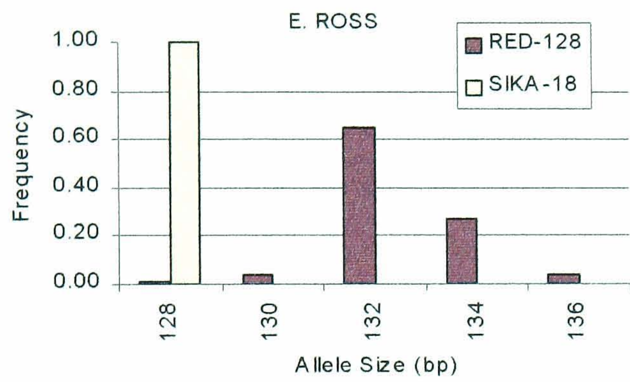

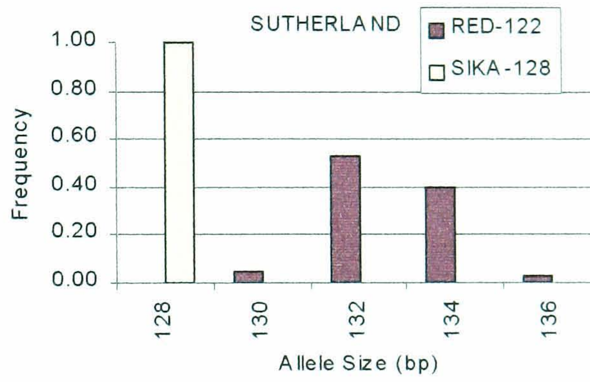

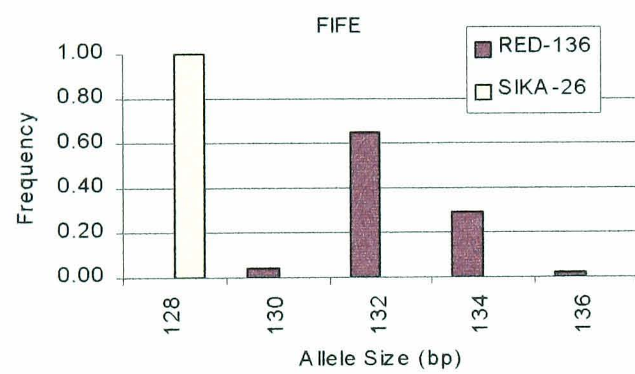

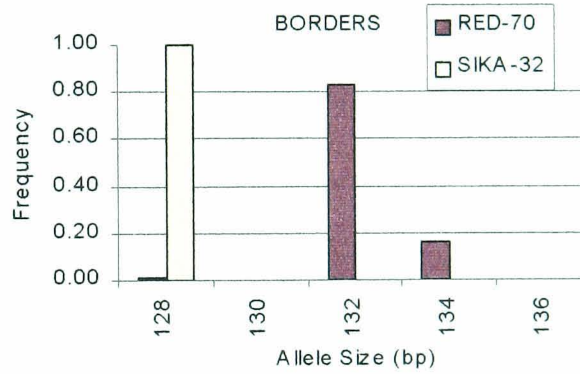

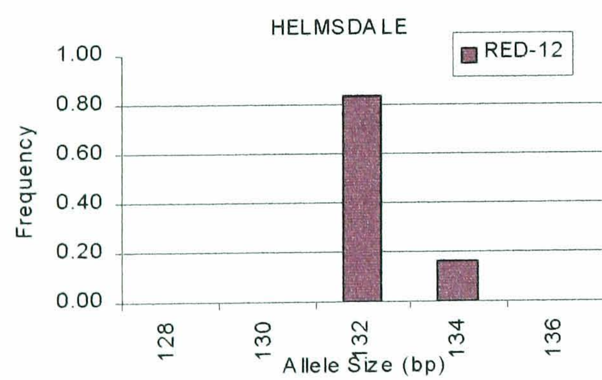

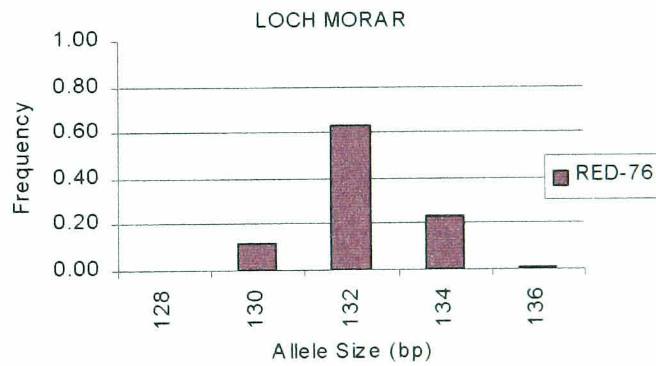

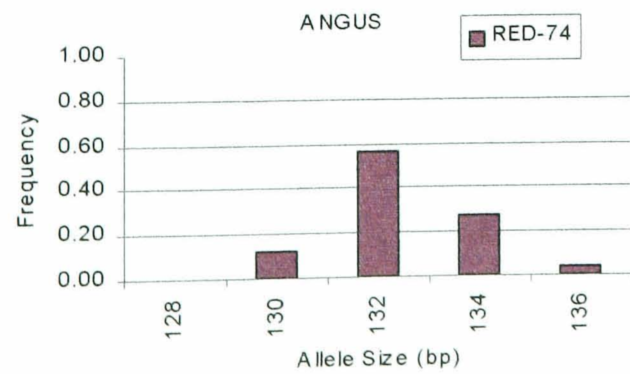

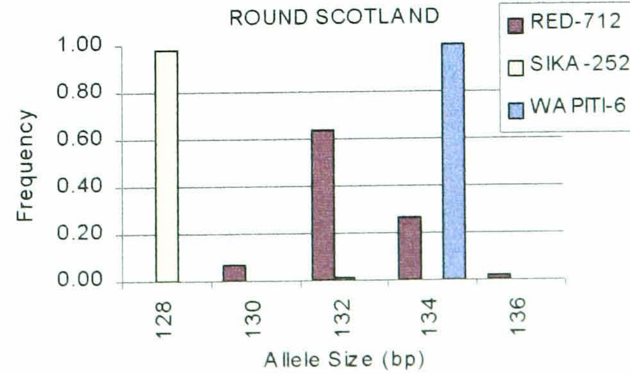

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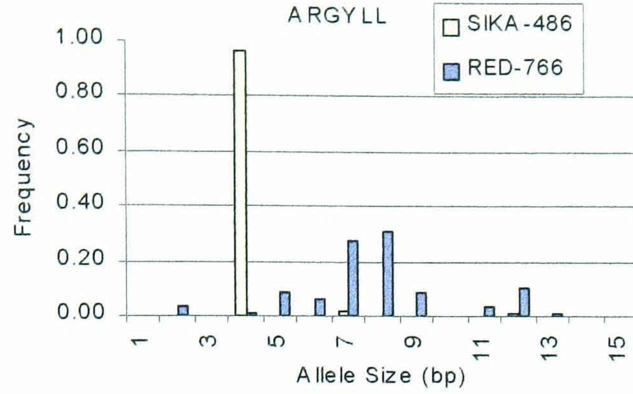
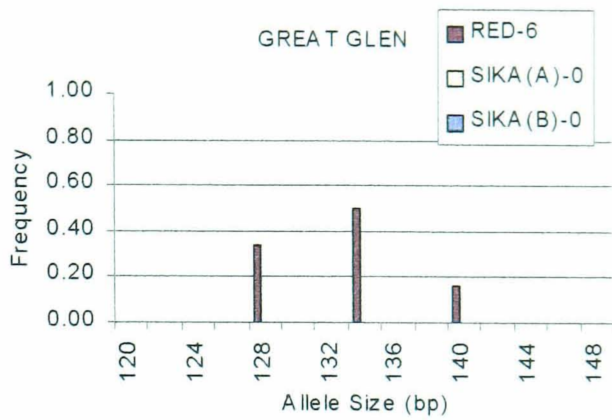
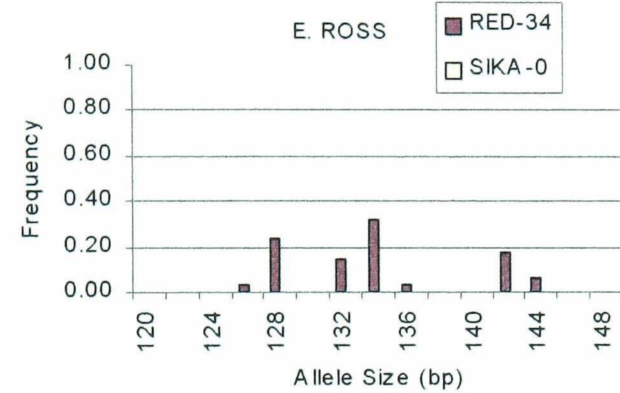
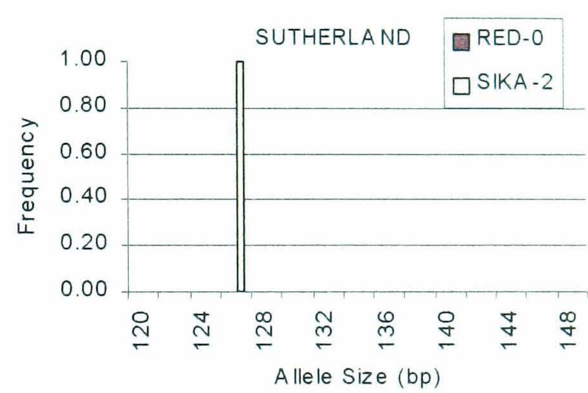
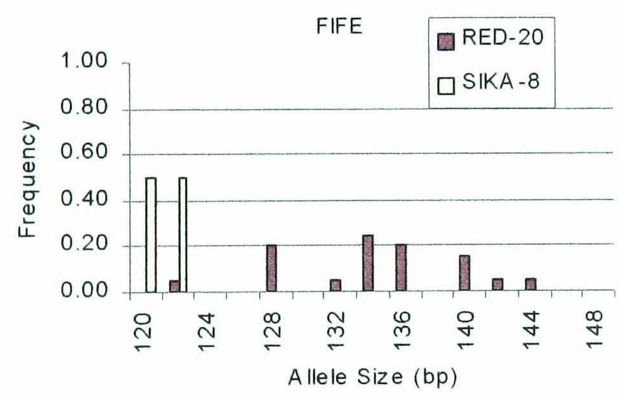
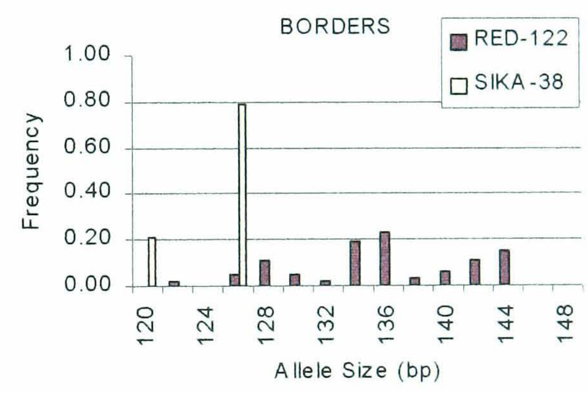


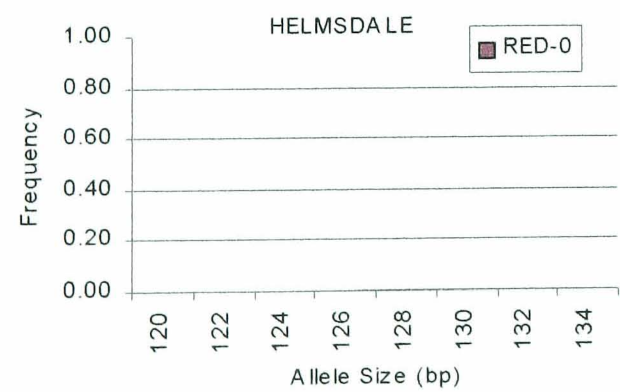


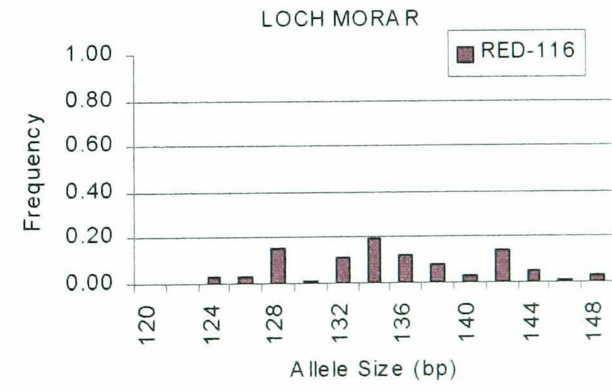


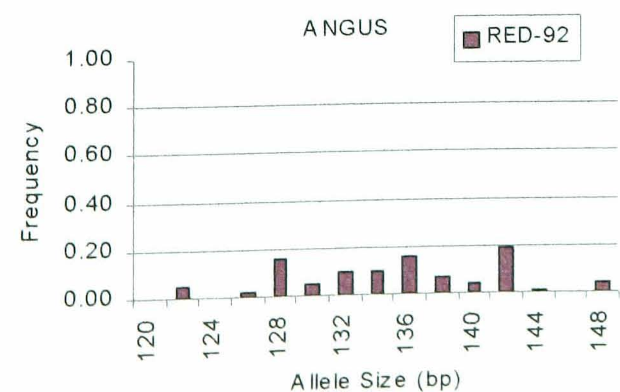












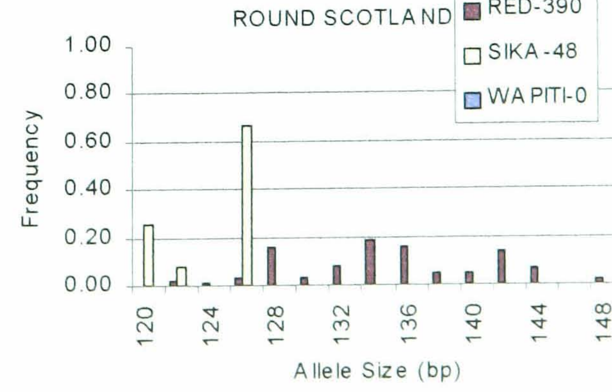


Table 3.1 Mitochondrial DNA haplotypes listed by population and phenotype in samples from Argyll.

	Phenotype					
	Red		Sika		Hybrid	
Mt DNA haplotype Population	red	sika	red	sika	red	sika
Carradale				26		
Lussa	2		5	76		
Tarbert South				17		
Tarbert North	8			2		
Achaglachgach	24			24		
Knapdale	25		4	31		
Kilmichael	59			44		
Inverliever	20				2	
Eredine	81			1		
Loch Avich	31		11		5	
Shira	22					

3.3.2 Heterozygosity

Population variability was assessed by calculating the expected heterozygosity (H_e). This measure gives the expected proportion of heterozygotes under H-W equilibrium (calculated from the allele frequencies) in the population sample. Table 3.2 shows individual population heterozygosity estimated for each locus and the average heterozygosity across all loci. Although there is considerable heterogeneity between loci, the pattern across populations is consistent within each taxon. Based on average expected heterozygosity, red deer populations were significantly ($p<0.001$; $t = 7.9$; d.f. 16) more variable ($H_e = 0.5$) than sika populations ($H_e = 0.23$). Within sika populations, the Borders sika had the highest heterozygosity ($H_e = 0.38$).

Table 3.2 Expected heterozygosity estimates (H_e) for each of ten microsatellite markers tested in the Argyll (*) and Scotland wide populations. Average heterozygosities across loci are given in the final column and average within locus estimates at the foot of the red and sika phenotype rows.

Locus / Population	RME025	BM6438	BOVIRBP	FCB193	MM012	BM4006	INRA005	RM188	INRA006	RM095	Average Heterozygosity	Standard error
LOR-RED*	0.33	0.59	0.68	0.83	0.48	0.20	0.00	0.71	0.48	0.79	0.51	0.08
COW-RED*	0.05	0.44	0.60	0.81	0.49	0.35	0.00	0.74	0.47	0.84	0.48	0.09
KINT-RED*	0.37	0.60	0.73	0.82	0.42	0.36	0.05	0.74	0.50	0.79	0.54	0.08
GRG-RED	0.27	0.69	0.71	0.86	0.47	0.25	0.05	0.72	0.54	0.73	0.53	0.08
ERS-RED	0.68	0.61	0.76	-	0.44	0.22	0.01	0.83	0.51	0.81	0.54	0.09
STH-RED	0.53	0.42	0.77	-	0.33	0.23	0.12	0.83	0.57	-	0.47	0.09
HEL RED	0.73	0.74	-	-	0.48	0.19	0.00	0.82	0.30	-	0.47	0.12
LMO-RED	0.57	0.61	0.81	-	0.46	0.40	0.00	0.68	0.54	0.88	0.55	0.09
ANG-RED	0.42	0.63	0.79	-	0.21	0.35	0.00	0.75	0.60	0.89	0.52	0.10
FIF-RED	0.34	0.51	0.64	-	0.42	0.23	0.02	0.80	0.49	0.87	0.48	0.09
BOR-RED	0.03	0.61	0.61	0.66	0.28	0.09	0.09	0.78	0.29	0.86	0.43	0.10
RED MEAN	0.39	0.59	0.71	0.80	0.41	0.26	0.03	0.76	0.48	0.83	0.50	0.08
KINT-SIKA*	0.09	0.38	0.05	0.02	0.11	0.10	0.10	0.57	0.05	0.06	0.15	0.06
GRG(A)-SIKA	0.00	0.12	0.00	0.11	0.10	0.00	0.03	0.65	0.09	-	0.12	0.07
GRG(B)-SIKA	0.00	0.40	0.00	0.00	0.00	0.00	0.18	0.61	-	-	0.15	0.08
ERS-SIKA	0.09	0.54	0.11	-	0.52	0.23	0.34	0.56	0.00	-	0.30	0.08
STH-SIKA	0.31	0.66	0.18	0.31	0.49	0.11	0.19	0.44	0.03	0.00	0.27	0.07
FIF-SIKA	0.31	0.09	0.52	0.00	0.00	0.00	0.00	0.54	0.00	0.57	0.20	0.08
BOR-SIKA	0.52	0.53	0.50	0.54	0.26	0.00	0.06	0.69	0.00	0.34	0.34	0.08
SIKA MEAN	0.19	0.39	0.19	0.16	0.21	0.06	0.13	0.58	0.03	0.24	0.22	0.05

3.3.3 Inbreeding Coefficient (F_{is})

The Hardy-Weinberg (H-W) equilibrium predicts that in large random mating populations neutral genotype distributions (e.g. AA, Aa, and AA) are expected in proportion to their respective allele frequencies (A = p and a = q) in the following ratio:

p^2	:	$2pq$	:	q^2
AA	:	Aa	:	aa

Deviations from equilibrium can be measured using Wrights's (1951) inbreeding coefficient (F_{is}) which gives the difference between expected and actual genotype frequencies [$(H_{exp} - H_{obs})/H_{exp}$].

The interpretation of any deviation must be made in the context of the sample population, especially when investigating two potentially hybridising taxa. The general approach to studies of hybrid zones is to calculate inbreeding coefficients for populations across the hybrid zone, regardless of phenotype, as hybridisation is either known or assumed to be common (Barton & Hewitt, 1985; Abernethy, 1994a,b). In this case, high F_{is} values could be interpreted as evidence of infrequent hybridisation, strong assortative mating, low fitness of hybrids, immigration of parental types (and migration of hybrids), or a combination of these factors. Conversely, low values would indicate random mating within the population, no selection against hybrids etc. Differences in H-W equilibrium between loci can arise through selection of specific genes or drift of neutral material.

However, in this study, hybridisation was thought to be rare (section 1.4.2), so geographical populations were subdivided by phenotype prior to the analysis (see Goodman *et al.*, 1999) and inbreeding coefficients were then calculated to test the assumption that the populations were discrete random mating units. Choosing to separate the populations by phenotype reverses the relationships between F_{is} values and interpretation described above. Heterozygote deficits may now be *caused* by hybridisation, especially if there is spatial

Table 3.3 Population- and locus-specific inbreeding coefficients (F_{is}). Positive values indicate a heterozygote deficit and negative values an excess. Statistically significant deviations from random gametic union are indicated for those p-values less than 0.0004 [α=0.05, k= 127; Bonferroni test, (Rice, 1988)]. SS indicates small samples (<10) and M indicates monomorphic locus.

Locus POPULATION	RME025	BM6438	BOVIRBP	FCB193	MM012	BM4006	INRA005	RM188	INRA006	RM095
LOR-RED	0.11	0.03	0.13	0.05	0.07	-0.09	M	0.04	0.16	0.11
COW-RED	-0.01	-0.01	0.17	0.23	0.35	0.09	M	0.01	-0.05	-0.07
KINT-RED	0.00	0.18	0.03	0.11*	0.05	0.03	0.14	-0.02	0.11	0.03
GRG-RED	0.20	0.25	0.10	0.22	0.08	0.06	M	0.34*	0.21	SS
ERS-RED	0.27*	0.37*	0.18	SS	0.00	-0.09	M	0.10	0.17	-0.10
STH-RED	0.32	0.12	0.19	SS	0.23	-0.04	0.88*	0.03	-0.05	SS
HEL RED	SS	SS	SS	SS	-0.05	-0.06	M	SS	SS	SS
LMO-RED	0.23	0.10	0.31*	SS	0.13	0.28	M	0.03	0.01	0.18
ANG-RED	0.19	-0.03	0.10	SS	-0.11	0.14	M	0.03	0.23	0.07
FIF-RED	0.10	0.04	0.35	SS	0.25	-0.03	M	0.23	-0.11	0.20
BOR-RED	0.01	0.33*	-0.04	0.12	-0.06	0.16	0.21	0.04	0.22	0.01
KINT-SIKA	0.18	0.12	0.14	0.33*	0.27*	0.11	0.20	0.13*	0.24	0.25
GRG(A)-SIKA	M	-0.05	M	0.38	0.71*	M	M	0.36*	1.00	SS
GRG(B)- SIKA	M	0.01	SS	M	M	M	-0.08	0.26	SS	SS
ERS-SIKA	M	SS	SS	SS	-0.06	0.65	SS	0.00	SS	SS
STH-SIKA	-0.11	-0.06	0.17	0.06	-0.05	0.52*	0.22	-0.10	M	SS
FIF-SIKA	0.43	M	-0.04	SS	M	M	SS	0.08	M	SS
BOR-SIKA	0.02	0.32	0.28	0.20	-0.16	M	M	0.33	M	0.39

variation in occurrence (for example in areas with some hybrids). Hybridisation and immigration will tend to

produce heterozygote deficits across loci, whereas population subdivision through drift or selection are more likely to produce differences between loci.

Table 3.3 gives locus- and population-specific inbreeding coefficients and shows where there was significant deviation from H-W equilibrium under the null hypothesis of random union of gametes. Given the large number (127) of comparisons made, a critical p-value of 0.0004 was chosen to reduce the chances of a Type 1 statistical error [α=0.05, k= 127; sequential Bonferroni test, (see Rice, 1988)].

No locus showed consistent heterozygote deficits across populations, suggesting that null alleles are not at high frequency at the study loci. Previous work by Goodman *et al.* (1999) had estimated null allele frequencies at BOVIRBP (0.057) in the Kintyre sika and red populations respectively, but there is no evidence of any significant effect at BOVIRBP in the larger samples presented here, although the Loch Morar red deer do have a deficit at this locus.

Only one red deer population (Easter Ross, ERS-RED) had a significant heterozygote deficits at more than one locus. In this population the samples originated from a small area and were unlikely to have any intra-population subdivision which could have explained this result. Furthermore, there were alleles present at low frequency that are characteristic of sika deer and in this population heterozygote deficit may be caused by hybridisation (see chapter 4).

One sika population (Kintyre) had a significant heterozygote deficit at three of the ten loci screened. This population contains individuals from across the range of a previously described hybrid zone (Abernethy, 1994a,b; Goodman *et al.*, 1999) and the pattern of a heterozygote deficit across several loci may reflect this (see chapter 4).

3.4 Conclusions

In most areas where they were sampled, phenotypic red showed higher levels of microsatellite variation than sika. None of the loci used showed clear evidence of widespread null alleles, and only one of the populations screened showed consistent departures from H-W equilibrium across several loci. It appears that except for the Kintyre sika, hybridisation is not disrupting H-W genotype frequencies. Mitochondrial DNA introgression was only evident in sika-like deer and one population, at the edge of the sika range (Loch Avich), contained entirely red mt DNA. The interpretation of these results will be discussed in the following chapter (4).

4 Hybridisation

4.1 Introduction

This chapter reports on the central theme of this study: the identification of hybrids between red and sika deer and the extent of subsequent introgression in each population. Ideally, all genetic markers used would be completely diagnostic between the taxa under investigation. However, following the wider screening of Scottish populations, a number of ambiguous alleles were identified that were not present in the initial test panel data. This chapter begins with some methodological points that arose from this discovery.

4.2 Analysis Methods

4.2.1 Identification of hybrids

Individual deer samples were defined as hybrid if they contained genetic material characteristic of both taxa. Specifically, hybrids were defined under any of the following circumstances: (a) the sample was heterozygous for alleles characteristic of red and sika at a microsatellite locus, (b) at a microsatellite locus the sample had two alleles characteristic of one taxon, in a background of alleles characteristic of the other taxon at other microsatellite loci, (c) the sample had mtDNA characteristic of one taxon, but microsatellite alleles characteristic of the other taxon. It follows that the attribution of alleles to taxon needs to be accurate and conservative.

As the Scotland wide survey proceeded, it became clear that loci that appeared entirely diagnostic in the original test panel of 44 red and 44 sika were not always so. The commonest kind of ambiguity arose when rare alleles in the red-like population could not be attributed to sympatric sika, because they were absent in the local sika-like population, but they were found in other Scottish sika populations. For example some northern red deer

populations such as Angus and Easter Ross had rare alleles at 211 and 213 bp at RME025, but the only sika population in which these alleles were present was the Borders population some 200 km away (Figure 3.1). Ambiguous alleles were most often defined as ones that were present in both red-like and sika-like deer across Scotland, but were not found in the local population of the opposite taxon. Another example is that of the allele at 89 bp at MM012 in the Borders sika. This allele was found in all red-like populations, but appeared at high frequency in the Borders sika-like deer. As this sika population only very recently (5 years ago) came into contact with red deer, it is unlikely that this allele reached such high frequency through recent hybridisation.

Table 4.1 lists the alleles used to assign taxonomic status at each of the 25 microsatellite marker loci and all cases of ambiguous alleles. The interpretation of specific ambiguous alleles will be discussed in the results section below (section 4.3).

As a result of the allele attribution shown in Table 4.1, two classes of hybrids were defined. Class A hybrids were defined on the basis of alleles which appeared completely diagnostic in that population, i.e. in the red or sika columns in Table 4.1. Class B hybrids were defined on the basis of alleles which appear in the "ambiguous alleles" column of Table 4.1. These definitions were population specific as some alleles were considered diagnostic in some populations, but ambiguous in others. No ambiguous alleles were identified for the additional 15 microsatellite loci screened in Argyll, because there was no round Scotland data to compare them to, and in Argyll all alleles were assigned to red or sika based on which phenotypic group they were most common in.

Six samples that contained entire discordant nuclear genotypes were re-assigned to the opposite phenotypic group and were assumed to have been mis-labelled.

Table 4.1. Attribution of alleles to taxa at 25 putatively diagnostic microsatellite markers (see section 4.2). See Chapter 3 for allele frequencies for populations. The lower 15 markers in italics were screened only in the red-sika test panel (44 of each taxon) and Argyll (by S. Goodman). Ambiguous alleles may occur at these loci upon wider screening.

Locus name	Sika alleles	Red alleles	Ambiguous alleles (population)	Reasons for designating ambiguous alleles
RM0E025	197, 203, 207, (211, 215, Borders) (213 Bord. & Fife)	147, 151, 153, 155, 157, 159, 161, 171, 173, 221	211,213, 215 (Argyll, E. Ross, Sutherland, Angus red)	Low frequency in red pops but not found in local sika, except Borders &/or Fife sika.
BM6438	259, 261, 267, 275, 277	251, 253, 255, 263	257 (Fife sika & G.G. red)	Rare in both Fife sika and Great Glen red, but not found in local opposite taxa
BOVIRBP	144,146	142, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 174		
FCB193	125, 129 (123, 139 Borders)	101, 103, 105, 107, 109, 111, 115, 117, 119, 121, 127	123 (G.G. red & Sutherland sika) , 139 (Argyll & G.G. red)	Rare in G.G. red, but not found in G.G. sika; rare in Sutherland sika, but not found in Suth. red; common in Borders sika
MM012	93	89,91,97	89 (Borders sika)	High frequency in Borders sika without other introgression
BM4006	89	91-99		
INRA005	146	128		
RM188	144 (Borders), 146, 152, 162, 164, 166, 168, 178, 180, 182, 184, 186, 188	118, 120, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142,	144 (Easter Ross red)	Low frequency in E. Ross red but not found in local sika: rare in Borders sika
INRA006	128	130-136		
RM095	126, (120 Borders), (122 Fife)	124, 128, 130, 132, 134, 136, 140, 142, 144, 146, 148	122 (Argyll red & Borders red)	Rare in Argyll red, but not found in Argyll sika; rare in Borders red, but not found in Borders sika; common in Fife sika
IDVGA055	213-215	191-207,221		
UWCA047	239	227-233		
BM757	175	163-167,177-207		
INRA131	107	93-105		
IDVGA37	218	198-216		
TGLA126	105	109		
IDVGA029	159	139-149		
TGLA040	108	94-104		
TGLA337	128	132-148		
TGLA127	157	165-189		
AGLA293	144	126-142		
RM012	115	123-163		
HUJ175	139	115-133		
FCB048	155,157	143,145		
FSHB	181	185-212		

4.2.2 Calculation of hybrid indices

Nuclear genotypes were classified as homozygous red (2), heterozygous hybrid (1) or homozygous sika (0) and mtDNA haplotypes as red (1) or sika (0). The proportion of red microsatellite alleles per individual was then calculated to give a Hybrid Index (HI); i.e. "pure" red samples were classified as 1, "pure" sika as 0 and hybrids intermediate. Indices were calculated on the basis of all hybrid alleles (both classes, A & B), as the majority of Class B alleles were thought to be introgressed (see discussion, section 4.4).

4.3 Results

4.3.1 Extensive Scotland wide survey

The number of hybrids identified from each of the Scotland wide populations is shown in Table 4.2. Small numbers of hybrids were found in all phenotypically red populations except Helmsdale. The majority of red-like class A hybrids were found in three populations (Borders, 13/144; E. Ross, 7/117; and Sutherland, 6/88) and almost half (9/21) of class B hybrids were found in just one population, Angus. All phenotypically red deer had red mtDNA, regardless of hybrid status.

Although hybrids were found in all sika-like populations, their frequency varied greatly between areas. 91% of all class A phenotypically sika hybrids were found in three of the sampled populations (Sutherland, 86/110; Borders, 11/38; and E. Ross, 10/11). A single class B phenotypically sika hybrid was identified in the Fife population, and a single phenotypically sika sample from Sutherland had red mtDNA (see below).

Only one of the eight phenotypic hybrids sampled in the Scotland wide survey was confirmed as a genetic hybrid. This sample was shot near New Galloway on the east edge of the Galloway Forest Park. It had red mtDNA and numerous microsatellite loci heterozygous for red and sika alleles.

Table 4.2 Distribution of hybrids in the Scotland wide survey. Class A hybrids are defined on the basis of alleles characteristic of the opposite taxon, while class B hybrids are defined on the basis of ambiguous alleles (see section 4.2.1). MtDNA hybrids contained mtDNA of the opposite to taxon to alleles present at microsatellite loci. The mtDNA haplotype is shown for individual phenotypic hybrids (R = red deer). Dashes indicate not tested.

Population	Phenotype	Total	Non-Hybrid	Hybrid Class A	Hybrid Class B	mtDNA hybrids
Great Glen	Red	91	88	1	1	0
Easter Ross	Red	117	109	7	4	0
Sutherland	Red	88	80	6	2	0
Helmsdale	Red	10	10	0	0	0
Loch Morar	Red	76	73	3	0	0
Angus	Red	63	52	2	9	0
Fife	Red	81	80	0	1	0
Borders	Red	144	127	13	4	0
	Total	670	619	32	21	0
Great Glen (A)	Sika	70	65	5	0	0
Great Glen (B)	Sika	22	18	4	0	-
Easter Ross	Sika	11	1	10	0	0
Sutherland	Sika	110	23	86	0	1
Fife	Sika	16	14	1	1	-
Borders	Sika	38	24	11	0	0
	Total	267	145	117	1	1
Great Glen (A)	Hybrid	3	3		0	-
Great Glen (B)	Hybrid	0	0	0	0	0
Easter Ross	Hybrid	0	0	0	0	0
Sutherland	Hybrid	1	1	0	0	R
Fife	Hybrid	2	2	0	0	-
Borders	Hybrid	2	1	1	0	R,R
	Total	8	7	1	0	N/A

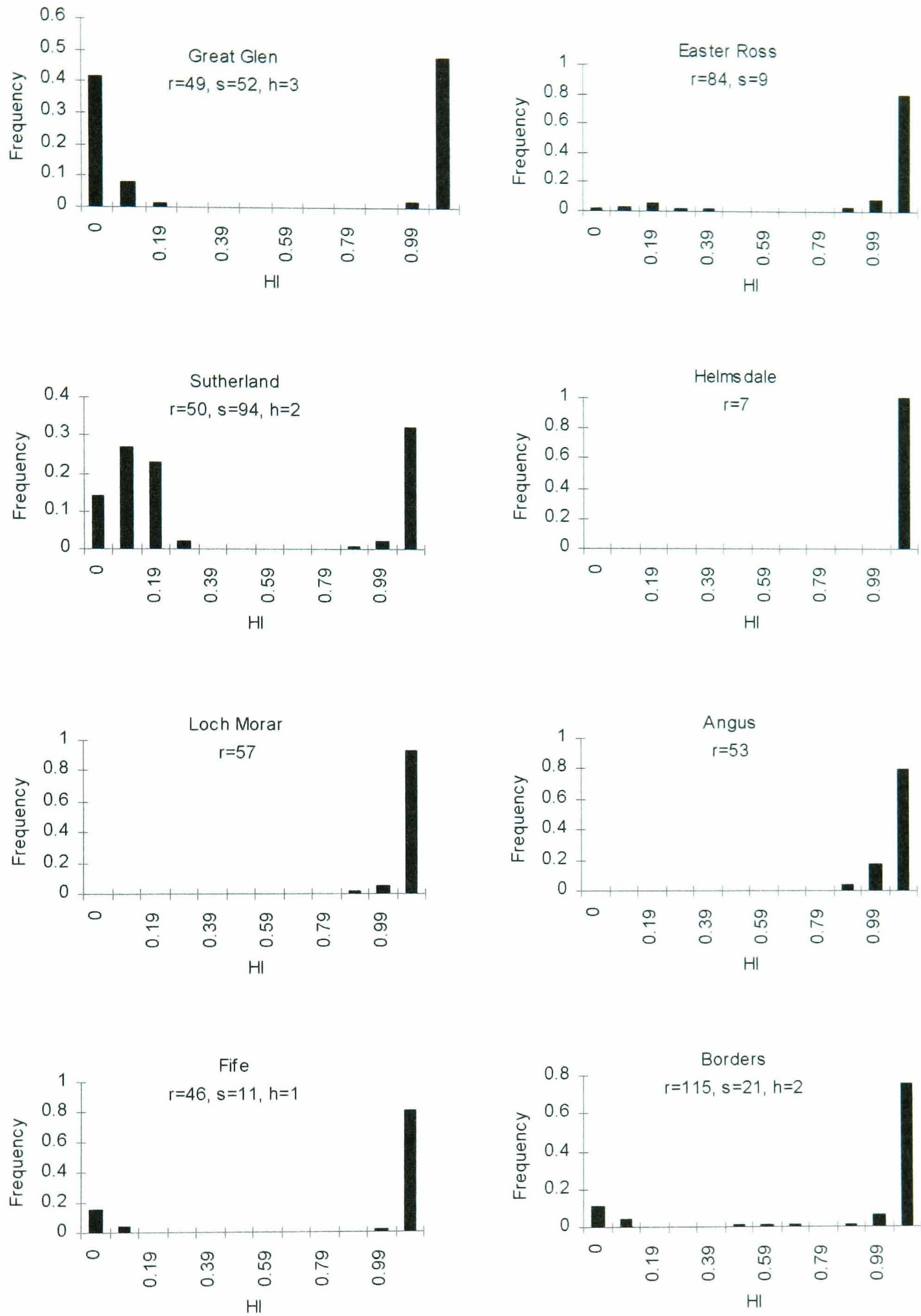


Figure 4.1 Frequency distributions of Hybrid Indices (HI) in populations in the Scotland wide survey. HI is the proportion of red alleles found in each sample at 6-10 microsatellite loci (both A and B hybrids included). 'Pure' sika is represented by 0 and 'pure' red by 1. Sample sizes are displayed under the population names (r = phenotypic red, s= phenotypic sika, h = phenotypic hybrids).

Table 4.3 Introgressed loci and alleles in each of the around-Scotland study populations. Ambiguous alleles are marked with an asterisk* (see section 4.2).

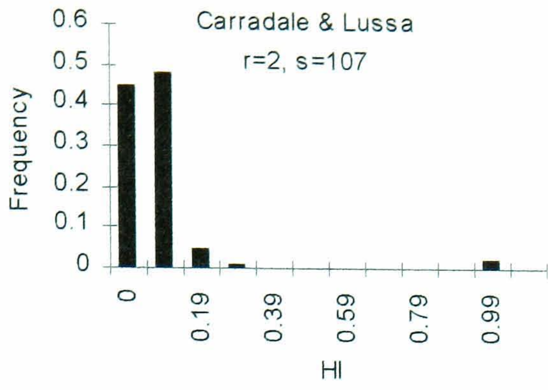
Population	Phenotype	Introgressed loci and alleles
Great Glen	RED	FCB193 (123*,139*), MM012 (93)
Easter Ross	RED	RME025 (197,211*,213*), BM6438 (277), BOVIRBP (144), MM012 (93), INRA005 (146), RM188(146), INRA6 (128), RMO95(126)
Sutherland	RED	RME025 (211*), BM6438 (267), INRA005 (146)
Helmsdale	RED	None
Loch Morar	RED	RMO95 (126)
Angus	RED	BOVIRBP (146*), RMO95 (122*,126)
Fife	RED	RM095 (122*)
Borders	RED	RME025 (197), BM6438 (275), BOVIRBP (144), FCB193 (123*), MM012 (93), BM4006 (89), INRA005 (146), RMO95 (122*,126)
Great Glen (A)	SIKA	MM012 (89), FCB193 (103), INRA005 (129)
Great Glen (B)	SIKA	INRA005 (129)
Easter Ross	SIKA	BOVIRBP (150,154), MM012 (89), INRA005 (129), RM188 (126,130,142)
Sutherland	SIKA	BOVIRBP (156,158), MM012 (89), BM4006 (99), INRA005 (129), RM188 (132,140,142)
Fife	SIKA	RM188 (124)
Borders	SIKA	RM188 (134, 140), MM012 (89)

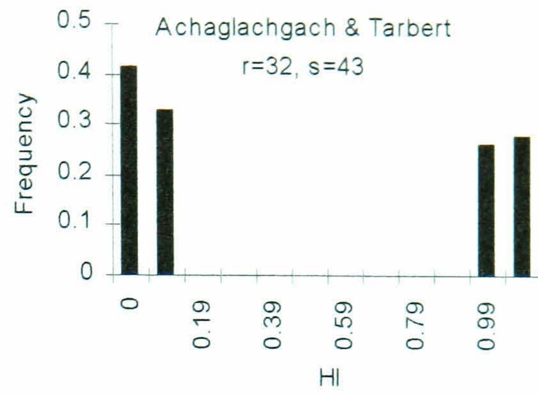
Across Scotland, the pattern of introgression varied greatly between populations (Figure 4.1). In most red-like populations introgression was rare (less than 10% of individuals) and restricted to the same one or two alleles at low frequency, many of which were of unclear taxonomic origin (Table 4.3). However, a small number of more complex hybrid samples, with multiple introgressed alleles, were found in two of the red-like populations (Borders, N=4 and Easter Ross, N=2); both of which were associated with reports of recent phenotypic hybrids.

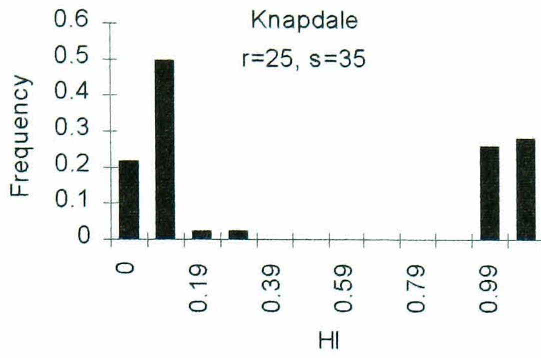
The pattern of introgression in sika like populations was less consistent with phenotype. Although there were no reported differences in the extent of phenotypic introgression between sika-like populations, genetic introgression varied greatly. The majority of samples from Easter Ross and Sutherland (more than 75%) contained introgressed alleles, although only five of the ten nuclear loci screened showed introgression. In the Borders, Great Glen, and Fife populations introgression was restricted to a small number of samples (five, four and one respectively), each containing a single characteristic red allele. One further Fife sika-like sample contained a single allele that was rare in both taxa.

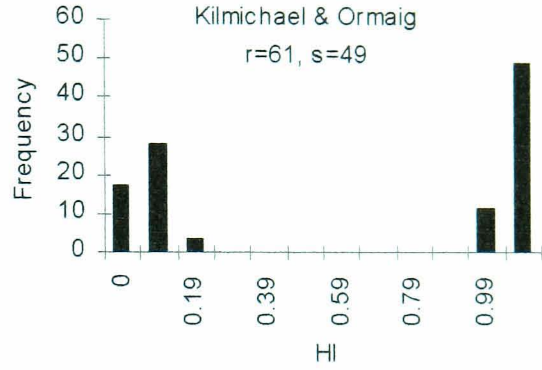
As mentioned in the previous section, an individual sample from Sutherland provided the only evidence for introgression of sika by red deer mtDNA. Although this sample had been supplied as a phenotypic red deer, it had been re-classified to sika as a consequence of genotyping homozygous sika at three nuclear markers. It is possible mtDNA haplotype may have been measured incorrectly, in either case the direction of introgression in this sample is unclear.

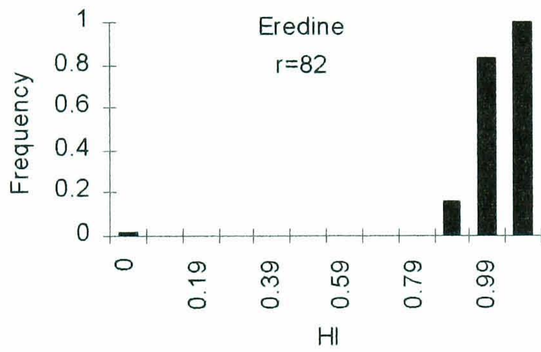
Figure 4.2 (over) Frequency distributions of Hybrid Indices (HI) in the Argyll population study area sampled in 1996/97. HI is the proportion of red alleles found in each sample at 25 microsatellite loci; "pure" sika represented by 0 and "pure" red by 1. Sample sizes are displayed under study site names. Sites are shown in order of distance from the Carradale introduction and should be read from top left to the most distant site at the bottom right The x-axis values show the upper bin range values, e.g. the figure 0.19 below an interval includes individuals with hybrid indexes between 0.1 and 0.19.

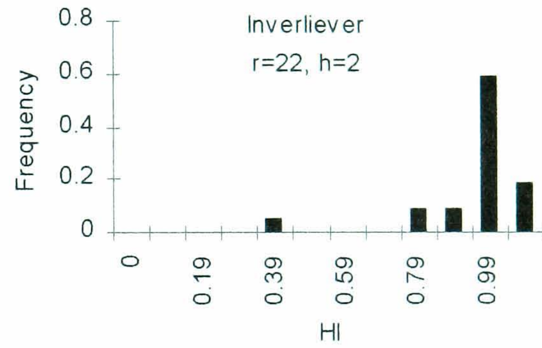

Carradale & Lussa
r=2, s=107
Frequency
HI

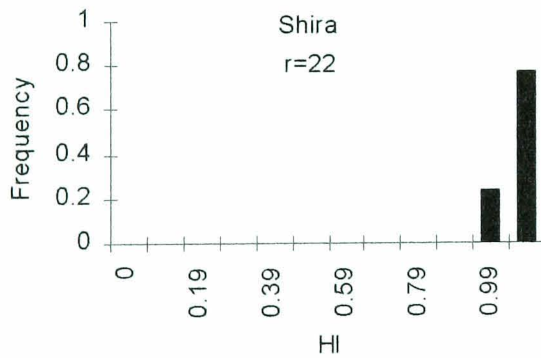

Achaglachgach & Tarbert
r=32, s=43
Frequency
HI

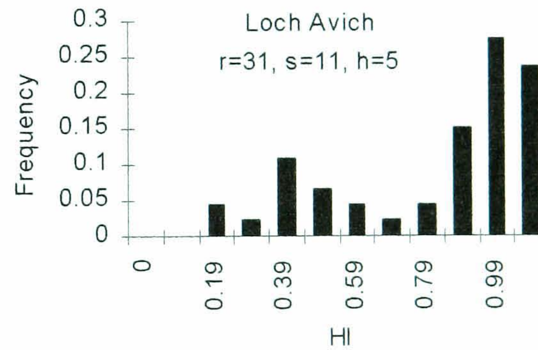

Knapdale
r=25, s=35
Frequency
HI

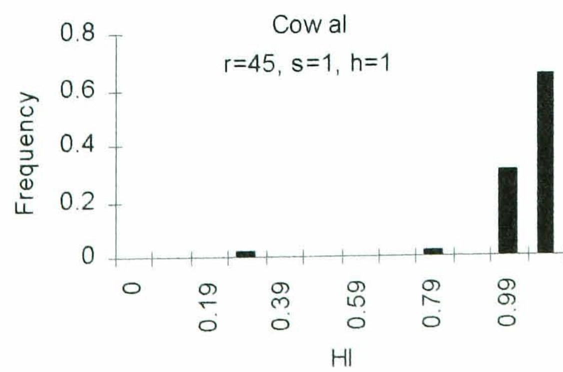

Kilmichael & Ormaig
r=61, s=49
Frequency
HI

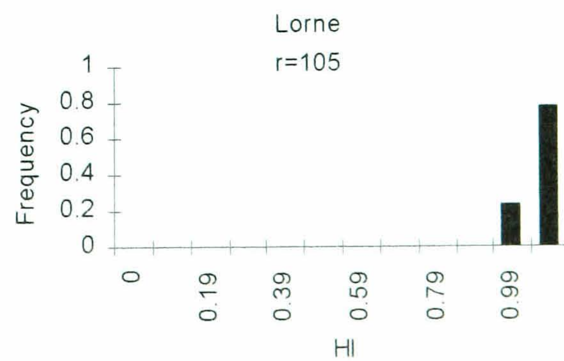

Eredine
r=82
Frequency
HI


Inverliever
r=22, h=2
Frequency
HI


Shira
r=22
Frequency
HI


Loch Avich
r=31, s=11, h=5
Frequency
HI


Cow al
r=45, s=1, h=1
Frequency
HI


Lorne
r=105
Frequency
HI

4.3.2 Intensive Argyll Study

The pattern of hybridisation and introgression across the intensive Argyll study area can be seen in Figure 4.2. A similar pattern to the Scotland wide study emerged, in which recent hybridisation was rare (except at site 10, Loch Avich) and introgression more extensive in sika like deer (62% of samples) than red-like deer (33%). The pattern of introgression changed with distance from the introduction site, with all intermediate hybrids (HI 0.2-0.8) found more than 70km from the introduction site (Inverliever, 76km; Loch Avich, 89km; and Cowal, 126km).

The Loch Avich population provided the best evidence for recent hybridisation with 14 (32%) samples having hybrid indices between 0.2 and 0.8, four of which were identified as phenotypic hybrids.

Introgressed mtDNA was found exclusively in phenotypically sika deer, and all recent hybrids in Argyll had red mtDNA.

4.4 Discussion

The previous sections have highlighted the problems caused by certain alleles, the taxonomic origin of which was unclear. At the local population level, rare alleles in one population that are not typical of the opposite local taxon would simply be identified as rare polymorphism within that particular population. However, when these alleles are shown to be characteristic of another opposite taxon population, it is not clear how to classify them. There are three possible explanations for the source of these ambiguous alleles.

First, in red deer low frequency ambiguous alleles may simply be taxon-specific polymorphism, reflecting the less restricted population genetic history of red deer compared with that of the sika introductions (as indicated by higher hetereozygosities in red deer, table 3.2) (see also chapter 1). In this case we would expect the loci concerned to be in linkage equilibrium, assuming they are selectively neutral and unlinked. In accordance with this explanation, ambiguous alleles occurred individually and not in association with other

ambiguous or sika alleles at other loci, i.e. they were in linkage equilibrium in the red deer populations where they occurred.

If ancient polymorphism was present in a historically large panmictic population the alleles should be uniformly diffused across subpopulations. Although no consistent geographical pattern was observed, several of the rare alleles did occur in local concentrations of homozygotes and heterozygotes. For example, the only red-like class B hybrid in the Great Glen population was a heterozygote for two ambiguous FCB193 alleles. Similar associations were found in Easter Ross red-like deer at RME025. In Argyll, several ambiguous alleles, at *different* loci, were found only in certain local red-like populations North East of the Kintyre peninsula (Kilmichael and Eredine) and Lorne (Glen Lochy and Glen Orchy) and were not more widespread across the survey transect. The distribution of these alleles does not, therefore, appear to be uniform as would be expected with background polymorphism within these populations.

Second, ambiguous alleles might be the result of recent mutation. Assuming that microsatellite alleles mutate in a step-wise manner, we would expect to see small size differences between rare ambiguous alleles and more common 'parental' alleles. Although some ambiguous alleles (e.g. BOVIRBP, allele 146 in Angus; RM095, allele 122 in Borders and Angus) fall into this category, the majority are very different in size to common alleles in either local taxa. While non-stepwise mutations do occur at microsatellites, they are relatively much rarer (Jarne & Lagoda, 1996) and seem an unlikely explanation for these ambiguous alleles.

The third and final explanation is that the ambiguous alleles have been introduced via translocation or hybridisation. Associations between introgressed alleles at different loci would be broken down through recombination and after a number of generations introgressed alleles would reach some low frequency locally or be lost through drift or selection. Introgressed alleles would also appear uncharacteristic in the context of the larger parental population. The most likely source of these alleles is from introduced *Cervus* deer,

of which there are three possible documented sources, other park and European red deer, sika deer and wapiti (Whitehead, 1964; Lever, 1977; Ratcliffe, 1987, see chapter 1). Chapter 3 described the population- and locus-specific allele distributions and included examples of wapiti allele distributions at nine of the ten loci studied (Figure 3.1).

The possibility of wapiti introgression makes the picture especially complicated because there is no genetic data for the introduced individuals, only for other wapiti samples. At eight of the nine microsatellite loci for which data was available, wapiti allele sizes overlapped with characteristic red (4), sika (1) or both (3). The most compelling evidence for wapiti introgression came from (a) rare alleles found in red-like deer that were not found in sika-like samples but which were typical of wapiti, e.g. BM6438 allele 263; and (b) sika/wapiti alleles that were found in red-like samples where sika were unknown locally, e.g. BOVIRBP allele 148 in Angus. It is therefore possible that some of the samples identified as non-hybrid red or class B red x sika hybrids may have been wapiti x red hybrids.

Although this discussion has been framed in the context introgression of red deer , the same possibilities exist for the sika-like populations. The genetic background of these populations is unclear (see section 1.2.3) and it is possible that some of the characteristically red alleles present in some sika populations were the remnants of past park hybridisation events, or polymorphism. In the sika-like populations, founder events have probably reduced variability relative to the native red deer populations (see chapter 3). The chance of wrongly classifying rare alleles is therefore reduced because most loci were monomorphic, and this is reflected in the absence of any class B sika-like hybrids. However, in the more polymorphic Borders sika the identification of several "hybrids" based on a single allele at MM12 (89) could be incorrect.

In the Sutherland and Easter Ross phenotypic sika populations introgressed alleles were found at a number of loci, suggesting recent hybridisation. However, the pattern of introgression was not consistent across loci and only five of the ten loci have red-like alleles. This pattern suggests that one or a few hybridisation events occurred at an early stage in the

establishment of the feral population and have not occurred since. Ongoing hybridisation would have introduced alleles across all loci.

The new study of Argyll has provided further evidence for a moving wave of hybridisation related to the expansion of phenotypic sika into resident phenotypic red deer areas (Abernethy, 1994a,b). The results concur with previous data from this area (Goodman, *et al.*, 1999) showing rare introgression at individual loci, but locally high proportions of the population carrying introgressed alleles. Introgression of nuclear alleles occurred in both taxa, but was higher in sika-like deer (62%) than in red-like deer (33%). Mitochondrial introgression was found in the sika-like population, but not in the red-like population. This bias between the rates of introgression could be caused by differences in the strength of assortative mating, fitness between backcross categories, and/or the direction of hybrid crosses. The absence of any F1, F2 or F3 hybrids with sika mtDNA indicates that female red deer are generally the dam in hybridisation events. These issues will be investigated further in a detailed analysis comparing hind and foetal samples by S. Goodman and N. Barton.

4.5 Summary

The complexity of allele distributions has made the interpretation of data difficult. Although the problems of identifying species-specific microsatellite markers in the genus *Cervus* have been reported elsewhere (Bruford *et al.*, 1998), the frequent translocations, rarity of hybridisation and geographical scale of this study have presented new challenges. The primary problem has been the classification of rare alleles and measuring introgression accurately. However, this has not prevented the identification of recent hybridisation, one of the principle aims of the study.

Across the country a definite pattern emerges of strong assortative mating, but occasional hybridisation where sika are expanding their range into resident red deer populations. Two local "hot-spots" of hybridisation were found near New Galloway (Galloway) and Loch Avich (Argyll). Evidence from Sutherland, Easter Ross and Argyll

suggests that once this initial wave has passed, there is little superficial phenotypic disruption but considerable low level genetic introgression. Introgression was consistently greater in sika-like populations than in red-like populations and this may be due to many factors, including biases in the direction of hybridisation and backcrossing. Contrary to a previous genetic study (Abernethy, 1994a,b) which used some markers now known to be non-diagnostic in Scottish populations (Goodman *et al*, 1999) no evidence for extensive hybridisation was found in the Great Glen samples, although phenotypic hybrids have previously been reported there (see Ratcliffe, 1987a).

The possible reasons why some populations appear to have hybridised and others have not will be discussed in the following chapters.

5 Genetic Variation Within Red & Sika

5.1 Background

The previous chapter indicated that hybridisation between red and sika deer has occurred in several different locations across Scotland. This chapter addresses one aspect of why some populations appear to have hybridised and others have not, genetic variation within species. As discussed in chapter 1, the genetic background of the populations of red and sika have been proposed as important in affecting the incidence of hybridisation (Harrington, 1973, 1979; Lowe & Gardiner, 1975). It is not so much argued that there is a gene specifically related to mate choice, more that pure populations are less likely to hybridise because of the relative physical size differences between taxa (see chapter 7). Although there may well be genetic variance for mate choice, studying this factor was beyond the scope of this study and would be better investigated under experimental conditions, where the potentially considerable environmental effects (e.g. relative densities of each taxa) on mate choice can be controlled for.

However, the data produced as a consequence of looking for hybrids (the primary aim of this thesis) could be used productively to establish whether populations of each taxon involved in hybridisation were genetically similar or not. For example, if the sika populations involved in hybridisation were genetically more similar than the those that were not, then this cold be taken as weak evidence for a genetic basis hybridisation and *vice versa*.

A genetic comparison may also confirm whether the population histories suggested for the sika introductions matched the genetic patterns seen between them, and also to estimate the extent to which hybridisation has affected the variability between populations.

5.2 Genetic variation

5.2.1 Introduction

The measurement of population differentiation within red and sika populations presented several problems in this study. First, the microsatellite markers employed were chosen for their diagnostic properties between taxa and not within. Nine of the ten markers tested in sika populations were monomorphic in one or more populations, and four of the ten markers had three or fewer alleles in most red populations. Therefore the resolving power between sika populations was likely to weak compared to that between red deer populations. Although the microsatellite variation within red populations was comparable with other large mammal microsatellite population studies (Goodman, 1998; MacHugh *et al.*, 1997), within sika the level of variation was more similar to that found in allozyme studies of the Cervinae (e.g. Emerson & Tate, 1993).

The association between genetic background and hybridisation is confounded by the very process of hybridisation. Establishing the initial genetic state of any population prior to hybridisation requires the removal of all suspected introgressed alleles to create a virtual population. Only then can populations which have hybridised be compared with those that have not equitably. However, by "filtering out" introgressed alleles from populations, important population information may be lost. For example, it would not be possible to differentiate between hybridisation prior to introduction from that which occurred subsequently, unless the population had provided stock for subsequent introductions; i.e. was used to found another sampled population.

A wide variety of techniques is available for estimating genetic differentiation and genetic distance between populations based on genotype data (Nei, 1987; Goldstein, et al., 1995; Shriver *et al.*, 1995; Slatkin, 1995 Takezaki & Nei, 1996; Goodman, 1997). The application of genetic distance measuresand genetic differentiation are however both problematic. For both approaches, the assumptions they make (eg. equilibrium populations)

are violated (see Goodman 1997), so neither of them will be true measures of genetic differences between populations. Furthermore, the application of a basic Fst analysis would be less desirable because F_{st} measures how the genetic variance is distributed among groups of populations that have developed from a common ancestral source. Therefore, as most of the sika populations were introduced, most F_{st} measures would be artefactual (pers. comm. S. Goodman). Genetic distance measures are however more conservative and simply employ a commonly used scale to make broad qualifications of the differences between populations. This analysis would also allow a graphical representation of the genetic differences between populations.

5.2.2 Methods

Two of the loci screened in the Scotland wide survey (RM095 and FCB193) were dropped from this analysis as they were not screened in some populations. Similarly the Helmsdale red deer population was omitted due to gaps at several loci. Introgressed alleles were removed from sample genotypes of the eight remaining microsatellite markers according to the criteria described for each population in chapter 4 (i.e. class A and B hybrid genotypes).

"Virtual" population allele frequencies were calculated using the remaining microsatellite alleles and used to estimate Cavalli-Sforza and Edwards' (1967) genetic distance (D_c) using the phylogenetic software package PHYLIP 3.5c (Felsenstein, 1993). Unrooted phenograms were then constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987) and the stability of the tree nodes was checked by bootstrapping over loci (n=100). Although more bootstraps were possible, this default value was chosen because a more precise estimate was not required for tests of significance, but it should still give a reliable estimate with the small number of loci used and their low polymorphism and and would save on computer time. Both procedures were performed using PHYLIP. For

Table 5.1 Cavalli-Sforza and Edwards' (1967) genetic distance (D_c) measures between red and sika populations. Measures in the top right triangle were estimated without introgressed alleles (class A & B hybrids, see chapter 4) and those at the bottom left were estimated using all alleles from 6-8 microsatellite loci.

Population	KINT SIKA	KINT RED	COW RED	LOR RED	GG RED	GG SIKAA	GG SIKAB	EROSS RED	EROSS SIKA	SUTH RED	SUTH SIKA	LOCHM RED	ANGUS RED	FIFE RED	FIFE SIKA	BORD RED	BORD SIKA
KINTSIKA	0	0.296	0.319	0.340	0.349	0.035	0.034	0.336	0.040	0.339	0.039	0.349	0.357	0.354	0.090	0.314	0.092
KINTRED	0.432	0	0.011	0.012	0.017	0.346	0.359	0.016	0.266	0.024	0.301	0.021	0.024	0.017	0.375	0.035	0.357
COWRED	0.432	0.009	0	0.014	0.025	0.364	0.376	0.024	0.285	0.030	0.322	0.027	0.032	0.018	0.392	0.027	0.374
LORRED	0.432	0.008	0.013	0	0.009	0.386	0.398	0.013	0.306	0.021	0.343	0.014	0.019	0.010	0.405	0.036	0.393
GGRED	0.432	0.013	0.023	0.008	0	0.393	0.406	0.011	0.316	0.022	0.349	0.009	0.014	0.014	0.414	0.035	0.401
GGSIKAA	0.029	0.432	0.432	0.432	0.432	0	0.019	0.381	0.068	0.379	0.051	0.393	0.399	0.398	0.048	0.356	0.066
GGSIKAB	0.027	0.432	0.432	0.432	0.432	0.015	0	0.393	0.054	0.392	0.040	0.405	0.411	0.411	0.064	0.372	0.076
EROSSRED	0.432	0.014	0.022	0.010	0.010	0.432	0.432	0	0.301	0.012	0.333	0.012	0.013	0.011	0.404	0.036	0.382
EROSSSIKA	0.020	0.422	0.420	0.422	0.423	0.043	0.025	0.422	0	0.302	0.016	0.316	0.315	0.314	0.130	0.282	0.103
SUTHRED	0.432	0.020	0.026	0.018	0.020	0.432	0.432	0.010	0.420	0	0.331	0.021	0.019	0.020	0.398	0.039	0.381
SUTHSIKA	0.025	0.431	0.431	0.432	0.432	0.037	0.022	0.432	0.008	0.431	0	0.349	0.345	0.350	0.106	0.313	0.073
LOCHMRED	0.432	0.017	0.026	0.012	0.009	0.432	0.432	0.010	0.422	0.019	0.432	0	0.013	0.016	0.415	0.044	0.405
ANGUSRED	0.432	0.019	0.029	0.017	0.013	0.432	0.432	0.011	0.422	0.016	0.431	0.012	0	0.015	0.412	0.042	0.396
FIFERED	0.432	0.012	0.015	0.008	0.014	0.432	0.432	0.010	0.420	0.017	0.432	0.016	0.014	0	0.419	0.037	0.405
FIFESIKA	0.084	0.432	0.432	0.432	0.431	0.044	0.060	0.432	0.105	0.432	0.088	0.432	0.432	0.432	0	0.380	0.088
BORDRED	0.426	0.035	0.025	0.034	0.033	0.426	0.426	0.034	0.417	0.036	0.426	0.041	0.039	0.035	0.426	0	0.359
BORDSIKA	0.086	0.412	0.413	0.413	0.413	0.065	0.071	0.413	0.085	0.411	0.066	0.413	0.410	0.412	0.089	0.404	0

comparison, the same operations were carried out on the actual population allele frequencies including introgressed alleles.

5.2.3 Results

Table 5.1 gives the genetic distances between populations, both with and without introgressed alleles. Figure 5.1 shows the phenogram based on the reconstructed non-hybrid Scottish red and sika population data. Genetic distances between red deer populations were greater than those between sika populations. The Borders sika population was the most distinct sika population, followed by the Fife population. The remaining sika populations shared a common node and were relatively closely related. Among red deer, the E. Ross and Sutherland populations were the greatest genetic distance from the sika population node.

The stability of the nodes varied within red and sika but the node between taxa was 100% stable. Fife and Borders sika populations were more consistently differentiated (Borders & Fife, 81%) than the other sika populations. Among the red populations the Borders (86%) and Easter Ross (78%) populations had the best supported branch nodes. There was no clear association between tree topology and the occurance of hybridisation measured in red and sika populations. In other words, sika populations that have hybridised were not clustered together, nor were the red populations involved in hybridisation more similar than those not.

The phenogram based on the actual population allele frequency data is shown in Figure 5.2. The red-sika node remained robust (bootstrap support 100%), but both red and sika populations have been rearranged relative to Figure 5.1. A well-supported node (80%) separates the sika populations of Kintyre, E. Ross and Sutherland from the rest. These three populations (which are the most hybridised; see chapter 4) moved so that they were adjacent to the red-sika node. Red deer populations changed little except that the E. Ross and Sutherland populations moved closer to the inter-taxa node. The Borders red population remained the most consistently separate (87% bootstrap support).

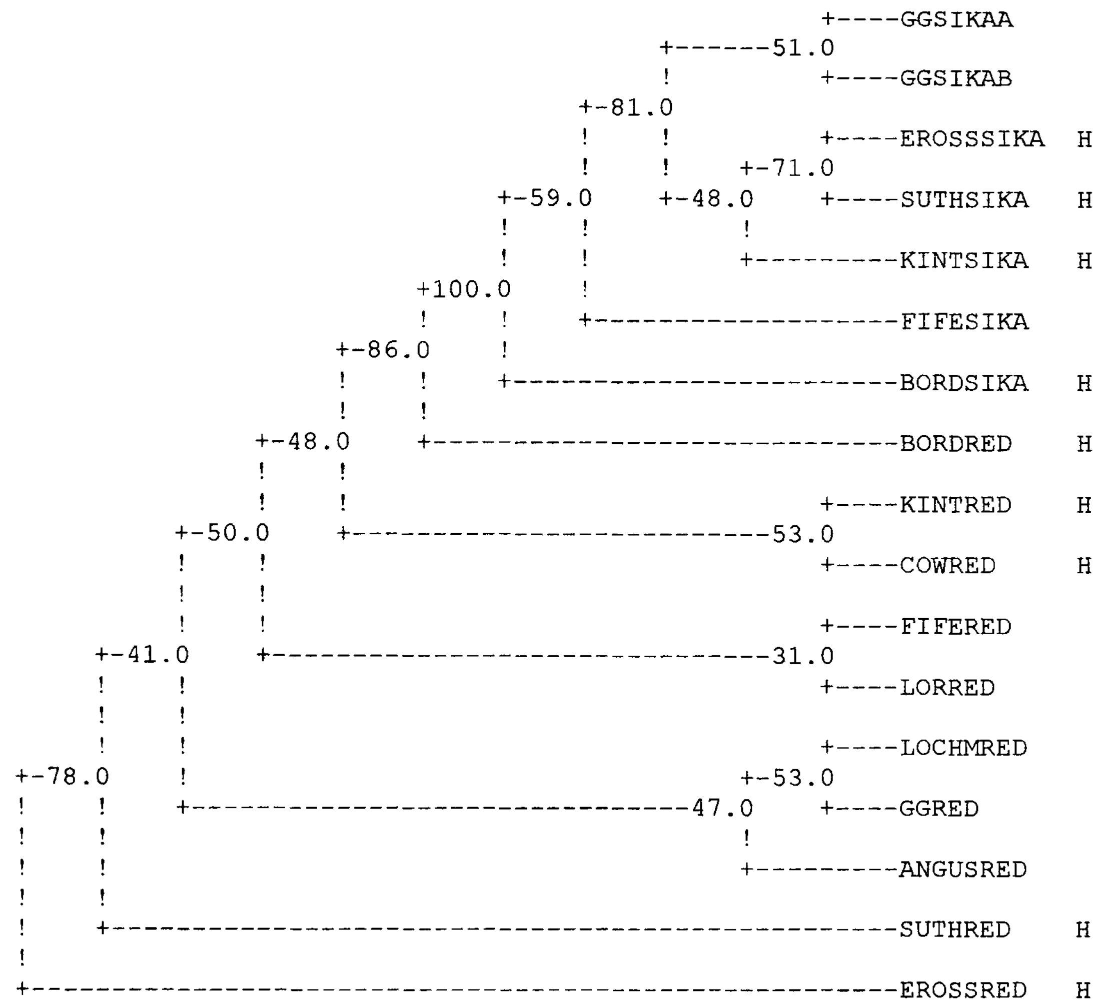


Figure 5.1. Neighbour joining phenogram of virtual* Scottish red and sika deer populations based on Cavalli-Sforza and Edwards' (1967) genetic distance. The number of times each node was constructed out of 100 bootstrap operations is shown at each node. Populations associated with hybridisation (class A & B hybrids , see chapter 4) are marked with an H. * Allele frequencies based on population data with introgressed alleles removed.

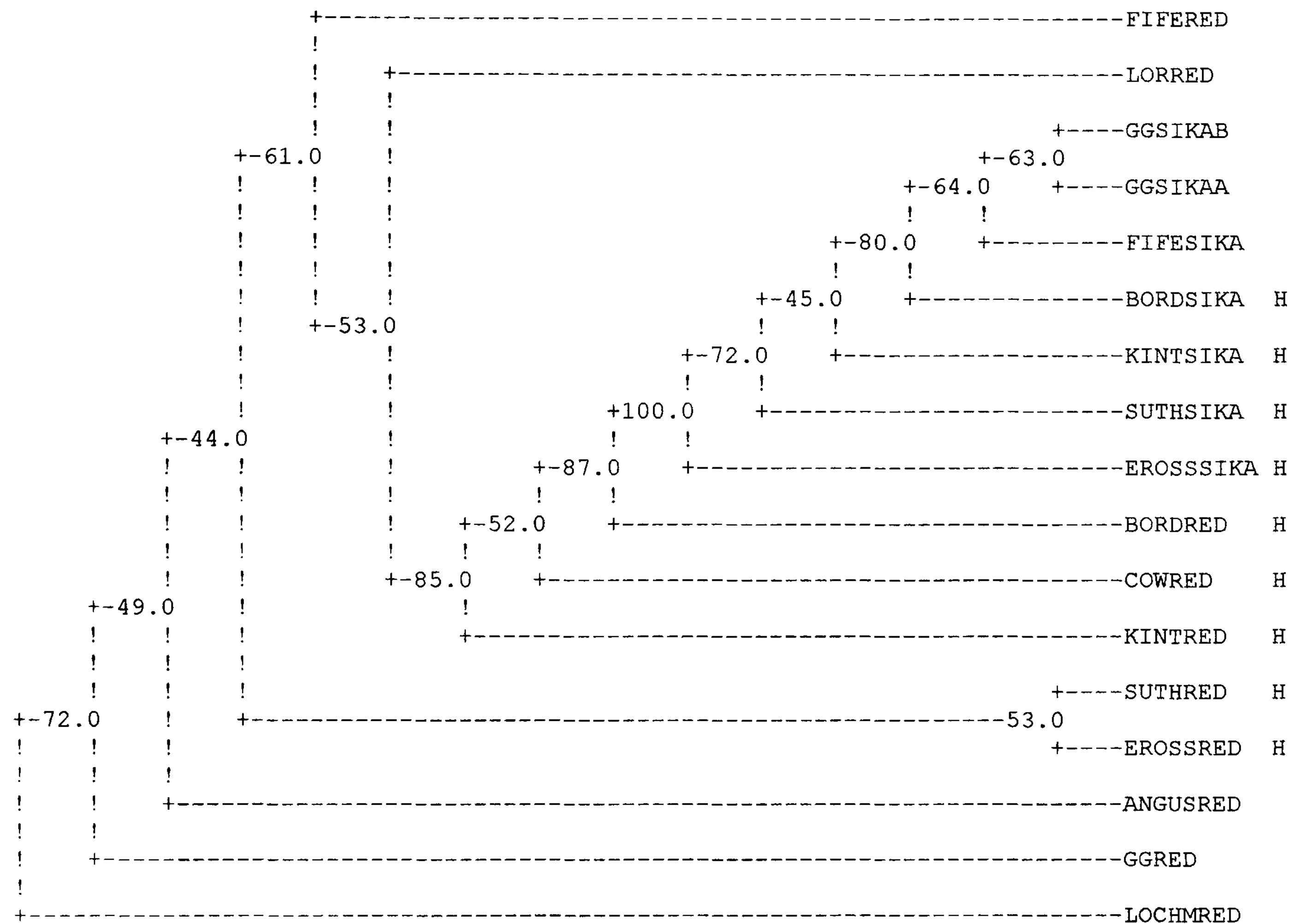


Figure 5.2 Neighbour joining phenogram of actual* Scottish red and sika deer populations based on Cavalli-Sforza and Edwards' (1967) genetic distance. The number of times each node was constructed out of 100 bootstrap operations is shown at each node. Populations associated with hybridisation (class A & B hybrids, see chapter 4) are marked with an H. * Real population allele frequencies used.

5.3 Discussion

The pattern of genetic variation between and within red and sika deer populations was compared using genetic distance measures and phenograms. Two phenograms were constructed, one using the actual sample allele frequency data and another using a modified version of the data set from which all putative introgressed alleles had been removed. The topology of the "non-hybrid" phenogram indicated that there was no simple relationship between the genetic background of the populations and the incidence of hybridisation.

5.3.1 Differences between sika deer populations

Within sika populations the deepest node separated the Borders sika from the other populations. This confirms skull morphometric comparisons carried out by Ratcliffe *et al.* (1992) and others (Lowe & Gardiner, 1975; Putman & Hunt, 1993). Ratcliffe *et al.* (1992) showed that the Borders sika are the most morphologically distinct population and that they match most closely a set of Japanese sika skulls from Honshu. The differences seen here, however, may simply reflect a separate introduction history and cannot be taken as evidence of a different phylogenetic origin. Further comparison with Asiatic populations will be required to resolve this issue.

Except for the node separating the Borders and Fife sika from the others (bootstrap support 81%), sika population nodes were relatively unstable, indicating that these populations were genetically similar at these loci. The differentiation of the Fife sika may indicate a different phylogenetic history for this population (source unknown; Ratcliffe, 1987a), or it may simply be an artefact of the longer genetic bottleneck the other populations experienced [longer time in captivity in small park populations and several founder events (Ratcliffe, 1987a)], which can exaggerate genetic distances due to the sampling effect it has (Takezaki & Nei, 1996). Alternatively it may simply reflect a chance founder effect in this particular population (i.e. by chance some alleles were sampled from the source population

in this case and not in the other translocations). A final explanation is that Borders sika (or related individuals from the same population) have mated with the Fife populations. Overall, it appears that the Borders sika represent a separate introduction from the others and that further comparisons with Asian populations may indicate whether they represent a separate phylogenetic group (and whether the sika in Scotland are less genetically variable than those in Asia).

5.3.2 Differences between red deer populations

Scottish red deer populations have also received little published genetic comparison. With the exception of some early allozyme work (see Dratch, 1983), the genetic investigation of Scottish red deer has been restricted to an intensive study on Rum (e.g. Coulson *et al.*, 1998) and screening for red x sika hybrids in Argyll (Abernethy, 1994a,b; Goodman *et al.*, 1999) and the Great Glen (Abernethy, 1994a,b,). The results from the present study show an approximate cline from south to north, with the Borders red placed nearest to the sika node and the E. Ross and Sutherland populations furthest away (see Fig. 2.2). Although the markers were more polymorphic in red populations (average red H_e = 0.5; sika = 0.23), the tree nodes were relatively unstable. This may reflect a lack of genetic differentiation between Scottish red deer populations at the scale of this study, or may indicate a lack of resolving power of these specific markers, which were chosen for their taxonomic information. Further comparisons using more polymorphic markers would allow a more accurate estimate of their relative genetic differences.

5.3.3 Genetic status and hybridisation

In this relatively crude analysis, there appears to be no obvious association between genetic background and hybridisation. In both the red deer and sika deer clades, the most genetically distant populations (red, E. Ross and Argyll; sika, Borders and Argyll) have been involved in hybridisation.

The more difficult issue to resolve is whether any of the populations were hybridised prior to introduction and if so, whether this predisposed them to further hybridisation (Lowe & Gardiner, 1975; Harrington, 1982). However, the chronological order of sika introductions presents an opportunity to infer the post-introduction hybrid status of some pairs of populations. Two such pairs were known to have shared a common ancestry: the Sutherland (SUTHSIKA) and Great Glen A (GGSIKAA); and Argyll (KINTSIKA) and Great Glen B (GGSIKAB) populations. In each pair one population contains introgressed alleles, but the other does not, indicating that the original source populations were not hybridised. Although it is not possible to reconstruct all populations in this way, the evidence from these pairs suggests that the Argyll and Sutherland sika populations were not hybrid at introduction, but this did not act as a barrier to subsequent hybridisation.

5.3.4 The effect of hybridisation on genetic distances

A phenogram was constructed with the full allele frequency data set including introgressed alleles so that the effect of hybridisation on the genetic relationships of the populations could be visualised. The most obvious effect was to re-shuffle the populations into an order corresponding to their degree of introgression (chapter 4). Indeed, the most consistent within-sika node was now between the hybrid and non-hybrid populations (80%).

Red deer populations were less affected by hybridisation, with the most obvious change resulting from the movement of the Sutherland and Easter Ross populations (both with low levels of introgression) closer to the red-sika node. The difference between sika and red deer in the amount of reshuffling on inclusion of introgressed alleles was consitent with the greater introgression of red alleles into sika than *vice versa*.

The limited genetic comparison carried out in this chapter suggests that there is no evidence to support the hypothesis that some Scottish populations were introgressed prior to their release and that this has not had an effect on their propensity to hybridise subsequently. Furthermore, it appears that the genetic background of the populations matches, to some

extent, the history of introductions summarised by Ratcliffe (1987a). Those introductions with unknown population histories (e.g. Carradale) appear to resemble the majority of other sika populations in Scotland (except of the Borders population) and seem likely to have originated from the same source: Powerscourt, Ireland.

6 Materials and Methods for Phenotypic study

6.1 Introduction

6.1.1 Background

The previous chapter investigated whether there was any simple realtionship between the genetic characteristics of a population and its involvement in hybridisation. Associated with this hypothesis is the idea that there is variation in body size between populations of both taxa, and that intermediate types (e.g. certain subspecies) are more likely to hybridise (Lowe & Gardiner, 1975; Harrington, 1973, 1982). Once hybridisation occurs, it is suggested that genetic and phenotypic introgression can quickly follow, resulting in extensively hybridised populations; e.g. south Lake District, England (Lowe & Gardiner, 1975), Wicklow, Ireland (Harrington, 1973, 1982) and Czechoslovakia (Bartos & Zirovnicky, 1981b).

Previous studies have, however, suffered from a lack of detailed genetic and phenotypic information for the same samples (see chapter 1). Populations have generally been described in terms of phenotype (Lowe & Gardiner, 1975; Bartos & Zirovnicky, 1981; Ratcliffe *et al.*, 1992; Putman & Hunt, 1993) or genotype (Abernethy, 1994a,b; Goodman *et al.*, 1999) and only Harrington (1979, 1982) has so far compared both in any detail at one site. There has also been a tendency to concentrate on the genetic component of phenotype and ignore the considerable environmental influence on body size in deer (Mitchell *et al.*, 1976; Mitchell & Crisp, 1981; Mitchell *et al.*, 1981; Mitchell *et al.*, 1986; Hewison *et al.*, 1996).

One aim of this study was to examine the possible phenotypic causes of hybridisation. By comparing adjacent red and sika populations from around Scotland it is possible to look for associations between the phenotype of one or both taxa and whether or not they have

hybridised. Specifically, one can ask whether the physical size of one sex in one taxon, or the differential in size between potential mates of the two taxa appear to act as a barrier to hybridisation. If such associations were found, then the source of the relevant variation in phenotype, i.e. whether it was genetic of environmental in origin, would be of great interest.

A second aim of the study was to examine the phenotypic consequences of hybridisation. The intensive Argyll study provides the opportunity to examine whether, in a region of extensive backcrossing, there are detectable phenotypic consequences in terms of size, condition and reproductive traits. Specifically, in Argyll, most animals retain the appearance of the parental taxa, and it is of considerable interest to know whether there are detectable phenotypic consequences of hybridisation, especially in terms of reproductive traits which might have management consequences

6.1.2 Size and propensity to hybridise - Scotland wide comparison

The principle trait of interest in the extensive around-Scotland comparison was body size. A small body size differential between red and sika populations has been cited as a probable factor in their predisposition to hybridisation (Lowe & Gardiner, 1975; Harrington, 1979, 1982). Body weight data was requested for all samples and where possible, sample providers were asked for a small (n = 20) sample of red and sika phenotypic material similar to that collected in Argyll (section 6.1.3). Although phenotypic material and background population data was requested for these populations, the small number of independent sites prevented any formal statistical tests.

Mean carcass weights of red and sika, adult males and females were calculated for each population to test whether populations of each species with larger or smaller body size were more likely to have hybridised. Carcass weight differentials were then calculated by subtracting mean sika male weights from mean red female weights and mean sika female weights from mean red male weights in each population. The differentials were used to test

whether hybridisation was more likely between sympatric populations with small relative size differences between the opposite sexes or not.

6.1.3 Phenotypic consequences of hybridisation – Intensive Argyll study

Comparisons of parental and hybrid phenotypes of red and sika deer have been carried out in New Zealand (Davidson, 1973), Czechoslovakia (Bartos *et al.*, 1981; Bartos & Zirovnicky, 1981), England (Lowe & Gardiner, 1975) and Ireland (Harrington, 1979) and are reviewed in chapter 1 (section 1.4). In these studies, post mortem measurements were taken, average measures were compared and individuals with medium or uncharacteristic traits were assumed to be hybrid.

In this project, the planned extensive sample collection operation restricted the quantity of phenotypic data and material to amounts that would be acceptable to the stalkers and rangers who would be collecting it. For decades projects have relied on the good will of Forestry Commission (now FE) rangers, so establishing a balance between practicality and biological enquiry was essential. As far as possible the project requests were tailored around the normal deer management operations of the FE rangers. Sample material was restricted to standard body parts that have previously been shown to be of functional or diagnostic importance: female reproductive organs [which give data on fecundity, foetal sex ratio & conception date (Albon *et al.*, 1986; Mitchell *et al.*, 1986; Hewison & Gaillard, 1996; O'Donohue, 1991)], skulls [which give data on skull morphology (Lowe & Gardiner, 1975; Bartos *et al.*, 1981; Ratcliffe *et al.*, 1992)], lower jaws [which give data on body size, age, incisor breadth, condition (Mitchell & Crisp, 1981; Suttie & Mitchell, 1983; Illius & Gordon, 1987; Hewison *et al.*, 1996)] and kidney and surrounding fat [which give data on condition (Caughley, 1971b; Mitchell *et al.*, 1976; Ratcliffe, 1987b)].

6.2 Sample collection and methods

6.2.1 General

Almost all phenotypic samples were collected by Forest Enterprise rangers during their annual culling operations. A small number of samples were also provided by individual sporting estates or conservation bodies, usually where there was a particular hybridisation issue.

All tissue was removed from the culled animal, placed in labelled plastic bags and frozen at –20°C until collection. Freezers were either already resident in larders, or were provided especially for this purpose. Most material was taken to The National Museum of Scotland's (NMS) maceration unit in Granton, Edinburgh for further treatment. The sections below outline the exact treatment given to each type of sample.

The number of samples provided with phenotypic material for the Argyll study are shown in Table 6.1. Most analyses were restricted to adult female samples, unless otherwise stated, as these provided the largest sample sizes.

Table 6.1. Number of phenotypic samples provided from the W. Argyll FE District sample sites. Samples numbers are shown by population, phenotype, sex and age (0=0-12 months, 1=13-24 months, 2+ = more than 24 months).

	Red				Sika			
	Male	Female			Male	Female		
Population	all	0	1	2+	all	0	1	2+
Carradale						5	4	17
Lussa		1		1	19	5	9	46
Tarbert South						5	2	10
Tarbert North		2	1	4		1		1
Achaglachgach	2	8	1	11		11	1	14
Knapdale	1	7	3	14	2	12	4	15
Kilmichael	1	22	4	33	6	9	6	22
Inverliever	2	3	1	17				
Eredine	12	21	5	43				1
Loch Avich	11	1	2	18	2	3	2	6
Shira		6	2	14		0		
Total	**29**	**71**	**19**	**155**	**29**	**51**	**28**	**132**

6.2.2 Kidneys

Rangers were asked to remove kidneys with any surrounding fat. In moderate and low condition animals this task was easy as the kidney and fat comes away as a single unit. However, in some very good condition deer the perinephric fat is attached continuously to the fat stores running along the spine and in these cases the point at which the kidney broke away from the wall determined the sample. Kidneys were defrosted and removed from their bags, weighed and then any surrounding fat and connective tissue and was removed. The remaining kidney was then weighed and a small 2cm^3 of tissue was sub-sampled, placed in a labelled bag and frozen at -20°C for future DNA extraction if required.

6.2.3 Heads

Once defrosted, the heads were tagged using either numbered FE plastic larder tags, or with a 1cm x 2cm aluminium tag showing a consecutive population number. The tags were attached to one of the lower jaws using thin metal wire. Lower jaw incisor breadths were measured to the nearest 0.1mm using Vernier callipers and a small portion of the tongue tip was removed (2cm^3) and placed in a labelled polythene bag before storage at –20°C for future DNA extraction. The heads were then placed in suitably sized plastic containers and covered in approximately 100g of biological washing powder per head and covered in water. The plastic containers were then immersed in water and heated to 80°C for around 14 hours in a large (2.25m^3) aluminium water bath.

After soaking the heads were retrieved and all flesh was removed using forceps and a strong jet of water. Initially great care was taken to retain all teeth and nasal bones, but as the volume of material increased less care was taken and juvenile skulls were eventually discarded, although their jaws were retained. Once clean, the skulls and jaws were kept together and submerged in a 1:50 bleach solution for one hour to denature the washing powder enzyme. Samples were then rinsed with tap water and left to dry. Both jaws and

skulls were then labelled with a permanent marker pen before storage at room temperature in the NMS storage facility.

6.2.4 Reproductive organs

Defrosted female reproductive organs were opened and inspected for small embryos or larger foetuses. Embryos were removed and if large enough weighed to the nearest gram and separated from maternal tissue to be frozen for DNA extraction. Foetuses were detached from the placenta, weighed (to nearest g), measured from rump to head (to nearest 0.1cm), sexed and then frozen whole or sub-sampled for DNA extraction.

6.2.5 Larder records/data sheets

Samples provided by FE were supplied with associated data collected as part of the normal deer management strategy. These records contained information regarding the date shot, location, sex, phenotype, estimated age, carcass weight (head, lower legs, organs removed; to nearest kg), reproductive state, lactation state and general comments. Although the accuracy of these data cannot be confirmed, standard procedures are followed carefully in most FE districts.

Where these records were not collected as standard practice, sample data sheets were provided for the ranger or stalker to complete. The completion and return of these sheets was not consistent and samples were supplied with different permutations of phenotype, shooting date, sex and weight.

6.3 Traits measured

6.3.1 Skull/jaw measurements.

Age and jaw length were assessed from the cleaned lower jaws. Age was estimated from tooth wear and eruption pattern (Lowe, 1967; Koike & Ohtaishi, 1985) and jaw length was measured from the posterior margin of the alveolus of the fourth incisiform to the process angularis (Ratcliffe, 1987b). Eighteen skull dimensions described by Ratcliffe *et al.*

(1992) and Putman & Hunt (1993) (appendix 5) were measured by R. Kernahan on 143 adult female skulls from the W. Argyll (121) and Cowal (21) sample areas.

6.3.2 Body condition

Individual body condition was assessed by two methods; carcass weight relative to skeletal size [carcass index (CI) (Ratcliffe, 1987b)] and Kidney Fat Index (KFI) as described by Mitchell *et al.* (1976).

CI was calculated as:

$$CI = \text{Carcass weight} / \text{jaw length}$$

KFI was calculated as:

$$KFI = \text{Perinephric fat weight} + \text{kidney weight} / \text{kidney weight}.$$

6.3.3 Conception dates.

Conception dates were estimated from the foetal weights at death and date shot. Foetal weights (W) were adjusted to foetal age (T) from published gestation length and foetal growth rate parameters for each taxon. The following equations were applied to each sample according to its phenotypic classification. Hybrid conception dates were estimated according to the phenotype provided by the FE ranger.

(i) Red: $T_r = \left(\sqrt[3]{W} + 3.2\right)/0.094$ Lincoln & Mitchell (1973)

(ii) Sika $T_s = \left(\sqrt[3]{W} + 2.7\right)/0.091$ Suzuki *et al.* (1996)

Conception dates were then calculated by subtracting T from the shooting date.

6.3.4 Hybrid Index classification

The relationship between sample genotype and phenotype was investigated by grouping samples with similar Hybrid Indexes (HI; chapter 5) into five categories reflecting

their extent of introgression (Table 6.2). This allowed statistical comparison of similarly introgressed samples and smoothes for missing genotypes (Arnold, 1997).

Introgressed and non-introgressed sample groups in each phenotypic category (red and sika) were then compared using t-tests.

Table 6.2 Relationship between individual Hybrid Index (HI) and Hybrid Index Class (HIC). "Pure" red are represented by 1, "pure" sika by 5, and intermediate samples 2-4 respectively.

HIC	**1**	**2**	**3**	**4**	**5**
HI Range	1	0.99-0.80	0.21-0.79	0.20-0.01	0

6.4 Statistical analysis

General descriptive statistical analysis and data management were carried out using Excel 97 and Access 97 (Microsoft® Corporation, USA). Principle Component Analysis (PCA) was carried out on skull measurements using MINITAB® v10.2 (Minitab Inc., PA, USA).

Body weight and KFI measured have been shown to vary over the shooting season in both red (Mitchell *et al.*, 1976; Ratcliffe, 1987b) and sika deer (O'Donohue, 1991). In the round Scotland samples, however, it was not possible to adjust for this as there were significant gaps in the shooting date data. In the Argyll samples only one significant temporal change was detected and in this case adult (≥ 2yrs) female red deer KFIs ($\log_e$ transformed) were adjusted to an estimated value at the start of the sampling period (1st November) using linear regression analysis, as follows:

Adjusted measure (Y) = lnKFI (X) + {-0.006 (L) x days from 1st Nov. (N)}

The variances of samples were compared using F-ratio tests (Sokal & Rohlf, 1997, pp. 189-190). Average sample values were compared using non-parametric Mann-Whitney tests (Sokal & Rohlf, 1997, pp. 427-431) and parametric t-tests (Sokal & Rohlf, 1997, pp. 223-227) depending on the data distribution and sample sizes.

7 Phenotypic Study: Results & Discussion

7.1 Introduction

This chapter describes the results from the extensive Scotland wide and intensive Argyll phenotypic investigations. The results are then interpreted in the context of other work and the phenotypic consequences of hybridisation between Scottish red and sika deer are discussed.

7.2 Scotland wide survey

Carcass weights of adult (>1yr old) red and sika deer were compared across Scotland (Table 7.1). Across Scotland, the mean adult male red carcass weight was 56.4 kg (s.e. = 2.0, n = 7) and ranged from 46.9 kg (Cowal) to 62.5 kg (Sutherland), almost double the mean adult male sika weight of 32.1 kg (s.e. = 1.5, n = 8), which ranged between 27.8 kg (G.G. A) and 36.0 kg (E. Ross). The mean adult female red carcass weight was 42.9 kg (s.e. = 1.3, n = 6), ranging from 38.3 kg (G.G. A) to 50.3 kg (Borders) compared with a mean adult female sika weight of 23.1 kg (s.e. = 0.8, n = 6). which ranged between 20.9 kg (G.G. A) and 26.5 kg (Fife). A comparison of mean adult carcass weight of populations with and without recent hybrids is shown in Figure 7.1. There were no significant differences between mean carcass weights of populations with and without hybrids in each phenotypic category (Mann-Whitney; $W > 12$, $p > 0.10$). There was no obvious relationship between carcass weight differentials between sexes of the opposite taxa and the presence of hybrids. (Figure 7.2). Populations with both below and above average differentials contained recent hybrids.

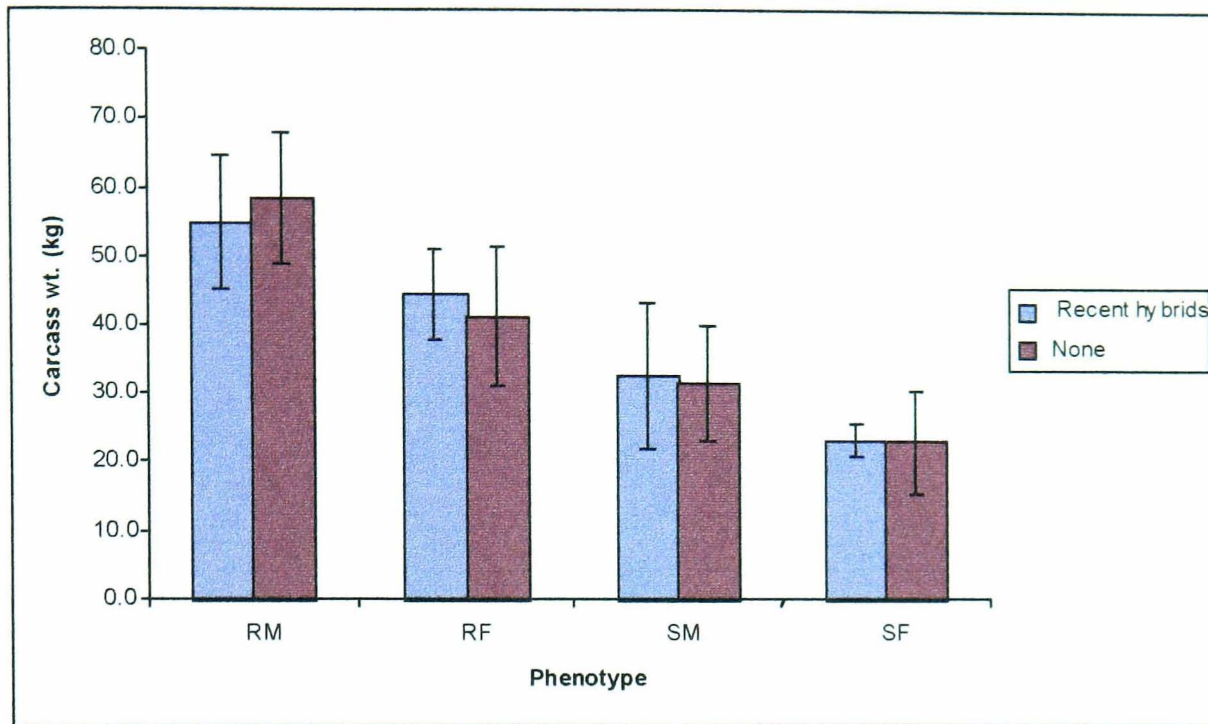


Figure 7.1 Mean adult carcass weights (kg) and standard errors of populations containing recent hybrids and those without. Phenotypic categories represent, red males (RM), red females (RF), sika males (SM) and sika females (SF).

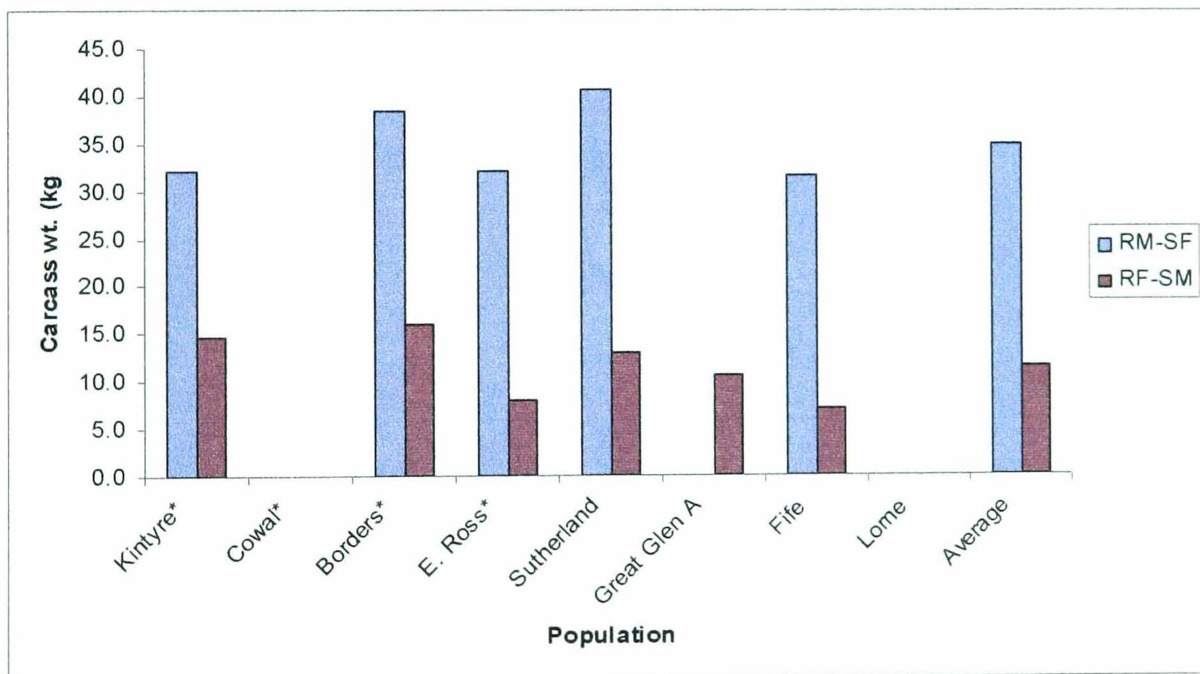


Figure 7.2 Mean adult carcass weight (kg) differential between phenotypic opposing sexes in various Scottish populations (*hybrids present). Phenotypic categories compared are red males (RM), red females (RF), sika males (SM) and sika females (SF).

Table 7.1 Mean carcass weight (CW) of adult (more than 1yr. old) deer taken from cull records in the Scotland wide survey. Weights are not adjusted for shooting date as data not available. Standard deviation (sd) and sample size (n) are given for each population and phenotype category. Populations containing recent hybrids in this study are listed first (*).

	Red						Sika					
	M			F			M			F		
	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n
Kintyre*	54.4	15.8	437	42.5	5.9	524	27.9	6.7	201	22.3	4.1	305
Cowal*	46.9	9.8	7	41.1	7.1	93	-	-	-	-	-	-
Borders*	61.8	22.3	37	50.3	12.6	27	34.4	16.5	7	23.3	8.4	13
E. Ross*	56.3	11.8	29	44.0	4.8	5	36.0	9.8	39	24.1	3.3	11
Sutherland	62.5	13.5	23	47.3	9.0	25	34.2	6.9	11	21.7	3.7	20
Great Glen A	-	-	-	38.3	4.0	7	27.8	6.6	4	20.9	2.9	10
Fife	58.1	19.1	127	39.6	8.4	94	32.5	9.3	11	26.5	0.7	2
Lorne	54.8	15.9	464	39.9	6.7	389	-	-	-	-	-	-
Pop. mean	56.4	5.3	7	42.9	4.1	8	32.1	3.5	7	23.1	2.0	6

7.3 Consequences of hybridisation: Argyll study

7.3.1 Skull Dimensions

Principle Component Analysis (PCA) of 18 skull dimensions, from adult female red (n = 89) and sika (n = 54) deer, revealed little variation between measurements, as 94% of the variation was described by the first principle component, which corresponded to the overall size of the skulls. The second principle component (PC2) accounted for a further 1.1% of measurement variability, consisting mostly of skull depth. Plots of PC1 against PC2 are shown in Figure 7.3. When plotted by gross phenotype (Figure 7.3a), the single stalker-identified hybrid skull (LGP-130, Loch Avich) included can be seen to fall midway between the two parental clusters. Figure 7.3b shows that several other intermediate skulls were genetically recent hybrids, but that more distant backcrosses are indistinguishable from non-hybrid samples.

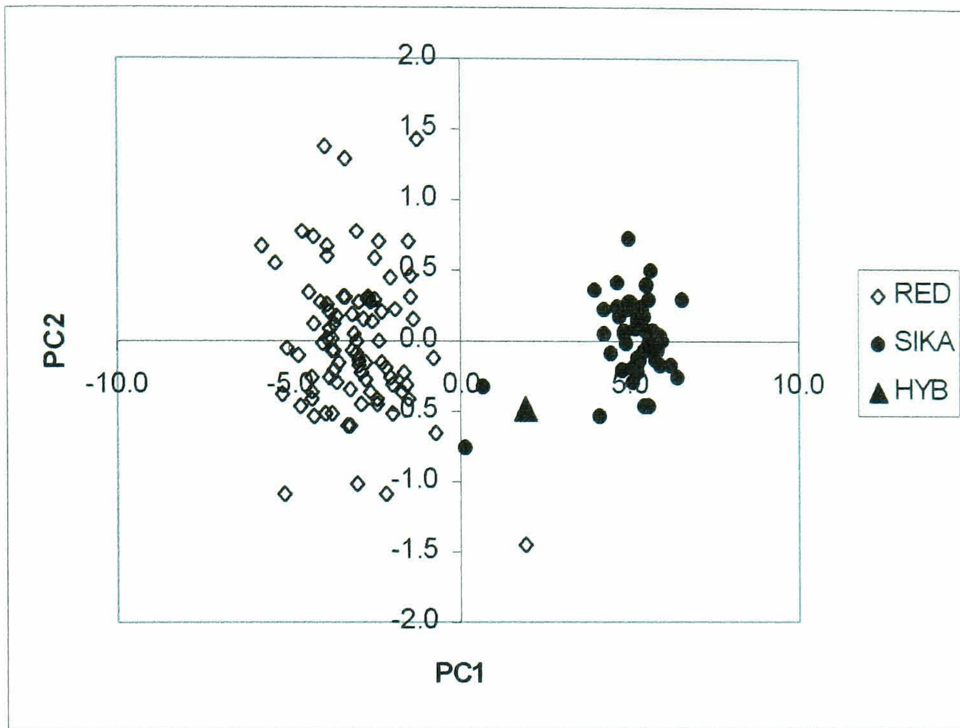


Figure 7.3a Plot of first two principle components of 18 skull measurements taken from 89 red and 54 sika adult female skulls from Argyll. Individual points are marked by phenotype assigned at time of shooting.

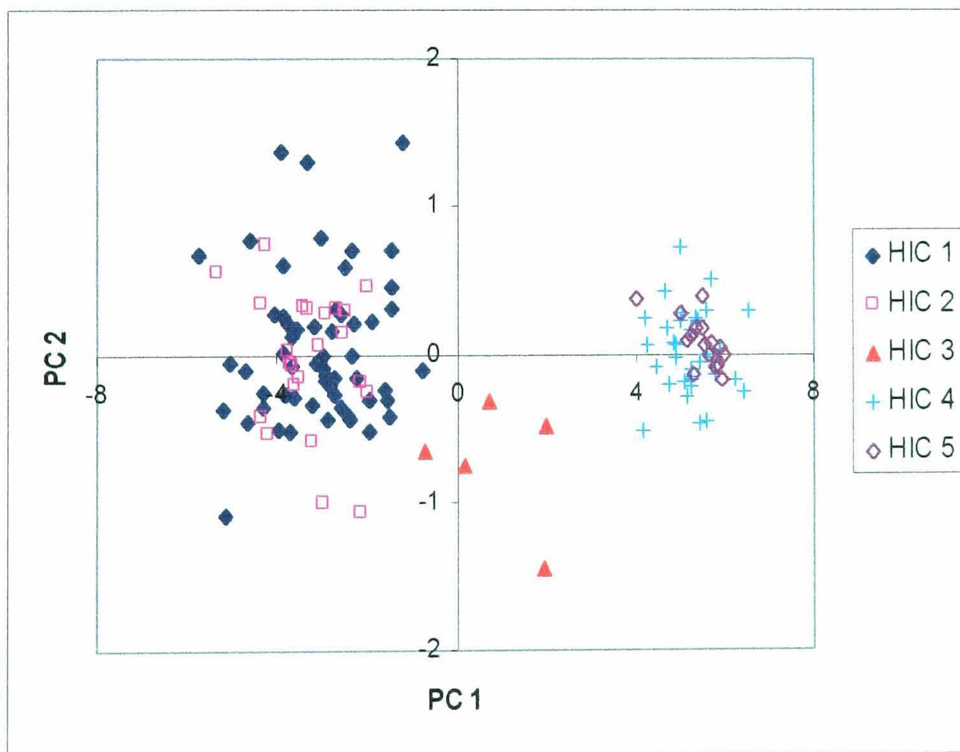


Figure 7.3b Same plot as 7.3a., except individual points are marked by Hybrid Index Class (HIC). The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

7.3.2 Jaw length

Adult female jaw length was compared between HIC categories (Figure 7.4a). There were no significant differences between the variances of HIC1 and HIC2 (*F*-ratio test; F = 1.26, p = 0.26, d.f. = 87 & 30) or HIC4 and HIC5 (F = 0.78, p = 0.18, d.f. = 58 & 43). Mean jaw length was not significantly different between HIC1 and HIC2 (t = -1.04, p = 0.15, d.f. = 117) but HIC4 jaws were longer than HIC5 (t = 1.75, p = 0.04, d.f. = 101). Median HIC3 jaw lengths were significantly different from both HIC1/2 (Mann-Whitney; W = 15, p < 0.001) and HIC4/5 (W = 355, p < 0.001). Jaw lengths were plotted against HI (Figure 7.4b) to show that the distribution of hybrid jaw lengths was not discrete and overlapped with a small number of non-hybrid red deer.

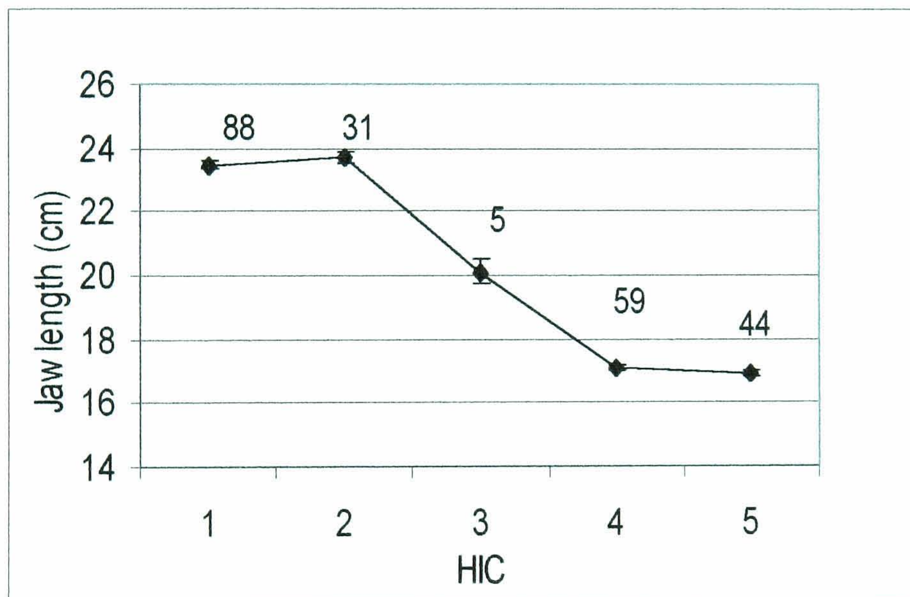


Figure 7.4a Mean adult female jaw length by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

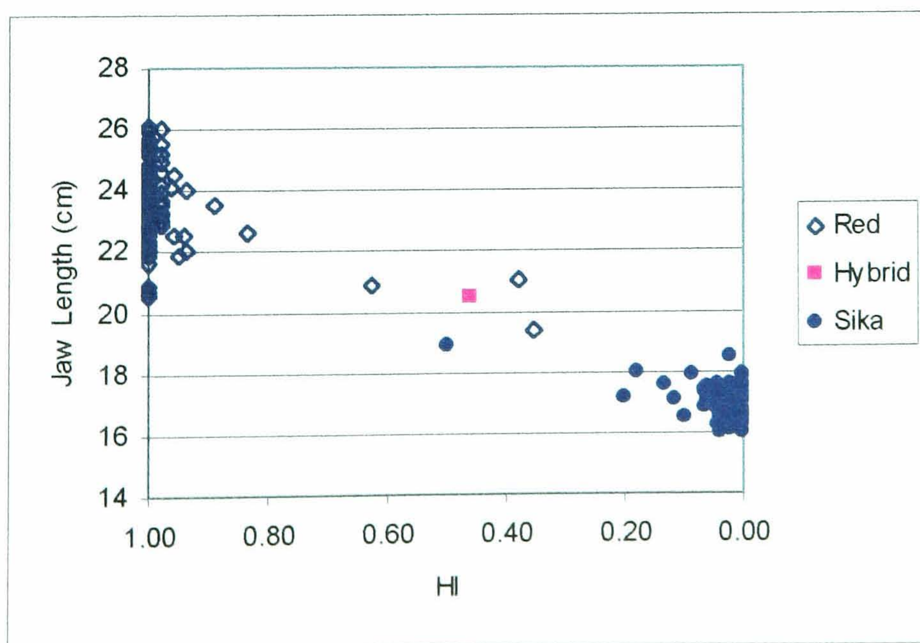


Figure 7.4b Adult female jaw length by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika.

7.3.3 Incisor Breadth

Mean adult female incisor breadths (IB) were plotted against HIC category (Figure 7.5a). The variances of HIC1 and HIC2 IBs ($F = 1.58$, $p = 0.07$, d.f. = 89 & 32) were not significantly different, but HIC4 IBs were significantly less variable than HIC5 samples ($F = 0.42$, $p < 0.001$, d.f. = 58 & 46). HIC1 IBs were not significantly smaller than HIC2 IBS ($t = 1.55$, $p = 0.06$, d.f. = 116), but HIC4 IBs were significantly larger than HIC5 IBs ($t = 2.15$, $p = 0.02$. d.f. = 71). Median HIC3 IBs were both significantly smaller than HIC1 and HIC2 IBs ($W = 8241$, $p < 0.001$), and significantly larger than HIC4 and HIC5 IBs ($W = 5460$, $p < 0.001$). IB was plotted against individual HI (Figure 7.5b); the five HIC3 samples lie between 29 and 35 mm, discrete from the main red-like and sika-like distributions.

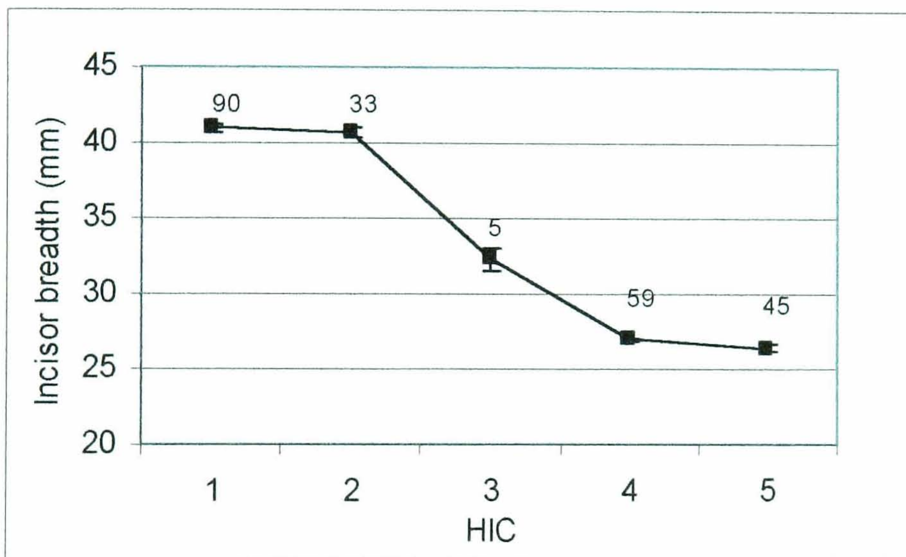


Figure 7.5a Mean adult female incisor breadth (IB) by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

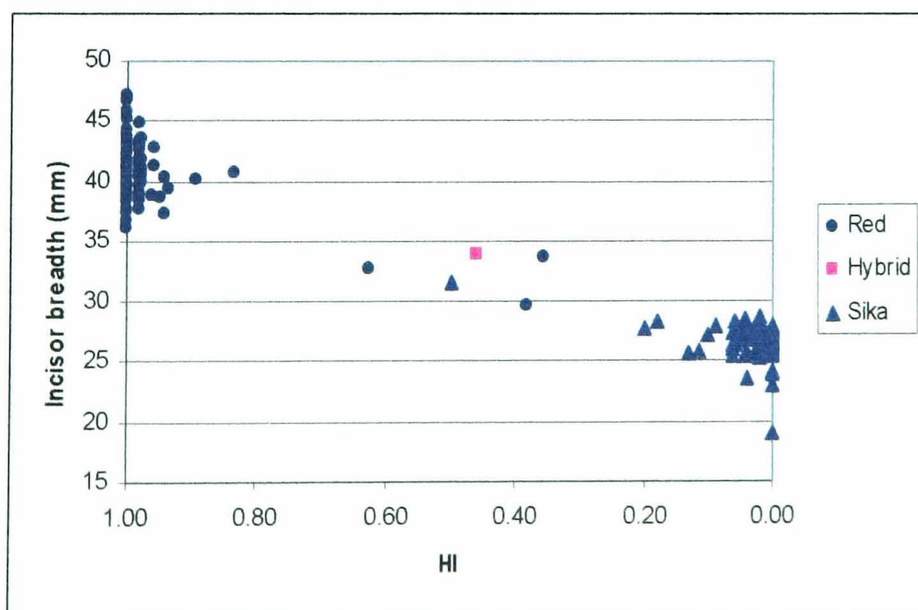


Figure 7.5b Adult female incisor breadth (IB) by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika.

7.3.4 Body weight

Adult female carcass weight did not change significantly with shooting date in either red-like deer ($R^2 = 0.01$, $F = 0.07$, $p = 0.79$) or sika-like deer ($R^2 = 0.03$, $F = 3.46$, $p = 0.07$). Mean adult female carcass weight and standard error is shown in Figure 7.6a for each of the five HIC categories. There were no significant differences between the variances of HIC1 and HIC2 samples ($F = 1.01$, $p = 0.50$, d.f = 92 & 34) or between HIC4 and HIC5 samples ($F = 1.39$, $p = 0.12$, d.f. = 60 & 45). Mean carcass weight was not significantly different between HIC1 and HIC2 samples ($t = -0.69$, $p = 0.02$, d.f. = 126), but HIC4 samples were significantly heavier than HIC5 samples ($t = 3.44$, $p < 0.001$, d.f. = 105). Median HIC3 carcass weight was significantly lower than combined HIC1 and HIC2 median weight ($W = 8815$, $p < 0.01$) and significantly higher than combined HIC4 and HIC5 ($W = 5790$, $p < 0.001$). Combined HIC1 and HIC2 sample mean weights were significantly greater than combined HIC4 and HIC5 ($t = 26.64$, $p < 0.001$, d.f. = 233). Individual HI is plotted against carcass weight in Figure 7.6b. Intermediate HI samples did not have a discrete weight range.

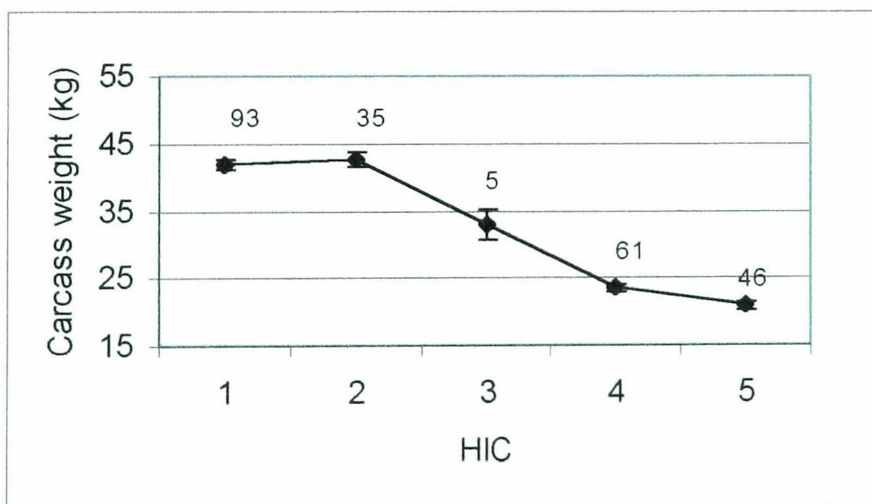


Figure 7.6a Mean adult female carcass weight (kg) by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

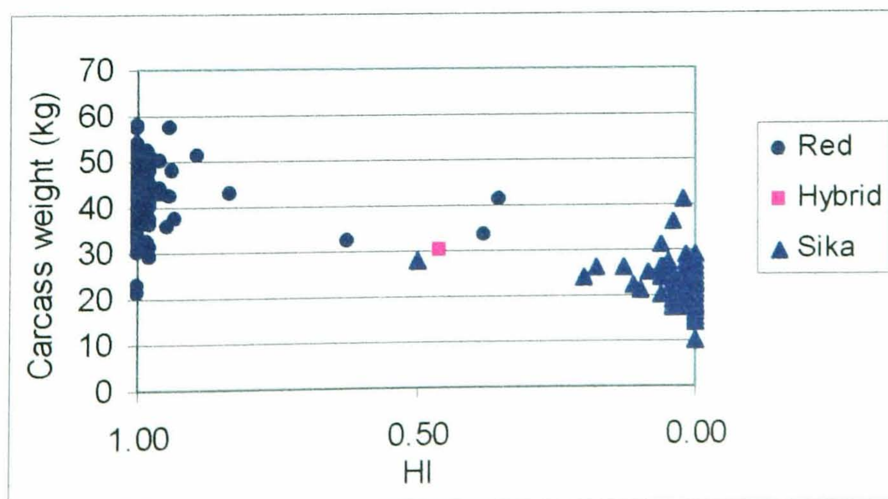


Figure 7.6b Adult carcass weight (kg) by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika.

7.3.5 Condition

7.3.5.1 KFI

Log transformed Kidney Fat Index (KFI) of adult red females varied with shooting date ($R^2 = 0.09$, $F = 11.96$, $p < 0.001$), but those of sika did not (sika; $R^2 < 0.01$, $F = 0.46$, $p = 0.50$). Mean shooting date adjusted KFI (AKFI) plotted by HIC is shown in Figure 7.7a. There were no significant differences between the variances of HIC1 and HIC2 ($F = 1.43$, $p = 0.15$, d.f. = 70 & 25) or between HIC4 and HIC5 ($F = 1.01$, $p = 0.49$, d.f. = 52 & 38) sample groups. There were no significant differences between the mean AKFI of HIC1 and HIC2 ($t = 0.41$, $p = 0.34$, d.f. = 111), or between HIC4 and HIC5 samples ($t = 1.10$, $p = 0.14$, d.f. = 100). However, HIC4 and HIC5 samples had a significantly higher mean AKFI than combined HIC1 and HIC2 samples ($t = -8.62$, $p < 0.001$, d.f. = 187). HIC3 median AKFI was not significantly higher than combined HIC1 and HIC2 median AKFI ($W = 4856$, $p = 0.06$), or significantly lower than combined HIC4 and HIC5 median AKFI ($W = 4543$, $p = 0.07$). Intermediate HI samples did not have a discrete distribution compared with non-introgressed samples (Figure 7.7b).

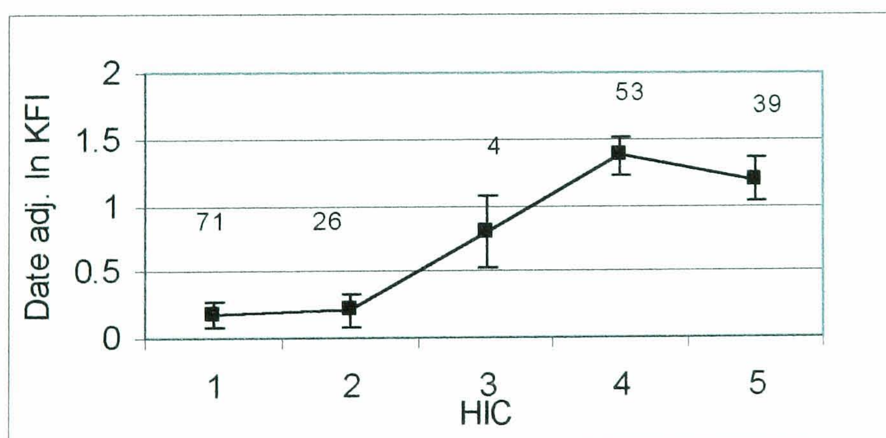


Figure 7.7a Mean adult female, date adjusted, ln Kidney Fat Index (AKFI) by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

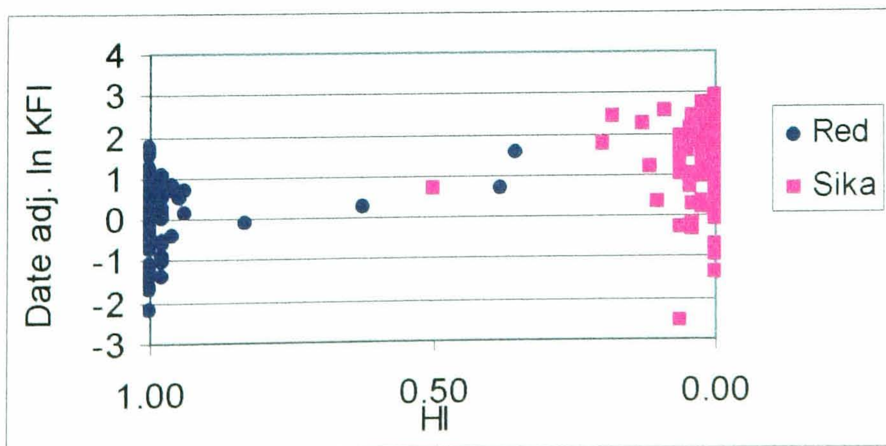


Figure 7.7b Adult female, date adjusted, ln Kidney Fat Index (AKFI) by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika. No phenotypic hybrids supplied.

7.3.5.2 Carcass weight by jaw length

Adult female carcass weights were adjusted by jaw length to give a carcass index (CI). CIs did not vary significantly with shooting date (red, $R^2 = 0.04$, $F = 3.63$, $p = 0.06$; sika, $R^2 = 0.01$, $F = 1.11$, $p = 0.29$). Mean CI for each HIC category is shown in Figure 7.8a. There were no significant differences between the variances of HIC1 and HIC2 ($F = 1.04$, $p = 0.46$, d.f. = 87 & 30) or between HIC4 and HIC5 ($F = 1.38$, $p = 0.17$, d.f. = 58 & 43) sample groups. There were no significant differences between the mean CI of HIC1 and HIC2 samples ($t = -0.63$, $p = 0.26$, d.f. = 58), but HIC4 samples had a significantly higher CI than HIC5 samples ($t = 3.34$, $p < 0.001$, d.f. = 101). Combined HIC1 and HIC2 samples had a significantly higher mean CI than combined HIC4 and HIC5 samples ($t = 14.77$, $p < 0.001$, d.f. = 220). HIC3 median CI was significantly lower than combined HIC1 and HIC2 median CI ($W = 7569$, $p = 0.04$) and significantly higher than combined HIC4 and HIC5 median CI ($W = 5402$, $p = 0.001$). Intermediate HI samples did not have a discrete CI distribution compared with non-introgressed samples (Figure 7.8b).

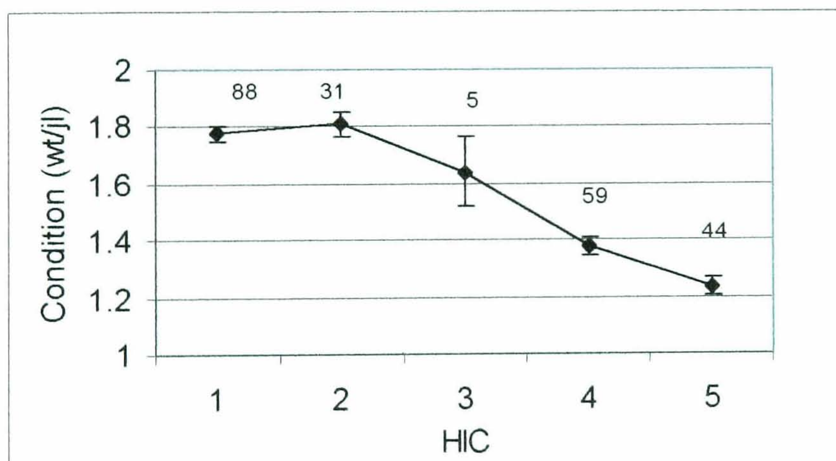


Figure 7.8a Mean adult female, carcass index (CI) by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

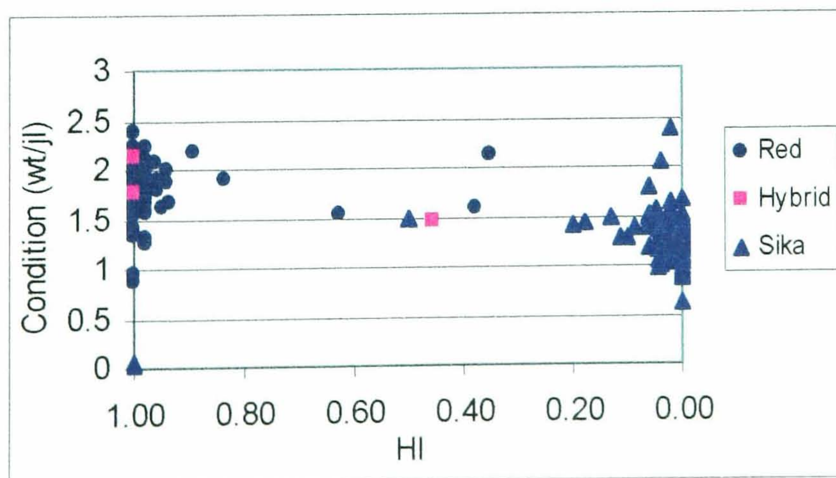


Figure 7.8b Adult female carcass index (CI) by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika.

7.3.6 Female reproduction

7.3.6.1 Conception date

Mean foetal conception date (CD; adult hind samples only) and standard error was estimated for each HIC category (Figure 7.9a). Variance estimates of HIC1 and HIC2 were not significantly different (F = 0.59, p = 0.06, d.f. = 54 & 21), nor were those of HIC4 and HIC5 (F = 1.04, p = 0.46, d.f. = 49 & 36). Mean CD was significantly earlier in HIC2 samples compared to HIC1 samples (t = 2.13, p = 0.04, d.f. = 75), but mean CD did not differ significantly between HIC4 and HIC5 samples (t = -0.03, p = 0.49, d.f. = 85). There was no significant difference between combined mean CD of HIC1/HIC2 samples and combined HIC4/HIC5 samples (t = -0.84, p = 0.20, d.f. = 162). Median CD of HIC3 was not significantly different from combined HIC1/HIC2 (W = 3107, p = 0.28) or combined HIC4/HIC5 (W = 3968, p = 0.52). No clear pattern between CD and HI was seen for intermediate hybrid samples (Figure 7.9b).

Figure 7.9a Mean foetal conception date by Hybrid Index Category (HIC), showing standard errors and sample sizes. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

Figure 7.9b Foetal conception date by Hybrid Index (HI) and phenotype. HI represents the proportion of red alleles genotyped in each sample, i.e. 1.0 represents all red and 0 represents all sika.

7.3.6.2 Fecundity

Fertility of adults and yearlings was estimated for each HIC class (Figure 7.10). The percentage of adult pregnant hinds was independent of HIC classification ($G = 4.08$, $p > 0.05$, d.f. = 4), however, yearlings pregnancies did vary with HIC class ($G = 25.24$, $P < 0.001$, d.f. = 4). Combined HIC1 and HIC2 yearlings had a lower percentage of pregnancies than combined HIC4 and HIC5 yearlings ($G = 9.81$, $p < 0.001$, d.f. = 1). There were no differences between HIC1 and HIC2 yearlings ($G = 0$, $p = 1$, d.f. = 1), but HIC4 samples had a lower percentage of pregnancies than HIC5 samples ($G = 9.69$, $p < 0.001$, d.f. = 1). Among phenotypic sika there were also five pregnant calves (two HIC4 and three HIC5) and one twin pregnancy (in a HIC5 individual).

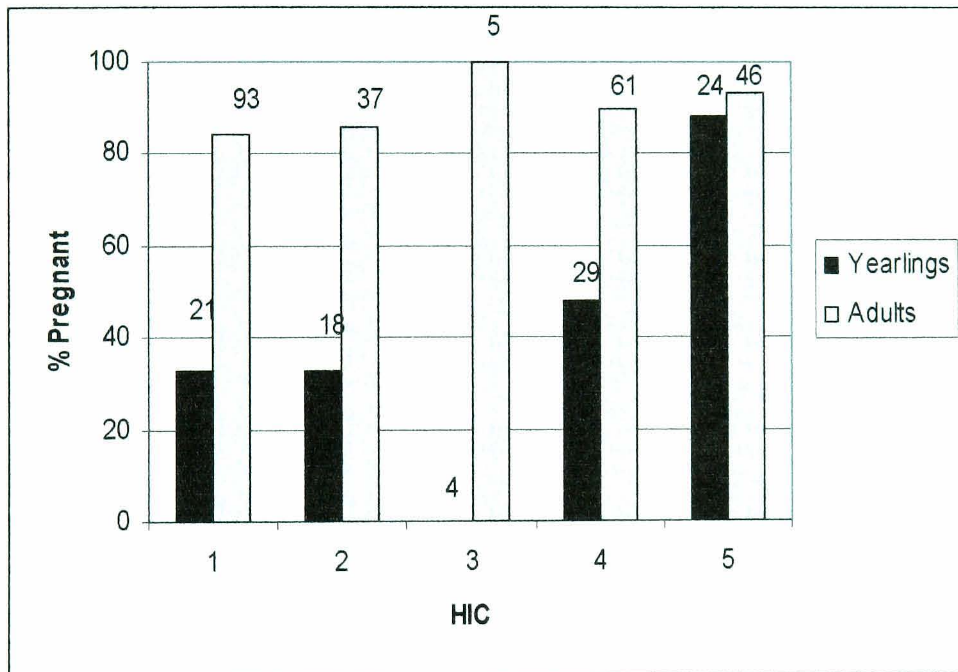


Figure 7.10 Percentage and sample size of pregnancies in yearling and adult deer by HIC category. The five HIC classes are individuals genotyped with: (1) no sika alleles, (2) 1-19% sika alleles, (3) 20-80% sika alleles, (4) 81-99% sika alleles and (5) 100% sika alleles.

7.4 Discussion

7.4.1 Carcass weight and hybridisation

A comparison of average taxon carcass weights revealed wide variation both within and between populations, and showed no association with the identification of recent hybridisation events across Scotland. As carcass weight is highly correlated with skeletal size (as measured by jaw length; $R^2 = 0.82$, $F = 1082$, $p < 0.001$: Argyll data), the hypothesis that small differences in average physical size between populations allows hybridisation (Lowe & Gardiner, 1975; Harrington, 1982) appears unlikely. Indeed, the population with the largest difference between red females and sika males, the Borders, has recent hybrids (chapter 4) and the population with the smallest differences, Fife, has none. However, body size may well be important in specific hybridisation events (Harrington, 1979), as within populations a wide variety of differently sized potential mates exist due to age and local environmental effects (Mitchell *et al.*, 1981; Mitchell & Crisp, 1981; Feldhammer, 1985; Ratcliffe, 1987b; Albon & Clutton-Brock, 1988).

The genetic evidence presented in chapter 4 indicated that the most probable direction of hybridisation is between red females and sika males. One stage at which red females may be more vulnerable to hybridisation is as yearlings. The average carcass weight of red yearling females (25.85 kg, s.e. = 1.49, n = 35: Scotland wide data set) was close to the average sika female weight in the Scotland wide study (23.1 kg, s.e. = 2, n = 6), and in forestry high rates of ovulation are common in yearling red deer (Ratcliffe, 1987b). Younger red deer have also been reported to ovulate past the main peak of the rut (Mitchell *et al.*, 1981; Ratcliffe, 1987b) when competition involving larger red males is likely to be reduced (Clutton-Brock *et al.*, 1982). Were the production of F1 hybrids frequent enough, the collection of mother-foetus pairs would allow the maternal age, condition and timing of such events to be investigated further.

7.4.2 Phenotypic effects of hybridisation

In Argyll the pattern of phenotypic changes associated with hybridisation was relatively consistent across measures. Initial hybridisation events and first backcrosses produced offspring with on average intermediate scores. However, in most cases the distribution of HIC3 samples was not discrete from either parental group and could not be used as tool for identifying hybrids in the population (e.g. carcass weight). Once further backcrossing had occurred, in either parental direction, the phenotypic consequences of introgression were more subtle and nearly always involved a shift towards red characteristics among hybrid sika-like deer (HIC4). There was little evidence of any change in red-like hybrid samples (HIC2) with the only significant difference being an earlier than average conception date, but this is a confusing result as sika-like deer did not have correspondingly earlier conception dates with the parameters used in this analysis.

There is no clear reason why introgression has affected sika-like hybrids more than red-like hybrids. One possibility is that the red phenotype is dominant to the sika phenotype but this seems unlikely from other hybridising populations where, if anything, the opposite has been found (Harrington, 1979; Bartos & Zirovnicky, 1981).

An alternative explanation is that there was a bias in the HIC1/HIC2 sample designation due to overestimation of introgressed alleles in red-like samples. Chapter 4 described the problems of specific microsatellite allele designation. It is possible that some rare alleles in red samples were not introgressed, but were due to taxon-specific polymorphism (Goodman *et al.*, 1999). If actual HIC1 samples were included in the HIC2 sample group, real differences between the two data sets may have been masked. A corresponding bias in HIC4/HIC5 samples was less likely because it was easier to characterise sika-like alleles due to low polymorphism in sika deer, probably caused by a genetic bottleneck (chapter 3).

Potential differences between HICs may also have been masked by the inclusion of two year-old females in the adult data set as this would have affected the precision of each

estimate. There were, however, no differences between the proportions of 2 year-olds in HIC1, 2, 4 and 5 sample groups ($G = 1.09$, $p > 0.05$, d.f. = 3), and therefore any bias should have been consistent between categories. Furthermore, across the traits studied, red-like deer were no more variable than sika-like deer (in coefficient of variation) so the ability to detect significant differences was not biased by this potential problem.

There were, however, differences between the proportions of HIC1 and HIC2 samples across sample sites ($G = 23.80$, $p < 0.001$, d.f. = 6), with fewer HIC2 samples collected at Kilmichael and Eredine. Indeed 34% of all HIC1 samples came from Eredine which showed below average red deer carcass weights (40.0 kg < 43.26 kg), so any effects of hybridisation may have been masked by local environmental effects. In sika-like samples there were no differences between proportions of HIC4 and HIC5 samples across sites so any such effects would have been equal between classes.

Although multiple comparisons were made, the consistency, direction and magnitude of the various phenotypic comparisons provides strong evidence for an increase in body size of hybrid sika-like deer compared to non-hybrid sika. The extent to which these small differences have any functional significance will depend on how the various trade-offs involved affect the fitness of differently sized deer. The same arguments will apply as to the larger scale differences between taxa. In essence, the advantages of smaller body size come from an increased reproductive capacity (Caughley & Krebs, 1983), lower absolute energy requirements (Clutton-Brock & Harvey, 1983), and improved diet selection (Illius & Gordon, 1987; Clutton-Brock *et al.*, 1987). In contrast larger individuals will be able to carry larger neonates, travel further between food sources, suffer relatively lower heat loss, and survive on a lower quality diet (Clutton-Brock & Harvey, 1983).

The observed relationship across species between fertility and body size appears to be maintained among Argyll deer, with the smallest sika-like females in HIC5 having the highest yearling fertility rates. The large difference in fertility rate between hybrid and non-hybrid sika-like yearlings may be explained by the fact that the small HIC5 yearlings

achieved a greater proportion of their full adult body weight as yearlings and therefore ovulated: there was no significant difference in yearling carcass weight or jaw length ($t = 1.41$, $p > 0.05$, d.f. = 51; $t = 0.30$, $p > 0.05$, d.f. = 49). However, in contrast to previous findings on red deer (Albon *et al.*, 1986) differences in adult carcass condition index (CI) between hybrid and non-hybrid sika-like deer did not translate into differences in adult fertility because nearly all adult sika-like hinds were pregnant regardless of condition.

The large differences in KFI and fertility between red-like and sika-like deer may indicate that the smaller sika-like phenotype is currently at an advantage in this habitat. Although larger red-like deer are able to survive on lower quality diets and suffer relatively less heat loss, they have absolutely higher energy requirements (Clutton-Brock & Harvey, 1983; Illius & Gordon, 1987). However, larger body size can confer another advantage in that it allows the birth of larger neonates, which can increase survival rate by lowered predation (Clutton-Brock & Harvey, 1983) and reduced vulnerability to starvation and disease (Clutton-Brock *et al.*, 1987). Although adult mortality is probably low in forest red- and sika-like deer, juvenile (0-1 yr. old) mortality can be considerable (Ratcliffe, 1987b; O'Donohue, 1991). Red deer calf summer mortality was estimated to range between 0 and 29% by Ratcliffe (1997b) and in one culled forest population in Ireland, 64% of sika calves were estimated to have died by the December following their birth (O'Donohue, 1991). In this case, the smaller body size and lower birth weights of sika-like calves may make them more vulnerable to predation (T. Birkitt, pers. comm.) or starvation.

As juvenile survival has been found to be the most important variable in regulating large herbivore populations (e.g. Gaillard, *et al.*, 1998), a more detailed knowledge of sika and hybrid juvenile mortality may provide a better understanding of the fitness consequences of different body sizes. Unfortunately, the sample collection in Argyll began in mid December, too late to estimate reliably juvenile mortality from biases in lactation to pregnancy rates (Ratcliffe, 1987b).

Differences in conception date can reflect variation in individual and population condition, with later dates corresponding to lower condition (Mitchell & Lincoln, 1973; Guinness *et al.*, 1978). In this study there was no such relationship, however the accuracy of the parameters used to calculate conception dates could not be guaranteed. All it is possible to say is that there was no association between condition (AKFI or CI) and conception date. The marginally significant difference ($p = 0.04$) between hybrid and non-hybrid red-like deer in conception date may be the result of spatial variation in habitat quality, or may represent a Type 1 error (Sokal & Rohlf, 1997) at the 95 % level and have no biological significance.

Differentiating anything other than F_1 or F_2 hybrids from non-hybrid deer on the basis of the phenotypic measures used here is clearly not possible. Once backcrossing has occurred in either direction the offspring quickly converge on the parental phenotype. As even detailed morphometric techniques were unable to differentiate between backcrossed and parental populations, it is not surprising that few hybrids were identified by rangers and stalkers compared to the large number of genetic hybrids found. What is perhaps more surprising is that only one of the five genetically and phenotypically intermediate hybrids described here was correctly identified by the ranger. Although retrospectively these five recent hybrids could have been diagnosed on the basis of jaw length, or incisor breadth, changes in population performance may alter the parental trait distributions and make identification by phenotype alone inaccurate. These results also raise questions regarding the general power of morphometric techniques used to detect hybrids of mammalian species (i.e.: French *et al.*, 1988; Ratcliffe *et al.*, 1992; Clutton-Brock *et al.*, 1994).

8 Discussion

8.1 Hybrid survey

Despite widespread overlap among Scottish populations, hybridisation between red and sika deer appears to have been relatively rare, but where it has occurred, it has resulted in considerable introgression locally. Populations can be characterised in three ways: (a) entirely red-like and sika-like populations with occasional rare alleles that may be introgressed (e.g. Loch Morar red, Angus red, Fife red and sika and Great Glen red and sika), (b) entirely sika-like and red-like populations with moderate to high frequencies of introgressed alleles at some loci (e.g. Sutherland sika and South Kintyre sika), and (c) mostly red-like and sika-like populations, but with some phenotypically hybrid deer and a proportion of genetically intermediate deer (Borders, north Kintyre, Ross-shire and Cowal). In each case, information relating to the history of translocations, distribution of allele sizes and the spatial pattern of introgression was used in the translation of genotype data into the population histories suggested below.

Goodman *et al.* (1999) discussed in detail the difficulties involved in analysing and interpreting molecular data from populations where hybridisation is rare. In the first group of populations identified above, the presence of rare alleles typical of the opposite taxon does not alone provide sufficient evidence for hybridisation, as these alleles may represent ancient polymorphism within the parental populations (Goodman *et al.*, 1999). The interpretation is further confused when these rare alleles are not present in the local opposite taxon, but are characteristic of other populations or taxa such as wapiti. In some circumstances it may be possible to attach a probability to each of the possible sources of variation (mutation and introgression), when well characterised parental reference populations are available and numerous diagnostic microsatellite markers are used (Boecklen & Howard, 1997; Tate *et al.*,

1998). However with the present data the complex admixture of taxa and populations. and the advanced nature of the backcrosses make such an analysis impractical.

Across Scotland the pattern of rare alleles found in red-like populations suggests that a proportion are likely to have introgressed from sika and or wapiti. In sika-like populations the pattern of variation was less complex and ambiguous rare alleles could usually be attributed to introgression with red deer (except that is, in the Borders sika where a common allele (89bp) at one locus (MM012) was ambiguous). It is also possible that some variation found in certain sika-like populations originated from the admixture of different sika populations or subspecies. For example, the Fife sika share a number of otherwise rare alleles with the Borders sika, even though they are thought to have originated from the same source as the other Scottish sika populations (Powerscourt, Ireland), and not from the same source as the Borders sika (direct from Japan) (see section 1.3.2).

In the second group of populations, the pattern of variation suggests successful hybridisation between red and sika several generations ago, resulting in considerable introgression across loci, especially in the sika-like population. Differences in patterns of introgression between loci and taxa can be explained by varying histories of hybridisation in each population. In Sutherland, introgression is rare in red-like deer, but common at a proportion of loci in sika-like deer. This suggests that the initial sika escapees from Rosehall hybridised with local red deer and that these hybrid offspring successfully backcrossed into the small remaining sika herd to produce a hybrid population. Following their establishment, strong assortative mating took place and little or no gene flow has occurred between the taxa. Introgression and ancient polymorphism could be differentiated in this population, because prior to escape a group of sika were taken from Rosehall to establish the Aldourie (Great Glen) population which does not contain the same pattern of variation.

In south Kintyre, the pattern of introgression was more evenly distributed amongst loci, but still rare at any one locus and more frequent in the sika-like population. In this case, the original sika population was able to establish a larger population before expanding into a

colonising red population from the north (Ratcliffe, 1987a). As both populations colonised through each other they formed a moving hybrid zone, behind which the red-like and sika-like populations mated assortatively. Re-analysis of Abernethy's (1994a) Argyll samples indicated average rates of hybridisation of 1/500 matings for sika-like deer and 1/1000 for red-like deer (Goodman *et al.*, 1999) (see section 1.4.2). Differences in these rates can be explained by variation in breeding success of red-like and sika-like hybrids. First and second generation hybrids are intermediate in size (Harrington, 1979; pers. obs.) and therefore larger than sika-like deer and smaller than red-like deer. Male hybrids are therefore likely to have higher breeding success by backcrossing into the sika-like populations because of the effects body size can have on male dominance and breeding success in polygynous ungulates (Clutton-Brock *et al.*, 1982). There may also be other differences in the fitness of red-like and sika-like hybrids as there is some genetic evidence for selection against hybrids in Kintyre (Goodman *et al.*, 1999) (see section 1.4.2).

The best genetic and phenotypic evidence for recent hybridisation in Argyll comes from the north of Kintyre. Significant linkage disequilibrium was found at a number of sites (Pemberton *et al.*, 1999) and in one population (Loch Avich), 14 (32%) of the sika-like deer contained multiple alleles normally found in red-like deer. Phenotypic hybrids were reported in Inverliever and 12% of the Loch Avich samples were identified as hybrid by the forest ranger.

In Galloway a pocket of hybridisation was identified in Dundeugh Forest, north east of the main Galloway forest, at the leading edge of sika colonisation. A number of phenotypic hybrids have been reported in recent years (J. Wykes, pers. comm.), one of which was sampled and genotyped as an F1 or F2 hybrid. A further three red-like samples from the same forest were genotyped as F2 or F3 backcrosses.

In Easter Ross, the majority of red-like samples showed no evidence of introgression and most sika-like samples had similar patterns of introgression to those found in the Sutherland population (common introgression at a subset of loci). However, one red-like

sample from the Kildermorie forest genotyped heterozygous red/sika at six loci (out of eight tests), indicating a recent hybridisation event. Though no phenotypic hybrids were reported in the sample cull year (1997), they have been seen in the area in recent years (W. Lamont, pers. comm.).

8.2 Causes of hybridisation

The reason why some populations have hybridised whilst others have not is of interest because it may provide a basis upon which management strategies can be formed. It may also provide an understanding of the factors involved in the process of speciation. Three main hypotheses have been put forward to explain why some red and sika deer populations are more vulnerable to hybridisation than others. The first two, which overlap, involve the genetic provenance of the local *Cervus* deer, and the relative body size of the populations (Harrington, 1979, 1982; Lowe & Gardiner, 1975). Past hybridisation was thought to have increased the body size of Asian mainland and deer park sika populations, making them more reproductively compatible with red deer and therefore vulnerable to further hybridisation. These theories were suggested following breeding experiments (Harrington, 1979) and skull morphometric analysis (Lowe & Gardiner, 1975).

In chapter 5, various Scottish red and sika deer populations were compared genetically to establish whether there was any association between genetic background and post-introduction hybridisation. The analysis revealed that genetically very similar populations showed different patterns of hybridisation. In those areas where it was possible to establish the pre-introduction hybrid status of the sika population through the chronology of introductions, there was no evidence that previous hybridisation led to more recent hybridisation events. In addition, the Borders sika were confirmed as the most genetically differentiated Scottish population, reflecting their documented history of direct introduction from Japan.

The carcass weights of culled deer were compared as a measure of body size to investigate whether populations with smaller body size differences were more likely to hybridise (chapter 7). Carcass weights varied greatly within and between populations and much of this variation was almost certainly due to age and habitat quality variation (Mitchell *et al.*, 1981; Mitchell & Crisp, 1981; Feldhammer, 1985; Ratcliffe, 1987b; Albon & Clutton-Brock, 1988). The comparison revealed no association between either current average size or current size differences between potential mates and recent hybridisation.

The final hypothesis is that hybridisation is more likely where sika or red deer males are colonising areas with resident populations of the opposing taxon (Ratcliffe, 1987a). The evidence from this study suggests that hybridisation does tend to occur in these circumstances as all three newly hybridised populations identified across Scotland (Argyll, Easter Ross and Borders) are at the leading edge of sika colonisation into resident red deer areas. The colonisation process may therefore be one factor involved in hybridisation, but hybridisation is clearly not inevitable, as some sika populations (L. Morar, Great Glen & Fife) have expanded without evidence of recent hybridisation in this survey.

Although there has been some debate regarding the direction of crossing between wild red and feral sika populations (Harrington, 1979; Abernethy, 1994a), it appears that in Scotland, initial hybridisation has occurred between red females and sika males. In addition to the geographical context of hybridisation events (see previous paragraph), strong evidence is provided by the pattern of mitochondrial DNA (mtDNA) haplotypes found. As mtDNA is inherited from the mother only, it provides a record of each deer's matrilineal taxon. Differences in the distribution of cytonuclear genotypes can therefore provide information about the direction of crossing (Arnold, 1993). Across Scotland, all hybrids of very recent origin (HI 0.2-0.8) contained red mtDNA and were therefore descended from a lineage of red females. Although some sika-like hybrids did contain sika mtDNA in the south of Kintyre, it is very likely that they are the result of backcrossing by hybrids into the sika population.

8.3 Phenotypic consequences of hybridisation

Body parts were collected from culled deer in Argyll and comparisons of phenotypic traits were made between five categories of deer reflecting their genetic status, based on genotypes from 25 microsatellite markers and one mitochondrial DNA marker: (1) 100% red, (2) 80-99% red, (3) 21-79% red, (4) 1-20% sika, and (5) 100% sika. Average values for very recent hybrids fell mid-way between the two parental groups. However, in backcrossed individuals (i.e. less than 20% introgressed DNA), the only evidence of phenotypic disruption was limited to small increases in trait measures in hybrid sika-like deer compared with those of non-hybrids. Sika-like hybrids had significantly longer jaws, wider incisor breadths and heavier adult carcass weights compared with non-hybrid sika, although no differences were found in their average condition (as measured by Kidney Fat Index). Corresponding differences between red-like hybrids and non-hybrids were not found, although this may have been due to the confounding effects of habitat quality variation.

The fitness consequences of these changes in phenotype are difficult to predict because of the various trade-offs associated with body size in ungulates. For example, across mammalian species, fecundity is negatively related to body size (Caughley & Krebs, 1983), both within and between species, but juvenile survival is normally positively related to body size at birth (Clutton-Brock & Harvey, 1983). The effects of body size on fecundity were reflected in a reduced yearling conception rate in hybrid sika-like deer, the same category which had larger carcass weights as adults. However, if birth weights are higher in sika-like hybrids the calves may suffer lower mortality than non-hybrids and therefore be at an advantage. Although the importance of natural juvenile mortality in regulating the population will vary with culling intensity, further investigation should be carried out to establish the importance of any effects.

Management implications

Given the overall similarities of hybrid and non-hybrid deer, it is not surprising that rangers found differentiating between the two almost impossible. In Argyll, only two percent of the genetically diagnosed hybrids were identified by the forest ranger as showing hybrid traits, and across Scotland only one out of the eight samples submitted as hybrid-like was confirmed genetically. Nevertheless, two pockets of recent hybridisation (Galloway and Loch Avich) were correctly identified and individual hybrid samples from one of these did have intermediate jaw lengths and incisor breadths.

As most hybrids and non-hybrids cannot be identified visually, the selective culling of hybrid deer is not a practical option, unless it is targeted at a local population containing obvious hybrids, rather than at specific individuals. Even then, it is only likely to slow down the rate of introgression rather than stop it entirely. Furthermore, little is understood about the effect heavy culling has on the dispersal patterns of culled populations, and this practice may push sika males into resident red populations, potentially increasing the chances of hybridisation. As the spread of sika, and subsequent hybridisation, is associated with the distribution of forests, it appears that the only practical way to slow down the spread of sika and their genes is to restrict afforestation in some areas as previously suggested by Ratcliffe (1987b).

If there are no practical ways of controlling introgression what are the likely consequences for the *Cervus* deer population in Scotland? In some areas introgression is already almost complete in the sika-like population, in the sense that most deer have some red material, and considerable in the red-like population, and many other red populations show some evidence of past hybridisation with sika and/or wapiti, although differentiating low level introgression from ancient polymorphism was difficult in this study. Despite this, phenotypic introgression has not followed (even with characters normally considered as diagnostic, e.g. metatarsal gland) apart from subtle changes in the average body size of sika-

like hybrids. Abernethy (1994a) suggested that the absence of obvious phenotypic introgression in Argyll was possibly only temporary, and that in time, following sufficient gene flow, assortative mating may break down, causing a chain reaction of introgression. The prime example of where this has occurred is in Wicklow, Ireland where introgression has resulted in the virtual extinction of completely red-like deer (W. Atkinson, pers. comm.) following approximately 20 more generations of introgression than in Argyll. However, the history of Wicklow red and sika deer [in which hybridisation occurred prior to release from a park (Delap, 1936; Mooney, 1952)] is not comparable to any population in Scotland, and the same outcome should *not* be considered inevitable. In Scotland, strong assortative mating predominates, hybridisation is relatively rare and there is a large, genetically diverse (see section 1.2.2 and chapter 3) red deer population.

In practical terms, it is unlikely that further hybridisation, and particularly introgression, can be prevented and only distant Hebridean island red deer are likely to remain protected (if they are currently unintrogressed). However, if stalkers are vigilant and manage to cull pockets of first generation hybrids, mainland red and sika should remain phenotypically distinct for the foreseeable future despite introgression. In terms of conservation, this may present problems for those concerned with protecting the genetic integrity of Scottish red deer, but the real opportunity to achieve this goal was probably lost some 100 years ago due to translocations of park red deer and wapiti!

The future management of sika should therefore not be based on vague goals that are unattainable, illogical and or ill-defined. Is it the red deer phenotype that is to be protected in Scotland and how should that be defined? Or is there some conservation value in the (already introgressed) red deer population? If so, are there limits to the amount of introgression (of all species) that are desirable and/or acceptable, and how might they be detected? Finally, is there any practical way of altering the rate of introgression anyway and if so, who's (financial and legal) responsibility is it to try? All of these questions (and no

doubt several others) should be addressed in an objective management plan for deer, genus *Cervus*, in Scotland.

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Appendix 1a. Scottish Red/Sika Hybridisation Project leaflet.

3) Lower hind leg sample

In addition to the above we would also like the lower portion of one of the animal's hind legs. The length of the cannon bone will be accurately measured to obtain information on the animal's size. We request that the skin is left on the leg portion to provide information on the appearance of the metatarsal gland. This part of the leg can be either *dried* or *frozen*.

4) Pregnant hinds

If your possible hybrid is pregnant, we would also like a tissue sample from the foetus. This can provide important information on the genetic status of the father. The same procedure should be followed as for (1) above (any small piece of tissue is acceptable, or if easier, freeze the whole womb).

Labelling and recording the samples

All the samples from each animal must be carefully labelled with the date and place where the animal was shot. Please also note the sex, larder weight and any special features about the animal's appearance. These details will ensure that the samples remain together for examination and thereby avoid wasting your valuable time and effort.

Results from the study

If you have sent some material to us for analysis, please be aware that it may be some time before we are able to process your sample. We will endeavour to report back as soon as possible to those who have been involved in the collection of project material, and will produce a report at the end of the study in which your help will be gratefully acknowledged.

Sample retrieval and information

If you would like a sample bottle sent out or require any samples collected, please contact:

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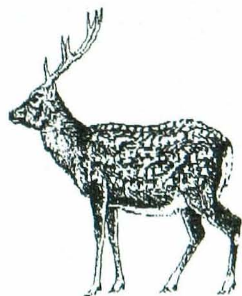
Front cover picture adapted with permission from the Handbook of British Mammals, second edition, (ed. G.B. Corbet and H.N. Southern). Blackwell Scientific Publications.

SCOTTISH RED/SIKA HYBRIDISATION RESEARCH PROJECT



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Appendix 1b. Scottish Red/Sika Hybridisation Project leaflet.

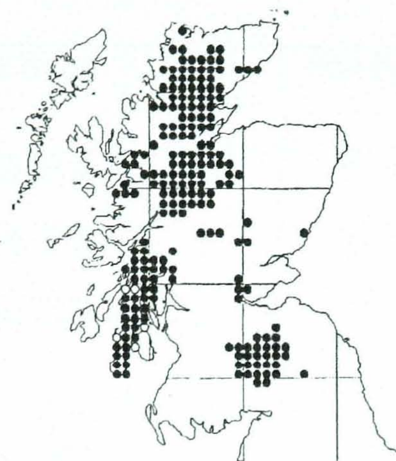

Figure 1. Current sika range in Scotland. Full circles represent sightings since 1960 in $10km^2$ grid (prepared by the Biological Records Centre from data supplied by The Mammal Society & The British Deer Society).

Sika deer in Scotland

Following their introduction in the late 19th century, sika deer (*Cervus nippon*) have now established several successful populations across Scotland (Figure 1).

Previous research by Kate Abernethy (Edinburgh University) has shown that two of these populations (Kintyre and Great Glen) have hybridised (interbred) extensively with the local red deer (*C. elaphus*), potentially altering the size and behaviour of red deer. Sika are smaller and may cause more damage to forestry. They are also thought to be more secretive and difficult to manage.

This project plans to build on Kate's assessment by carrying out a nationwide survey of red, sika and hybrids.

Project aims

The project aims to:

(1) Investigate whether the appearance, size, genetic ancestry, or local habitat of the deer is important in hybridisation.

(2) Predict the likely outcome of hybridisation, with special reference to the implications for deer management.

(3) Map the distribution of hybrid populations.

Collection of material

The project will involve the collection of a small amount of material from shot deer over the next three seasons (1996-1999).

Areas where red and sika overlap

We would like to hear from anyone interested in helping us by collecting samples and data from the various overlapping red and sika populations. This information will be used to find out why hybridisation appears only to occur in certain areas.

Argyll study

For our investigation of the Argyll population we will initially develop Abernethy's work using her samples from 1992. However, later in the study we will require some more samples, linking the genetics and appearance of the animals. If you are interested in helping, please contact us on the numbers shown on the back of this leaflet.

National distribution of hybrids

Have you shot a hybrid?

Over the period of the project we would like to hear from anyone who thinks that they have shot a hybrid outside their known range in Argyll and the Great Glen.

What to do

To establish whether the animal you have shot is a hybrid or not, we require as much of the following as possible:

1) Tissue sample

We require a small piece of flesh, about the size of a one pound coin. We suggest that you collect the tip of the tongue. There are two ways to preserve the samples:

a) Place the sample in a clean, carefully labelled plastic bag and freeze it.

b) Keep the sample as cool as possible (larder or fridge) in a labelled plastic bag and contact us for a sample bottle. When the bottle arrives, follow the instructions and return it to us as soon as possible (see below).

2) Skulls and jaws.

Ideally, we would like to obtain a matching skull with lower jaw, so that we can record the age and skeletal dimensions of the animal. However, if the skull is unavailable, the lower jaw is still informative alone.

For these materials, please remove as much flesh as possible before *drying* or *freezing*.

Appendix 2a. Microsatellite markers and mitochondrial marker used in Argyll and Scotland wide survey and primer sequences and reaction conditions.

Locus	Deer Linkage Group	Sika Alleles	Size Range	Red Alleles	Size Range	Primer Sequence	Mg2+	DMSO	Aneallin g temp	Reference
RME025	12	5	197-213	2	171-173	AGTGGGTAAAGGAGCCTGGT TTATTGATCCCAGCCTGTGC	2.0mM	5%	48-50	Grosse et al (1995) Ani Genet. vol. 26 p126
BM6438	31	3	267,275,277	4	251-263	TTGAGCACAGACACAGACTGG ACTGAATGCCTCCTTTGTGC	2.0mM	5%	52-54	Bishop et al. 1994
BOVIRB P	15	3	142-146	8	148-166	TGTATGATCACCTTCTATGCTTC GCTTTAGGTAATCATCAGATAG C	2.0mM	10%	52-54	
FCB193	5	4	123, 127, 129, 139	12	97-121	TTCATCTCAGACTGGGATTCAG AAAGGC GCTTGGAAATAACCCTCCTGCA TCCC	2.0mM	10%	52-54	Buchanan & Crawford 1993
MM012	26	1	93	2	89-91	CAAGACAGGTGTTTCAATCT ATCGACTCTGGGGATGATGT	2.0mM	10%	48-50	Mommens et al (1994) Ani Genet. vol. 25 p368
BM4006	-	1	89	2	97,99	CAATGTGCATTATTTCCAAAGTG AGAAATAACTCTTTCTCCTTGGA GG	2.0mM	-	50-52	Bishop et al. 1994
INRA005	30	1	144	1	128	CAATCTGCATGAAGTATAAATAT CTTCAGGCATACCCTACACC	3.0mM	10%	50-52	Vaiman et al.1992
RM188	18	7	146,162,182, 184, 186, 190, 196	9	120-142	GGGTTCACAAAGAGCTGGAC GCACTATTGGGCTGGTGATT	2.0mM	10%	50-52	Barendse et al. 1997
INRA006	20	1	128	3	130,132,134	AGGAATATCTGTATCAACCTCA GTC CTGAGCTGGGGTGGGAGCTAT AAATA	2.0mM	-	52-54	
RM095	31	3	120, 122, 126	12	122-148	TCCATGGGGTCGCAAACAGTGG ATCCCTCCATTTGTTGTGGAGTT	2.0mM	5%	52-54	(Bishop et al., 1994)
mtDNA	-	1	430bp (3 repeats)	1	350bp (1 repeat)	TTAAACTATTCCCTGACGCTT CCTGAAGTAGGAACCAGATG	2.0mM	-	50	Cook, 1993

Appendix 2b. Microsatellite markers used in Argyll survey and primer sequences and reaction conditions.

Locus	Deer Linkage Group	Sika Alleles	Size Range	Red Alleles	Size Range	Primer Sequence	Mg2+	DMSO	Anealling temp	Reference
IDVGA055	4	1	213	5	193-201	GTGACTGTATTTGTGAACACCTA TCTAAAACGGAGGCAGAGATG	2.0mM	-	52-54	Vaiman et al.1994c
UWCA047	-	1	239	2	227, 233	GGAAAGTCCTTAGATGGAGGATTGT TTGAGAACTTGTCCCGAGAGAA	2.0mM	-	50-52	Barendse et al. 1997, Kirkpatrick et al. 1995 Mammalian Genome 6:526-8
BM757	28	2	175, 177	12+	163,165, 177+	TGGAAACAATGTAAACCTGGG TTGAGCCACCAAGGAACC	2.5mM	-	48-50	Bishop et al. 1994
INRA131	11	1	107	6	93-105	GGTAAAATCCTGCAAAACACAG TGACTGTATAGACTGAAGCAAC	2.0mM	-	53-55	
IDVGA37	8	4	>=218	5	<=216	TAACAGGACAAGTCTTCAGGTG CCTCTCTTTCTCTATGCTCACA	2.5mM	-	50-52	
TGLA126		2	105,106	1	109	CTAATTTAGAATGAGAGAGGCTTCT TTGGTCTCTATTCTCTGAATATTCC	3.0mM	-	48-50	
IDVGA29		1	159	2	139, 147	CCCACAAGGTTATCTATCTCCAG CCAAGAAGGTCCAAAGCATCCAC	2.0mM	-	52-54	
TGLA040	10	2	108, 110	4	94, 100, 102, 104	GCTTCTCTGCCAACTAATATTATCC CACCAGGTAAGCCCCTTATATATGT	2.0mM	-	54-56	Georges and Massey 1992
TGLA337	13	1	128	5	132+	TTTGTTAAGGATAGTAGGCTACT GCTCTTCCCTTGGTTTCCTTG	2.0mM	-	50-52	Geroges & Massey 1992
TGLA127	-	1	157	8+	165+	CAATTGTGTGGTAGTTTGGACATTC ACACTATTGCAAAAGGACCTCCAATT	2.0mM	-	52-54	
AGLA293	3	1	144	2	126, 142	GTCTGAAATTGGAGGCAATGAGGC CCCAAGACAACTCAAGTCAAAGGACC	2.0mM	-	52-54	Georges and Massey 1992
RM012	9	1	115	12	>=123	CTGAGCTCAGGGGTTTTTGCT ACTGGGAACCAAGGACTGTCA	2.0mM	-	53-55	
HUJ175	24	1	139	7	115-133, +null?	CTGAAGTTATCCTGGAAGGG AACAAGAATGGCTGGTCACC	2.0mM	-	50-52	Shalom, A. et al. 1994. Dinucleotide repeat polymorphism at the bovine HUJ246, HUJII77, HUJ223, HUJVI74 and HUJ175 loci. Animal Genetics 25, 56
FCB048	-	3	155-161	2	141-143	GAGTTAGTACAAGGATGACAAGAGGCAC GACTCTAGAGGATCGCAAAGAACCAG	2.0mM	10%	52-54	Buchanan et al. 1994
FSHB	1	1	181	12+	185+ (mixed odd & even)	CAGTTTCTAAGGCTACATGGT TGGGATATAGACTTAGTGGC	2.0mM	-	52-54	Crawford et al. 1995

Appendix 3. Laboratory protocols.

3a. Phenol-Chloroform DNA extraction (modified from J. Slate).

1. Label up a set of 1.5 ml Eppendorf tubes and aliquot into each 0.55 ml of digestion solution [10μl proteinase K (@ 10mg/ml) in 25% sodium dodecyl sulphate (SDS) solution].

2. Cut a small piece of tissue from the main sample (not more than 2 x 2 mm), mascerate and immerse in digestion solution at 65^0C for 15 mins, then overnight at 37 ^{0}C. If working from samples preserved in salt / DMSO preservative, soak overnight in DD H_20 then blot as much of this from the sample as possible before immersion in digest solution.

3. Remove tubes from 37 C and add 0.55 ml of phenol equilibriated to pH 8. Mix phases vigorously, place tube in microfuge and spin at 13,000 rpm for 12 sec.

4. Label up another set of Eppendorf tubes.

5. With P1000 Gilson, remove 0.5 ml of the aqueous (upper) phase to the appropriate fresh Eppendorf. Avoid picking up the interface.

6. To supernatant add 0.5 ml chloroform (i.e. an equal volume to what is already in your tube). Mix phases vigorously and spin as before.

7. Remove 0.45 ml of the aqueous (upper) phase to a new tube, as before. Add 150 ul of 10 M LiCl. Mix gently and place in a -20 freezer for 20 min.

8. Remove tube from freezer and spin in microfuge at 13,000 rpm for 5 min.

9. After spinning, pipette 0.5 ml of sample to a new, prelabelled Eppendorf tube, carefully avoiding any precipitate.

10. Add 1.0 ml of 100% EtOH, mix gently, then microfuge at 13,000 rpm for 5 min.

11. Making sure the deposit stays there, gently pour away the fluid and replace it with 1 ml of 70% EtOH. Mix gently and leave to stand at room temp for 20 min.

12. Spin again at 13,000 rpm for 5 min and tip away the 70% EtOH. Blot excess EtOH from around the rim of the tube. Leave to dry, then add 1 x TE buffer. Add 250 ul to large visible DNA pellets or less respectively (min ~ 50ul. Place the tube in 65 C waterbath for 10 minutes or as long as it takes to dissolve the pellet. Then label up the tube with Time tape and an INDELIBLE pen.

Appendix 3b. Chelex DNA extraction method (modified from D. Coltman).

1. (In advance) prepare a 5% w/v solution of Chelex-100 resin in ddH_2O. Autoclave in a small glass bottle (20-100mL) containing a magnetic stir bar. Solution should be stored at 4°C. Always pipette Chelex as a slurry <u>while</u> it is stirring to ensure that resin is taken up.

2. A) IF your tissue samples have been stored in a preservative solution containing salt or other chemicals which may inhibit PCR, snip off a small piece of tissue (approximately $5mm^2$ or about 5mg) and place in a 1.5mL labelled eppendorf tube containing 1mL ddH_2O. Label tube directly with permanent marker clearly, but do not use labelling tape until after the boiling step (5.). Use clean surgical scissors to snip bits of tissue, and try to get either muscle or epidermis. Connective tissue and hair are okay, but adipose tissue or bits which have become rancid do not work well as the lipids in adipose and secondary compounds produced by bacteria in rancid samples can inhibit PCR. Rinse and wipe visible bits off the scissors between samples to limit cross-contamination. Let stand at RT for 1 hour then evacuate ddH_2O using a pipette. Now add 200uL of 5% Chelex slurry to the sample. When pipetting 5% Chelex, use a 1000uL tip which has a wide enough bore to ensure that the resin is taken up. Go to 4.

3. OR
4. B) IF your tissue/blood sample is fresh, frozen or dried thus containing no PCR unfriendly preservatives, snip off a small piece of tissue as described above or pipette 5uL of whole blood into a labelled tube containing 200uL of Chelex resin. Go to 4.

5. Incubate for 1 hour or more in a water bath at 60-65°C (I've left this step over the weekend with no adverse effects).

6. Shift tubes to a boiling water bath for 8 minutes.

7. Vortex briefly then spin in a microfuge for 3-5 minutes. Supernatant is ready to use as DNA template for PCR even though it may look like tea (or even soup!). Be very careful not to take up any resin when pipetting supernatant for PCR as the resin will kill your PCR by binding the Mg^{2+}. I transfer about 20uL of supernatant from each sample to a well on a microtitre plate and dilute 1:1 with ddH_2O for short-term storage where it can be then be aliquoted by a multichannel pipettor for PCR. Use 2uL of the diluted supernatant as template per reaction. Plates may be good for a week or so at 4°C if covered with parafilm, lid and cellowrap to prevent evaporation. Store extractions at −20°C where they should last indefinitely. To use again just thaw, vortex briefly, then spin before taking supernatant.

Appendix 3c. PCR reaction (modified from J. Pemberton).

1. For phenol choloroform extraction dilute template DNA concentration to working concentration following agarose visualisation (1/10 ~1/100). e.g. add 10ul of conc DNA to 90ul of BDH water in 96-well PCR. For Chelex extraction, vortex for 3-5 mins then use supernatant either directly, or as a 1/10 dilution.

2. Using the multichannel pipette transfer 2ul of DNA into a new PCR tray.
MAKE SURE YOU USE A DIFFERENT TIP FOR EACH SAMPLE HERE.

3. Make up PCR reaction cocktail for each locus.
   e.g. For 96 reactions.
   In a 1.5 ml eppendorf tube mix.

•720 ul BDH water
•96ul dNTP's (1mM dGTP, dATP, dTTP and 0.1mMdCTP) Less dCTP here as the radioactivity is incorporated into dCTP and we make up the deficit on addition of nukes.
•96ul PARR buffer. Note this contains $MgCl_2$ making the PCR cocktail 1.5mM $MgCl_2$.
•If we require 2.0mM $MgCl_2$ then add 9.6 ul of $MgCl_2$.
If 2.5mM required then 19.2ul
If 3.0mM required then 28.8ul.
• 60ul of TMAC
• 24 ul of formamide. If DMSO is required add 96ul, and reduce the H_2O accordingly.
• Add 36ul of primers if they are combined in one tube, or 18ul of each if separate.
• 8ul of QAT.
• Add 2ul dCTP-P32.

4. Using the multi-dispensing pipette mix the PCR cocktail and P32, and then dispense 10ul of PCR mix into each well in the microtitre plate and add 1 drop of mineral oil to each well.

5. Set appropriate PCR programme on machine and run.

Appendix 3d. Polyacrylamide gel electrophoresis

Pour gel

1. Clean plates and silicanise back plate, then assemble rig (2 plates, 2 spacers and 2 clamps).

2. Seal, then pour acrylamide gel and remove bubbles before leaving to dry with combs inverted.

3. Fill rig with 1 x TBE and pre-heat to 50^{0}C ready for PCR products after removing comb.

Preparing PCR

1. Remove PCR tray from machine, add 5ul STOP solution then return to PCR machine and denature at 95^{0}C for 5 minutes.

Loading gels

1. Denature the SS m13 size marker on the hot block (at 94°C).

2. Turn off the gel, re-insert combs and load 2 ul of each PCR or sequence.

3. Run the gel.

Removing gel

1. Remove electrodes and dismantle gel rig.

2. Remove gel on to blotting paper and dry.

3. Insert gel into X-ray cassette with film and expose overnight.

4. Develop film in developer, 2 hours-2 weeks later depending on signal.

Appendix 4a (i). Kintyre (West Argyll District) allele frequencies.

Locus: IDVGA37

	198	204	206	208	210	212	214	216	218	
Sika	0	0.009	0.026	0	0.019	0.067	0.002	0.002	0.876	466
Red	0.002	0.174	0.28	0.002	0.288	0.19	0.004	0.053	0.007	546

Locus: RM012

	115	123	125	127	129	131	135	137	139	141	147	163	
Sika	0.964	0.023	0.002	0	0	0.002	0	0.006	0.002	0	0	0	472
Red	0.026	0.236	0.041	0.031	0.057	0.317	0.028	0.12	0.103	0.011	0.028	0.002	542

Locus: UWCA047

	227	233	239	
Sika	0	0.01	0.99	498
Red	0.004	0.973	0.023	554

Locus: BM757

	163	165	167	175	177	183	187	189	191	193	201	203	205	207	
Sika	0.012	0.028	0	0.948	0	0	0	0	0	0	0.004	0.002	0.006	0	502
Red	0.046	0.467	0.004	0.024	0.007	0.064	0.117	0.046	0.007	0.002	0.011	0.082	0.089	0.035	548

Locus: INRA131

	93	95	99	101	103	105	107	
Sika	0	0	0.02	0.01	0.002	0	0.968	500
Red	0.033	0.002	0.485	0.369	0.081	0.024	0.006	544

Locus: IDVGA55

	191	193	195	197	199	201	205	207	213	215	221	
Sika	0	0	0	0.01	0.023	0.01	0	0	0.877	0.08	0	486
Red	0.004	0.086	0.145	0.335	0.297	0.101	0.005	0.005	0.018	0	0.004	546

Locus TGLA126

	105	106	109	
Sika	0.736	0.171	0.092	444
Red	0	0.004	0.996	546

Appendix 4a (ii). Kintyre (West Argyll District) allele frequencies.

Locus: TGLA337

	120	128	132	134	136	138	140	144	146	148	
Sika	0	0.905	0.002	0	0	0.016	0.071	0	0.004	0.002	494
Red	0.002	0.017	0.204	0.243	0.002	0.227	0.05	0.002	0.214	0.039	538

Locus: HUJ175

	115	121	123	129	131	133	139	
Sika	0.014	0	0	0.014	0.01	0.006	0.956	496
Red	0.399	0.076	0.021	0.156	0.175	0.148	0.025	474

Locus: IDVGA29

	139	147	149	157	159	193	
Sika	0.014	0.028	0.002	0	0.955	0	492
Red	0.676	0.311	0	0.002	0.009	0.002	546

Locus: TGLA040

	94	100	102	104	108	110	
Sika	0.002	0.024	0	0.015	0.957	0.002	466
Red	0.279	0.522	0.019	0.161	0.019	0	534

Locus: TGLA127

	157	165	169	171	173	175	177	179	181	185	187	189	
Sika	0.889	0.008	0.071	0	0.028	0	0	0.004	0	0	0	0	504
Red	0.015	0.506	0.063	0.007	0.156	0.107	0.002	0.035	0.026	0.052	0.03	0.002	540

Locus: FSHBA

	181	185	186	188	189	190	191	192	193	194	195	196	197	198	199	2(
Sika	0.958	0	0.004	0	0	0	0	0.002	0	0	0	0	0	0.002	0.004	0
Red	0.015	0.016	0.271	0.007	0.095	0.082	0.005	0.066	0.055	0.002	0.073	0.002	0.002	0.013	0.092	0.

Appendix 4a (iii). Kintyre (West Argyll District) allele frequencies.

Locus: AGLA293

	126	142	144	
Sika	0.002	0.036	0.962	500
Red	0.147	0.737	0.116	544

Appendix 4b(i). Allele frequencies for Cowal and Lorne FE Districts.

Locus: IDVGA37

	190	202	204	206	210	212	214	216	218	
COW	0.000	0.000	0.069	0.397	0.293	0.103	0.017	0.121	0.000	58
LOR-	0.022	0.005	0.092	0.364	0.386	0.043	0.005	0.076	0.005	184

Locus: RM012

123	125	127	129	131	135	137	139	141	147		
COW	0.232	0.049	0.000	0.098	0.390	0.024	0.085	0.110	0.012	0.000	82
LOR-	0.250	0.049	0.088	0.025	0.373	0.039	0.078	0.054	0.010	0.034	204

Locus: UWCA047

	227	233	239	
COW	0.000	0.988	0.013	80
LOR-	0.005	0.990	0.005	200

Locus: BM757

	163	165	175	177	179	183	187	189	191	193	201	203	205	
COW	0.049	0.427	0.012	0.012	0.000	0.122	0.098	0.000	0.000	0.000	0.183	0.098	0.000	82
LOR	0.067	0.438	0.000	0.000	0.005	0.082	0.144	0.041	0.010	0.005	0.108	0.082	0.015	194

Locus: INRA131

	93	95	99	101	103	105	
COW	0.107	0.000	0.625	0.179	0.089	0.000	56
LOR	0.017	0.017	0.575	0.230	0.103	0.057	174

Locus: FCB048

	143	145	
COW	0.924	0.076	66
LOR	0.939	0.061	196

Appendix 4b(ii). Allele frequencies for Cowal and Lorne FE Districts.

Locus: IDVGA55

	193	195	197	199	201	205	213	221	223	225	
COW	0.125	0.075	0.313	0.412	0.050	0.000	0.013	0.013	0.000	0.000	80
LOR	0.026	0.105	0.295	0.384	0.142	0.021	0.000	0.011	0.011	0.005	190

Locus: TGLA126

	109	
COW	1.000	64
LOR	1.000	168

Locus: TGLA337

	128	132	134	136	138	140	144	146	148	154	
COW	0.014	0.097	0.139	0.000	0.292	0.000	0.014	0.417	0.028	0.000	72
LOR	0.000	0.209	0.122	0.014	0.250	0.068	0.000	0.297	0.034	0.007	148

Locus: HUJ175

	115	121	123	129	131	133	139	241	
COW	0.218	0.167	0.000	0.141	0.038	0.064	0.026	0.346	78
LOR	0.255	0.054	0.005	0.127	0.147	0.103	0.000	0.309	204

Locus: IDVGA29

	139	147	
COW	0.563	0.438	64
LOR	0.505	0.495	186

Locus: TGLA040

	94	100	102	104	
COW	0.359	0.590	0.000	0.051	78
LOR	0.287	0.465	0.035	0.213	202

Locus: TGLA127

	157	163	165	169	171	173	175	179	181	183	185	187	189	
COW	0.013	0.013	0.575	0.025	0.000	0.175	0.038	0.100	0.038	0.000	0.013	0.000	0.013	80
LOR	0.000	0.010	0.446	0.025	0.005	0.299	0.039	0.054	0.049	0.010	0.039	0.025	0.000	204

Appendix 4b(iii). Allele frequencies for Cowal and Lorne FE Districts.

Locus: FSHBA

	181	182	183	186	188	189	190	191	192	193	194	195	196	198	199	2(
	201	202	203	204	205	206	207	208								
COW	0.014	0.000	0.000	0.457	0.000	0.129	0.014	0.057	0.143	0.000	0.043	0.014	0.000	0.000	0.014	0.[illegible]
	0.000	0.029	0.029	0.014	0.000	0.043	0.000	0.00	70							
LOR	0.005	0.010	0.005	0.339	0.010	0.063	0.073	0.073	0.036	0.036	0.005	0.010	0.016	0.010	0.042	0.[illegible]
	0.057	0.005	0.010	0.026	0.021	0.099	0.005	0.026	192							

Locus: AGLA293

	126	142	144	
COW	0.115	0.667	0.218	78
LOR-	0.207	0.683	0.111	208

Appendix 5. Skull measurement taken for morphometric analysis of Argyll samples after Ratcliffe *et al.*, (1992).

Variable	Skull measurement
A	Condylo-basal length. The posterior surfaces of the occipital condyles to the most anterior point of the premaxillae (prosthion).
B	Basilar length The median position of the ventral margin of the foramen magnum to the prosthion
C	Rostrum length. Orbital margin of the lachrymal to prosthion of the longer side.
D	Palatal length. Posterior junction of the palatines (staphylion) to prosthion.
E	Distance between the prosthion and the alveoli of the second premolars.
F	Premaxillary length. Posterior to anterior points of longer premailla.
G	Nasal length. Posterior to anterior points of longer nasal.
H	Maximum breadth of nasals.
I	Minimum width of maxillae.
J	Maximum breadth of frontals across orbital process.
K	Minimum inter-orbital width.
L	Maximum breadth of brain case.
M	Maximum condylar breadth.
N	Nose depth 1. Minimum depth between the nasal at the proximal end of the maxillae and inter-maxillary suture.
O	Nose depth 2. Between the inter-nasal / frontal suture and the inter-maxillary / palatine suture.
P	Palatal depth. Minimum depth between staphylion and the inter-frontal suture.
Q	Rear length 1. Inter-nasal / frontal suture and posterior of the skull.
R	Rear length 2. Inter-maxillary / palatine suture to median position of the ventral margin of the foramen magnum.