1. General introduction

1.1 The problem of pigmentation

Dark fibres in white wool become a highly visible fault in a light-coloured fabric. This simple statement of fact belies the complexity of issues that surround the various sources of dark fibre contamination, its detection and its consequences.

Pigmented fibres have not always been perceived as a problem. In medieval times, the fine Spanish Merino wool was famous throughout Europe and the Mediterranean for its superior quality – both brilliant white wool and lustrous black wool. In Australia as late as the 1960s, black wool was marketed alongside white wool at auction; pigmented sheep were generally not considered for breeding but were tolerated in the flock (Hayman and Cooper 1965). It was uncommon for light coloured woollen fabrics to be produced, but when they were the top was carefully selected for visually low prevalence of dark fibres. Where a light-coloured fabric was inadvertently produced with unacceptable dark fibre fault, it was possible to hand-pick the offending fibres, or to re-dye the fabric to a deeper shade.

Progressively since the 1950s, attitudes to dark fibres have hardened and practices throughout the wool industry have been modified. Competing fibres, generally with fewer contamination problems, have raised the bar for wool. Processors demand the flexibility to direct a processing batch to any final colour, pushing down the tolerance levels for pigmentation. On-farm procedures have focussed on minimising the risk of contamination from all sources, including stronger efforts to cull animals showing any fibre or skin pigmentation. Quality assurance programmes have directed best practice, while vendor declarations have encouraged wool growers to identify wool lots at elevated risk of contamination.

Dark fibres can originate on the sheep as naturally pigmented fibres or as urine stain, or from non-sheep sources, for example baling twine. Cross-contamination of white ewe fleece when mated to pigmented rams or when rearing pigmented lambs has been identified as a potentially damaging source of contamination (Hatcher 1995). The source of contamination will obviously affect the distribution of dark fibres through the sale lot.

Dark fibre contamination is difficult to detect in raw wool. In part this is due to the extremely low frequency of pigmented fibres which exceeds consumer tolerance – as few as one dark fibre per million can cause the rejection of tops (Fleet and Forrest 1984). Quite recently, machine detection of dark fibres has become feasible. However sampling procedures for routine wool testing are restricted; dark fibres distributed throughout a bale can be reliably detected, but clumps of contamination remain an insoluble problem – often missed by the sampling procedure but occasionally included in the sample and giving a very high reading. By contrast, the mixing

that occurs during early stage processing means that all dark fibres become distributed throughout the top, where machine measurement is reliable. (Prior to machine recognition of dark fibres, contamination might pass undetected until the fabric stage.) Unfortunately, because processing batches are typically made up of around 1000 bales, by the time dark fibres have been detected in top, it is too late to trace the property of origin responsible for the contamination.

Over the same period, products and processes have been developed to exploit the properties of wool (e.g. Sportwool^{TM}) and to increase consumer acceptability (e.g. machine washing, permanent press). Obviously, these market expansions are not compatible with any restrictions on the colour of the end product imposed by contamination.

Furthermore, economic pressures within the wool processing sector have increased the significance of contamination. The traditional wool processing pipeline extends for 20 – 24 months from raw wool auction until garments are sold off the retail shelf. While ownership of the wool may change several times during that period, at each stage the stock on hand must be financed by borrowings. The cost of finance has driven many industries to move towards a shortened processing pipeline and reduced inventory; all production is to order and consignments are delivered just-in-time. In order to meet specialised end-uses, wool orders are also becoming more highly specified.

In this environment, detection of contamination can have devastating results. If a processing batch is no longer suitable to fill an order then the owner of that batch faces multiple losses. First, it will often be impossible to process a replacement batch in time to meet the order, so there is loss of business, a potential claim for failure to fulfil a contract, and loss of reputation. Second, alternative markets for the contaminated batch will be limited, depending on its exact specifications — the higher the level of specification, the more valuable it will be, but the less likely that there will be alternative markets close to the original value. Third, machinery must be decontaminated to ensure that subsequent processing batches are not affected.

All of these aspects of wool contamination seriously undermine the notion of a value-adding chain. In reality processing companies lose substantial value through no fault of their own. Ultimately these losses must be recouped from elsewhere — affecting the price of raw wool paid to growers. At the other end of the chain, if a retail line of wool fashion garments destined for the boutiques of New York in spring is delayed or not delivered then other brands and other fibres will be substituted.

1.2 Inherited pigmentation

The phenotypic spectrum of mammalian pigmentation is hugely diverse, as attested by many children's picture books — through numerous colours, schemes and camouflage devices, and from intricately precise to apparently random. Unravelling of the genetics of pigmentation has

revealed that a great many genes are involved, often with complex interactions (Majerus and Mundy 2003). From an evolutionary perspective these phenotypic and genetic observations underline the importance of pigmentation as a component of fitness in a natural environment — a masking coat colour is critical to both parties in a predatory engagement, and very few species are neither predator nor prey; the elephant is a rare example (Searle 1968). In addition, coat colour functions in social cohesion and mate attraction may also relate to fitness.

According to Searle (1968), fibre pigment in mammals is the result of either eumelanin (brown-black) or phaeomelanin (in the range from pale cream to deep red). Melanosomes in certain follicle cells, called melanocytes, produce granular melanin and the cells grow elongated branches toward the centre of the follicle, facilitating the transfer of melanin to the growing fibre. Lack of pigmentation results from either the relative inactivity or the absence of melanocytes (Sponenberg 1997).

True randomness of pigmentation patterns may be apparently incongruous with deterministic Mendelian genetics. A classic example of randomness in pigmentation of tortoiseshell (calico) cats, outlined by Searle (1968) may assist. The gene in question is located on the X-chromosome. In a female heterozygous for orange and black alleles at this locus, random X-inactivation in skin cells results in some cells expressing one variant and some the other. X-inactivation in skin cells occurs quite early *in utero*, such that subsequent proliferation produces more-or-less discrete colour patches. Incidentally, a third colour may be due to an overlay of positional white patches under separate genetic control.

Two forms of inherited fleece pigmentation have been documented for Australian Merinos. Symmetrical pigmentation can take the form of self-colour (solid or baldy), badger face or reverse badger face patterns (Brooker and Dolling 1969a). These patterns are believed to be controlled by a single locus — thought to be *Agouti* (Parsons *et al.* 1999) — with multiple alleles, and transmission has been observed to conform with simple recessive inheritance. White is dominant to a hierarchy of the other alleles. These patterns can be overlaid by random white spotting. Work within the Australian Sheep Industry CRC has localised the gene to a small region on chromosome 14 and is working towards identification of the causal mutation/s.

An asymmetrical pigmentation pattern characterised by one to a few rounded fleece spots varying widely in size is known as piebald among Australian Merinos. Location of spots is apparently at random but with a degree of familial resemblance. Fibres in the patches can be black or brown, either uniformly pigmented or mixed with white fibres. Current knowledge holds that the *Australian Piebald* locus is responsible for this form of pigmentation (Sponenberg 1997). Brooker and Dolling (1969b) observed that inheritance did not conform to simple recessive, and the exact mode of inheritance remains uncertain. A fresh examination of this problem in the light of additional field data promises new insights to the possible mode of inheritance (Chapter 3). Modes of inheritance found to be plausible for piebald will then be

modelled in subsequent chapters. The location of the gene responsible for piebald is entirely unknown.

Major parts of the literature pertinent to pigmentation in mammals generally and in Merinos have been thoroughly covered elsewhere. Fleet and Langford (2005) provide a review of dark fibre contamination and related issues, along with an extensive bibliography. Meanwhile, a review of the physiological and developmental processes involved in pigmentation of wool fibres was being undertaken elsewhere within the Sheep CRC. Rather than duplicate these efforts, I have devoted my literature review to the gene discovery technique, homozygosity mapping. Other literature relevant to the experimental chapters will be addressed in the particular chapter.

Molecular genetics offers the opportunity to move beyond the restriction of observed phenotypes and deal directly with DNA. To harness this possibility involves two main steps. First, to discover the gene/s responsible and their chromosomal position, facilitating development of a diagnostic test. Second, to apply diagnostic testing to the wool industry by the most effective and efficient means.

1.3 Gene discovery

One may observe over the last twenty years in particular that molecular genetic techniques have steadily increased in power and reliability, at the same time as many of the costs have decreased. The reasons for these trends include refinements to techniques, economies driven by throughput, and the ever-expanding knowledge bank reflected in denser and more accurate genetic maps and understanding of gene function. While work in humans and laboratory species has led the way, the same trends are evident in many other mammals including livestock.

Genomic investigations which would once have been unimaginable can within a brief time become commonplace. For livestock species, techniques which would have been orders of magnitude beyond the scope of research budgets are becoming feasible. For example SNP chip technologies allow for genotyping at high speed and with great detail. Budget constraints must always be with us however, and it is not unreasonable that research funders seek to maximise returns. Design of genomic experiments will continue to be crucial to maximising experimental power, minimising risks and achieving efficiency.

For gene discovery, homozygosity mapping (Lander & Botstein 1987) offers the promise of a quantum leap in experimental power and efficiency, compared to conventional linkage mapping. This technique has been widely applied to Mendelian characters in humans with high levels of success — the majority with simple recessive inheritance, but a small number are controlled by other modes of inheritance. However the many methodological insights gained by the numerous practitioners have never been thoroughly examined, catalogued and brought

together into a single body of work. The literature review (Chapter 2) specifically aims to address this lack.

While gene discovery for Mendelian characters in humans has become routine, the state of knowledge of the genome in other species may not only prolong the process of gene discovery, but also change the dimensions of the problem. Thus techniques currently at the vanguard of human genetics may be technically difficult or financially prohibitive in other species. Conversely, a technique such as homozygosity mapping, which appears to be experiencing declining use in humans, may be worth reviving and/or reworking to suit livestock. In particular, the ability to construct specific matings could be used to maximum advantage. The design of gene discovery in livestock by homozygosity mapping is considered in Chapter 4, including mating design, marker spacing and experimental size, while assessing the impact of inheritance model, allele frequency, and population parameters.

The term "gene discovery" is used throughout this volume, adopted for its simplicity and because it is in common use. The literature related to Mendelian traits reveals that such discoveries are usually genes, or mutations within coding regions. It is recognised however that mutations in non-coding regions can also be determinative, and so the term "gene discovery" is intended to be inclusive of any mutation or polymorphism responsible for the character of interest.

1.4 Selection strategy

Few precedents exist for applying molecular techniques to eliminate undesirable genes from livestock production, and in addition the breeding structures vary widely between species and industries. The breeding structure of the Australian Merino is highly dispersed with a large number of seedstock establishments breeding rams for home use and/or sale. Artificial insemination is quite common in ram-breeding flocks but is rarely used in commercial matings.

Little exists in the way of modelling or analysis which would guide breeders to appropriate action on pigmentation genes.

Clearly, the elimination of inherited pigmentation would be seen as a desirable outcome for the wool industry. Conventional selection – phenotypic culling – has not led to eradication after several decades. Modelling of conventional selection strategies would shed light on this problem and may point to more effective alternatives (Chapter 5).

Consideration of optimum selection strategies incorporating DNA testing should involve the rate of reduction of allele frequency, the impact on inbreeding and selection intensity, along with the economic consequences at flock level, particularly for a ram-breeder (Chapter 5). At the industry level, benefits and costs of eliminating inherited pigmentation are relevant for setting policy and research priorities (Chapter 6).

1.5 Aims

This work examines aspects of inherited pigmentation in Australian Merino sheep.

Chapter 2 will review gene discovery by homozygosity mapping, its scope, strengths and weaknesses, consolidating the insights, shortcuts and pitfalls from the literature. Potential for exploitation of HM in livestock will be examined.

Chapter 3 will consider the inheritance of *Australian Piebald* and test potential models of inheritance which best explain all of the field data.

Chapter 4 will examine the consequences of mating design factors and assumptions on the efficiency of gene discovery for Mendelian characters in livestock. The applicability of and the most favourable conditions for homozygosity mapping will be clarified, and possible refinement assessed. Experimental power will be predicted.

In Chapter 5, various selection strategies for reducing or eliminating pigmentation alleles, including the potential role of DNA gene testing, will be assessed on cost and effectiveness. A scientific framework for guidance to breeders will facilitate choice of efficient and cost-effective selection strategies, reducing the impacts on the remainder of the breeding objective, on inbreeding and on financial viability.

Chapter 6 will clarify the economics of inherited pigmentation for the wool industry as a whole. These considerations may guide wool industry policy and protocol covering inherited pigmentation, as well as influencing research priorities.

This thesis will test the hypothesis that DNA technology can be a viable method of eliminating inherited pigmentation from the Australian Merino flock.

1.6 References

Brooker, M.G. and Dolling, C.H.S. (1969a). Pigmentation of sheep II. The inheritance of colour patterns in black Merinos. *Australian Journal of Agricultural Research* **20**: 387–394.

Brooker, M.G. and Dolling, C.H.S. (1969b). Pigmentation of sheep III. Piebald pattern in Merinos. *Australian Journal of Agricultural Research* **20**: 523–532.

Fleet, M.R. and Forrest, J.W. (1984). The occurrence of spots of pigmented skin and pigmented wool fibres in adult Merino sheep. *Wool Technology and Sheep Breeding* **32**: 83–90.

Fleet, M.R. and Langford, C. (2005). Contamination — dark and medullated fibres and vegetable matter. In: University of New England, WOOL 412: Sheep production (course notes). University of New England.

Hatcher, S. (1995). The potential of Awassi sheep and their Awassi x Merino progeny to cause fibre contamination. PhD Thesis, University of Western Australia.

Hayman, R.H. and Cooper, D.W. (1965). The frequency of pigmented sheep in the Australian Merino. *Wool Technology and Sheep Breeding* **12**: 81–85.

Lander, E.S. and Botstein, D. (1987). Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* **236**: 1567–1570.

Majerus and Mundy (2003). Mammalian melanism: natural selection in black and white. *Trends in Genetics* **19**: 585–588.

Parsons, Y.M., Fleet, M.R. and Cooper, D.W. (1999). The *Agouti* gene: a positional candidate for recessive self-colour pigmentation in Australian Merino sheep. *Australian Journal of Agricultural Research* **50**: 1099–1103.

Searle, A.G. (1968). Comparative genetics of coat colour in mammals. Academic Press, New York.

Sponenberg, D.P. (1997). Genetics of colour and hair texture. In: The Genetics of Sheep, Piper L.R. and Ruvinsky A. (Eds.), C.A.B. International, UK, pp. 51–86.

2. Literature review: homozygosity mapping in theory and practice

2.1 Introduction

Lander and Botstein (1987) devised the concept of homozygosity mapping of genes and established the cardinal points of the methodology for the discovery of genes responsible for recessive Mendelian characters. There can be little doubt that this was a significant insight and breakthrough. However in a curious footnote to history, they revealed that a referee of their manuscript pointed out that the eminent (later Professor) C.A.B. Smith had stated the principle in a much earlier era — long before it could be practically applied (Smith 1953).

Homozygosity Mapping (HM) makes use of the expectation that individuals affected by a rare recessive condition, who are known to be inbred, are likely to have inherited both copies of the rare allele from a common ancestor — to have identity-by-descent (IBD) at the causative locus. Moreover, adjacent chromosomal segments inherited along with the responsible allele result in a length of homozygosity flanking the locus of interest. The extent of homozygosity can be predicted according to the degree of inbreeding, and the expected length of homozygous fragments can be determined from the number of meioses between common ancestor and affected individual (Genin *et al.* 1998). In most practical cases the length of the homozygous segment is sufficient to make detection possible by screening with widely-spaced markers. Scanning for segments homozygous across several affected individuals can rapidly narrow the gene search. This makes HM a powerful tool for screening the entire genome, in cases where there is no prior information as to gene location. Once a critical region has been established, HM can give way to other techniques for fine mapping or mutation analysis.

Farrall (1993) was an early commentator on the bright prospects for homozygosity mapping. In concert with other developments, he saw HM contributing to great leaps in gene lab productivity, that would allow gene discovery to devolve into a "cottage industry" accessible by almost any life scientist and at any laboratory bench.

Technical respects of homozygosity mapping have been developed in a small number of methodological papers generally covering narrow aspects. Very few authors have catalogued significant deductive errors (Miano *et al.* 2000; Laurier *et al.* 2006) but many minor technical observations have been documented in individual studies, without ever being brought together in a logical or accessible form.

This review surveys the contribution of homozygosity mapping to mammalian gene discovery, especially for Mendelian characters, and documents its scope, strengths and weaknesses. The consolidated insights, shortcuts and pitfalls of homozygosity mapping will be gathered into a

compendium. In particular, the potential for further exploitation of homozygosity mapping in livestock species will be examined.

2.2 Theoretical development

The original description of HM by Lander and Botstein (1987) set out not only the principles but also a statistical framework. For a bracket of consecutive markers in an affected individual, the possible genotype outcomes in terms of homozygosity and heterozygosity can be listed — 16 combinations for a span of four markers, 64 combinations for six markers and so on. Taking account of the degree of polymorphism (the chance, h, of homozygosity at random) at each marker, the level of inbreeding and the map distance between markers, it is relatively straightforward (for a simple pedigree) to calculate two probabilities for any given bracket of consecutive markers: first the probability of each (multi-locus) genotype outcome given the disease locus lies in the centre of the region (P_1); and second the probability of each genotype outcome when the disease locus is unlinked (P_2). For each genotype outcome, the log of odds is calculated LOD = $\log(P_1/P_2)$.

In an individual with first-cousin parents, consider six consecutive markers at 10cM spacings. Homozygosity across all six markers with h = 0.5 gave P_1 = 0.215, P_2 = 0.033 and LOD = +0.81, naturally the most positive result possible. Complete heterozygosity gave P_1 = 0.0007, P_2 = 0.013 and LOD = -1.24 (Lander and Botstein 1987). Markers up to 50cM distant from the putative location could be included, after which contributions to P_1 and P_2 would be equal.

The expected LOD for each affected individual may be calculated by summing over all possible genotypes, as ELOD = $\sum P_1 \log(P_1/P_2)$ and this result can be used to determine the number of individuals required such that the expected aggregate LOD score will pass a certain threshold when the marker bracket encompasses the disease locus. Lander and Botstein (1987) based their calculations on h = 0.5, quite a conservative value for RFLP or microsatellite markers. They showed for the situation described above that the ELOD was 0.31 so that on average a positive LOD threshold of 3.0 would be exceeded by using ten such individuals. Conversely a negative LOD threshold of -2.0 could be exceeded by using six individuals, for statistical exclusion of linkage to that interval.

Lander and Botstein (1987) explored the link between marker spacing and the number of affected individuals required to surpass LOD = 3.0. They charted this relationship for several levels of h and inbreeding coefficients of F = 1/4, 1/16 and 1/64. In all cases the line curved upwards indicating that for each incremental increase in marker spacing, a progressively greater increase was required in the number of patients to compensate. More informative markers (lower h) allowed for both a wider marker spacing and fewer patients.

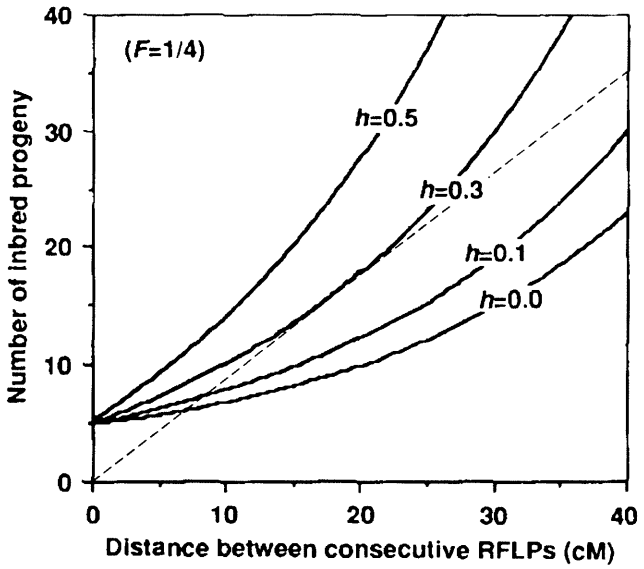


Figure 2.1 *The number of affected inbred progeny required to surpass LOD = 3.0 according to marker spacing and the chance of marker homozygosity at random, h, and for inbreeding coefficient F = 0.25 (Figure 2A of Lander and Botstein 1987). The dashed line has been added, positioned tangential to the curve for h = 0.3, showing the point of minimum genotyping effort at approximately 20cM marker spacing and 17 affected individuals.*

On each of these graphs (one of which is reproduced in Figure 2.1), points of equal genotyping effort may be connected by any straight line passing through the origin. For any level of h the point of minimal genotyping effort may be given by the line from this array which just touches the curve. Determining these points showed that with infinitely informative markers (h = 0.0) the most efficient marker spacing was around 25cM. At higher values of h the optimum spacing was reduced, especially at lower values of F. For h = 0.3 (a reasonable figure for livestock), Table 2.1 shows the approximate marker spacing and number of affected individuals to minimise genotyping effort, across inbreeding levels. The relative genotyping effort (number of affected individuals / 0.85 x marker spacing) suggests that maximum efficiency can be achieved at intermediate or low levels of inbreeding, provided that the disease is sufficiently rare (see below), but these figures are only approximate.

Table 2.1 *Approximate optimum number of affected individuals and marker spacing to minimise genotyping effort for homozygosity mapping at three levels of inbreeding. Values read from Figure 2 of Lander and Botstein (1987). The relative genotyping effort compares the other rows to F = 1/4. Marker homozygosity h = 0.3.*

Inbreeding coefficient *F*	Number of affected individuals	Marker spacing cM	Relative genotyping effort
1/4	17	20	1.00
1/16	8	14	0.67
1/64	7	11	0.75

Lander and Botstein (1987) went on to point out the problem of population frequency of the disease allele: for lower levels of inbreeding, the expectation of homozygosity through identity by descent (rather than identity by state) is only sustainable if the disease is progressively more rare in the population: allele frequency $q < 0.07$ for $F = 1/4$ (e.g. full sib mating), $q < 0.02$ for F

= 1/16 (e.g. first cousin mating), and $q < 0.008$ for $F = 1/64$ (e.g. second cousin mating). Finally they considered disease heterogeneity and concluded, in a simplified case at least, that despite a loss of power the technique was still capable of locating genes.

Linkage likelihood calculation

Homozygosity in association with a disease phenotype is quite easy to detect in marker genotype data, even visually or by a simple statistic such as X^2 — as have been used for preliminary identification of potential regions of linkage. However proper establishment of linkage for publication requires a formal LOD score. Although HM often uses relatively simple pedigrees, multiple inbreeding loops and many missing genotypes greatly increase the computational demands — a problem which was recognised quite early by Farrall (1993). The limited computer power available to the first users of HM meant that pedigree truncation was the only practical option. Computer power has of course increased dramatically over time, along with possibilities for parallel processing; however these gains on their own could not keep pace with the increasing demands of linkage problems — for example through extended and more complex pedigrees and greater marker density.

Several statisticians developed separate improvements to the calculation of linkage likelihood to address this problem. Kong (1991) devised a new statistical representation for efficient calculation of approximate likelihoods. Thompson (1994) developed an approach employing Monte Carlo approximate likelihoods with specific reference to HM problems. Kruglyak *et al.* (1995) developed their own rapid multipoint linkage analysis, using hidden Markov models. This method is particularly suitable to pedigrees with inbreeding loops and missing data — as are commonly found in HM situations. By commercialising their algorithm in the MAPMAKER/HOMOZ software package they ensured wide availability.

Meanwhile, adaptations to the LINKAGE software package (Lathrop and Lalouel 1984; Lathrop *et al.* 1984, 1986) through algorithmic modification resulted in the FASTLINK software (Cottingham *et al.* 1993; Schaffer *et al.* 1994). This also has been made widely available in a user-friendly form and has enjoyed much use.

Agarwala *et al.* (2000) approached the problem of excessive pedigree complexity from a different perspective. They reasoned that DNA sampling generally only extends to affected individuals and their parents. Thus the individual identities of ancestors are not important, only their combined contribution to the genotypes of those sampled. It would be expedient therefore to re-write the pedigree in the simplest possible configuration that gave equal coefficients of inbreeding in the final generation. While this approach appears to have potential, the paper shows zero citations on ISI Web of Science to October 2007; perhaps its publication in the mathematics literature left it off the radar of geneticists, or its complexity could have been an impediment to implementation.

IBD probabilities

A number of theoretical papers have dealt with calculation of identity-by-descent (IBD) probabilities. Guo (1997) described the calculation of prior probabilities of IBD in complex pedigrees, and Schaffer (1999) developed further aspects. Genin *et al.* (1998) calculated the average length of IBD segments according to pedigree structure, along with the probability of an affected individual showing homozygosity at a marker located any given distance from the disease locus. They aimed to provide guidance on an appropriate marker spacing and also on the setting of criteria for homozygosity screening. Clark (1999) determined the length distribution of homozygous segments in inbred individuals.

Inbreeding

Miano *et al.* (2000) reported a false positive result which was attributed in part to an underestimate of inbreeding in the affected individuals of their study; although the shortest inbreeding loop was soundly established, the possibility of more remote pedigree loops was initially ignored. They proposed a scheme of regarding founder individuals as first cousins, which in their case doubled the inbreeding coefficient of the latest generation. This seems arbitrary and may over- or under-compensate because the true level of inbreeding is unknown and cannot be generalised. Libiger and Schork (2005) assessed the inbreeding coefficient of early members of their pedigree by counting the proportion of random markers typed as homozygous. They then added an extra generation to their pedigree to create full-sib relationships between all of the founders, a strategy they admit was likely to be conservative with respect to LOD score estimation. Clearly, it also hugely increased the pedigree complexity.

Liu *et al.* (2006) attempted to quantify the risks of underestimated inbreeding. From studies of a well-documented founder population in The Netherlands, they showed that ancestral inbreeding accounted for 18–75% of the total inbreeding coefficient; in other words, using only the most recent 3–4 generations of pedigree risked massive underestimation of the total inbreeding over 10–15 generations in the full pedigree. Although the more distant loops contributed tiny amounts to the coefficient of inbreeding, these loops were so numerous (up to 677) that on average they accounted for 58% of total inbreeding. The implications of this for HM, they found, were greatest when the population disease allele frequency was very low, and at the extremes could inflate the incidence of type I error by orders of magnitude. Their proposed solution recognises the potential difficulties of tracing and analysing complex ancestral pedigrees. They suggest that the pedigree be reconfigured to the simplest alternative which delivers equal or slightly greater inbreeding levels. While similar to the approach of Agarwala *et al.* (2000) above, this solution is even simpler because a conservative rounding of inbreeding coefficient replaces the former's exactitude. Liu *et al.* (2006) acknowledged however that dramatic pedigree simplification will lead to underestimation of recombination

probabilities, in turn reducing the power for linkage detection. They went on to suggest that the separate calculation of recombination probabilities may provide a feasible work-around.

Leutenegger *et al.* (2003) reported a method for estimation of inbreeding from genomic data using a hidden Markov model. Leutenegger *et al.* (2006) developed this idea further and demonstrated its exciting possibilities: homozygosity mapping using affected individuals without any knowledge of pedigree. This has fundamental implications for the use of HM in domestic, and indeed in wild, species.

As originally proposed, HM was envisaged to use Restriction Fragment Length Polymorphism (RFLP) markers, but these were rapidly overtaken by the convenience of microsatellite markers. The advent of genotyping by Single Nucleotide Polymorphism (SNP) chip has massively increased the rate at which genotype data can be produced, and some recent papers announced software packages that provide a range of tools for collating and visualising data (Woods *et al.* 2004; Forshew and Johnson 2004; Carr *et al.* 2006).

Gibbs and Singleton (2006), in a review of recent developments in genome-wide SNP typing, explored the possibilities arising for the field of homozygosity mapping. A 100 000 SNP chip clearly has the power to detect much shorter homozygous regions, so that the accepted fundamentals of HM, such as pedigree structure, are now open to re-examination.

Relationship to other techniques: QTL mapping

Homozygosity mapping for the discovery of quantitative trait loci (QTL) was developed and applied to serum insulin levels (Abney *et al.* 2002) and serum triglyceride levels (Newman *et al.* 2003). Ewald *et al.* (2005) applied conventional homozygosity mapping to search for genetic risk factors to bipolar disorder, allowing for a phenocopy rate and incomplete penetrance. These authors commented on the advantageous power of HM to detect QTLs, although this only applied to consanguineous pedigrees and only to QTLs of recessive inheritance.

In the broad sense, HM may be thought of as a special case open to generalisation in a number of dimensions. First, as described, the extension from binary to quantitative traits. Additional considerations are required to allow for uncertainty in allocating phenotypes and genotypes – for example random effects in the model. Second, the explicit requirement for inbred individuals can be relaxed, in parallel to a shift of emphasis from homozygosity *versus* heterozygosity to any contrast in allele frequency distribution between phenotypic groups. Third, the relationship between genotype and phenotype could be relaxed, for example to polygenic risk factors underlying a threshold model of expression.

Many other papers used homozygosity mapping or similar techniques as part of a broader strategy for discovery of QTLs. To analyse and describe fully all of these approaches to QTL detection would be a considerable work in itself and was therefore considered to be beyond the scope of this review.

2.3 Terminology

The term "autozygosity" has been applied as short-hand for "homozygosity-by-descent" so some researchers have referred to "homozygosity mapping" as "autozygosity mapping." However the majority of practitioners have retained the name given by those who first described the concept in detail, Lander and Botstein (1987).

Puffenberger *et al.* (1994) reported the results of a study including "identity-by-descent" mapping of a Mendelian condition, in conjunction with linkage disequilibrium mapping. Their approach used the major tenets of homozygosity mapping but was apparently conceptualised independently of Lander and Botstein (1987). A few further instances of the term "IBD mapping" appeared in the literature related to Mendelian characters. However this description has more typically been associated with the discovery of genes affecting quantitative traits; this might be due to an extension of the terminology associated with linkage disequilibrium. Discovery of QTL is outside the scope of this review.

Throughout this review "homozygosity mapping" will be used to describe the equivalent autozygosity mapping and identity-by-descent mapping strategies related to Mendelian characters.

The nuance of some authors reveals their understanding of homozygosity mapping as simply a form of linkage mapping. For others it would appear that HM is regarded as a special case of linkage mapping, while others again seem to make a distinction between conventional linkage mapping and HM. These distinctions can be semantic; the standard linkage software is capable of handling data from HM studies and generating appropriate LOD scores, so it makes little difference whether locus probability is tested by homozygosity criteria or by LOD-scores. However those workers who saw HM as a distinctly different technique were more likely to take advantage of the particularly favourable aspects such as DNA pooling.

There also exists an area of overlap between HM and linkage disequilibrium mapping, as exemplified by Houwen *et al.* (1994). Where patients are related, chromosome segments around a disease locus can match across individuals. This creates non-random associations between a marker genotype and disease phenotype — linkage disequilibrium (LD). For recessive conditions and where disease alleles have been inherited from a common ancestor, this will clearly also involve homozygosity. No attempt is made here to create exclusive definitions of HM and LD mapping and in practice the techniques are often used together.

2.4 Homozygosity mapping in the literature — statistics

Searches were conducted in ISI Web of Science and SpringerLink to detect the terms "homozygosity mapping" and "autozygosity mapping." These searches returned a total of 213 references where the search term was included in the paper title or keywords, or a substantially

similar description was included in the title. Of these, at least 197 were obtained for study, and for most of the remainder the abstract was considered.

The search also revealed a further 202 papers which included the search term in the abstract. These were examined for their relevance to this review in terms of being the first report of a whole-genome search in which homozygosity mapping was declared as the primary methodology. This resulted in around 45% of these papers being excluded from further study.

Another large cohort of papers included the search term elsewhere in the full text. I considered that only a small proportion of these papers were likely to be relevant as described above, and because considerable time would be required to determine relevance, these papers were excluded *en masse*, except when citation elsewhere indicated points of interest.

A total of 188 papers reported on gene discovery by whole genome scan and primarily HM methods. Twenty-eight papers reported DNA pooling for an initial scan, and all but three of these papers reported on a whole genome scan.

This statistical summary indicates the extensive employment of HM in gene discovery, over a period approaching twenty years. The simple quantity of literature would present significant issues for a practitioner to keep abreast of all of the intricate observations, insights and considerations as they are reported. For a scientist new to HM, like myself, consolidating the mass of literature has been a significant work — many others may not have the time to repeat this process and might therefore miss out on some useful detail, or risk entrapment in one of the documented pitfalls.

The large majority of reports were of work in humans. Some instances were for other species which provided models of human diseases. A small number of papers related to domestic or livestock species on a condition of primary interest in that species. The text of this review will be expressed in terms relevant to human studies unless the point being made pertains specifically to another species.

Reports of gene discovery were characterised by long author lists and multiple institutional affiliations. For all the efficiency of homozygosity mapping, gene discovery remains a multi-disciplinary enterprise spanning at minimum fields of medical diagnosis, medical research, DNA manipulation and bioinformatics. Sheffield *et al.* (1995) commented specifically on the importance and magnitude of the work preceding laboratory DNA analysis.

2.5 Purposes for homozygosity mapping

The 361 papers which received at least an initial examination revealed a breadth of uses and purposes for the application of homozygosity mapping, which I have grouped into seven categories. Of course, any one paper may well fall into multiple categories.

1. **Gene discovery.** Locating chromosomal units responsible for a genetic condition is perhaps the most powerful application of HM. In the literature this search may have targeted candidate regions based on documented gene function or an established role in related conditions. In many cases HM has taken the form of a whole genome scan, either because the strong candidate genes have already been excluded from consideration, or because there were no candidate genes making a sufficiently strong claim, or because there were too many possible candidate genes. This purpose of HM is the primary focus of this review.

2. **Locus verification.** Where an earlier study had provided evidence for the involvement of a locus in a disease in one pedigree or in pedigrees from one geographic locale, confirmation in other pedigree/s or in geographically remote pedigree/s was considered valuable in definitively establishing the locus.

3. **Locus refinement.** Homozygosity mapping was used to refine a previously claimed locus by genotyping of additional pedigree/s. Particularly if the earlier study had relied upon conventional linkage, HM allowed for outer limits of the locus-containing region to be conclusively defined by informative recombination events.

4. **Disease homogeneity or heterogeneity.** Many diseases are defined as syndromes of a collection of symptoms which may vary widely in presentation, severity, age of onset, rate of progression, etc. Once a locus has been established, genotyping of diverse patients proceeds to establish by HM whether the one locus is responsible in all cases. If the locus has been excluded from pathogenicity in some cases, these patients may be suitable material for discovery of a second locus and so on (for example Bouchlaka *et al.* 2003). Alternatively, re-examination of the patients may reveal symptomatic differences between those cases linked to the locus and those unlinked, allowing for the disease definition to be subdivided into types (for example Saar *et al.* 1999). If a common locus is responsible for different disease presentations (or even conditions regarded as different diseases) then discovery of the pathogenic mutations may explain this variety.

5. **Cancer.** Comparing the genomes of healthy and tumour tissue from the same individual can reveal the mutations responsible for the growth characteristics of the cancer. Often these mutations are large deletions, so that a marker which was heterozygous in the healthy tissue presents as homozygous in the tumour. Termed "homozygosity mapping-of-deletion" this is a specialised field outside the range of this review.

6. **Diagnostic.**
    a. **Interim.** When a locus has been reported in a chromosomal region but prior to mutation/s being sequenced, homozygosity mapping can be used as a diagnostic tool in suitable pedigrees. This has implications for genetic counselling, but also

for understanding the spectrum and possible treatment, particularly of a highly variable disease. For these reasons, some essentially diagnostic work is published in the scientific literature, although often in the form of a brief report. See for example Mizrachi-Koren *et al.* (2006) and Peretz *et al.* (2007).

b. **Routine.** Strauss *et al.* (2005) reported on a unique use of HM for diagnosis. As paediatric specialists, they faced a large number of patients referred after diagnosis of developmental delay. Many syndromes have been described, each including a long list of essential and variable symptoms. Even if a systematic approach were adopted, the process of conventional diagnosis would regularly continue over several years and include considerable expense for various forms of testing; critically, this course was often inconclusive. They proposed for patients known to be inbred, and particularly for multiple related patients, that HM offered a cheaper and much faster alternative. Genotyping by 10k SNP chip can rapidly expose target region/s of homozygosity for which a database search could identify mapped disease loci, or examine gene function. At a cost of $US2000 per patient and taking around one month to complete, HM has the potential to revolutionise early diagnosis and treatment of these patients.

7. **Other.** Some papers included the term "homozygosity mapping" when they referred to some other body of work, or in announcing that they were commencing a homozygosity mapping study, or as part of a call for patients, or by flagging a certain condition and/or pedigree as suitable candidates for HM.

2.6 Inheritance models

In almost all cases the disease in question was described as following autosomal recessive inheritance. In a few cases the inheritance was uncertain or was not stated, and sometimes the condition under study was known to have dominant and recessive forms which may have both been present among the patients. Sironen *et al.* (2002) studied a sperm defect with autosomal recessive inheritance but sex-limited expression, and identified a highly significant locus.

In two cases the HM study highlighted two regions of homozygosity, leading workers to suspect digenic inheritance (Chen *et al.* 1997; Roscioli *et al.* 2003). In both cases relatively small numbers of individuals were genotyped and it would appear far more likely that a second region of homozygosity occurred due to chance. Ewald *et al.* (2003) searched for risk loci for the psychological affective disorder and located four suggestive regions, pointing to the possibility of oligogenic inheritance. Cavanagh *et al.* (2000) reported on the incomplete dominant condition bovine chondrodyplasia which is lethal in homozygotes but carriers can show a very mild disease phenotype — clearly very similar in presentation to autosomal recessive.

Sheffield *et al.* (1995) regarded HM as suitable for mapping conditions with dominant inheritance and also complex inheritance. To date however, no reports could be found of HM applied to dominant characters. The mapping of genes associated with quantitative traits receives a brief comment above.

The disease was almost always described as rare but this situation ranged from there being less than 50 cases documented in the world, through to certain types of deafness which occur as frequently as one in one thousand in some large populations. Often the condition was rare in the global sense but distinctly not rare in the genetically isolated population under study.

2.7 Advantages of homozygosity mapping

1. **Power.** Conventional linkage mapping requires upwards of ten nuclear family pedigrees each including at least two affected individuals (and preferably three or more) with all individuals genotyped (Farrall 1993). For very rare conditions, the requirements may exceed the known global population of affected individuals. Happily, very rare recessive conditions also tend to occur in consanguineous families which are excellent material for homozygosity mapping. Single-patient families are not only informative for HM, they are highly informative — contributing a potential LOD score of 1.2 if the parents were first cousins (Farrall 1993). Thus small experiments are capable of exceeding the typical genome-wide LOD score threshold of 3.0 or 3.3. Similarly, modestly-sized experiments are capable of exceeding the customary LOD score threshold of -2.0 for exclusion of linkage (Lander and Botstein 1987).

2. **Insensitivity to pedigree structure.** Various pedigree structures are suitable. Lander and Botstein (1987) recommended the use of consanguineous families where the parents were no more distantly related than first cousins. However researchers have successfully used more distant parental relationships — this may be because the ancestral (unobserved) pedigree also contributed to the level of inbreeding, or because markers were more informative and/or closer together. Importantly, it is not necessary to have more than one affected individual in each family. This flexibility could be particularly important in non-human species where full-sib families may be rare. Data can easily be combined from different family structures, whether related or unrelated. The inability to sample unaffected members of the pedigree is only a minor issue.

3. **Efficiency.** The genotyping effort required for HM, and therefore the financial resources required, can be a fraction of that needed for conventional linkage mapping, because:
   a. Fewer individuals are used.
   b. Knowledge of marker allele frequencies in the wider population is not particularly important and can often be ignored altogether or at least until the confirmation of a tentative locus. Some software packages automatically required estimates of

marker allele frequencies but Shalata *et al.* (1998) observed that LOD scores were insensitive to these values.

c. HM is generally amenable to DNA pooling prior to genotyping. This can make further dramatic cuts in the genotyping requirements.

4. **Defining the critical region.** Where a locus is located by conventional linkage, a LOD score peak is observed at the most likely position and often the curve declines over a chromosome segment, asymptotically approaching the background LOD score value. HM assists in the identification of recombination events through the discontinuity of homozygosity, without full haplotype analysis. Individual recombination events are easy to detect and recombination in affected or unaffected individuals can definitively limit the range of the locus (subject to some conditions as explained below under pitfalls).

2.8 Disadvantages of homozygosity mapping

1. **Location of suitable pedigrees.** Close inbreeding is not common in most western countries, so the patients for most HM studies have been ascertained from other parts of the world. Accuracy of pedigree information is also a clear advantage.

2. **Rarity of condition.** The more common the condition, the greater the possibility that affected individuals are not IBD for the causative locus (Lander and Botstein 1987). In addition, if a relatively common condition is genetically heterogeneous within a pedigree then HM attempts may fail.

3. **Knowledge of inheritance.** The power of HM is greatest for autosomal recessive conditions and may be much less for other inheritance patterns (Sheffield *et al.* 1995). If the mode of inheritance is unknown then it may be prudent to determine this first.

4. **Existence of a reliable map.** As the number of individuals used for HM is generally insufficient to infer marker position by linkage, it is preferable to use markers for which map position is already well known. The map of the human genome reached this point perhaps 15 years ago but this has been much more recent for many other species.

2.9 Suitable pedigrees

The original concept of homozygosity mapping (Lander and Botstein 1987) called for the use of patients of known consanguinity or inbreeding. This type of family is the most commonly used for HM in humans. In most western cultures marriages between known relatives are uncommon. This means that while a large part of human molecular genetics research takes place in Europe and North America, domestic resources for HM are rare in these regions. For this reason many of the families used in HM are located in defined parts of the globe, where either small communities are relatively isolated (eg St Helena in the South Atlantic or the

Amish populations of USA) or where cultural practices favour consanguineous marriages (eg Japan, Tunisia, many parts of the Middle East).

There is a risk that a family with a recent inbreeding loop recruited from such a population would also have additional accumulated inbreeding relating to more distant ancestors. This will lead to an overestimation of LOD scores derived from HM (Miano *et al.* 2000). On the other hand, exhaustive tracing of pedigrees to uncover all possible relationships results in pedigrees that are beyond the computational capabilities of linkage software — and the pedigrees must be trimmed for analysis (Ghiasvand *et al.* 2000).

Some families used for HM were sourced from genetically isolated populations but without identified inbreeding loops (see for example Winick *et al.* 1999). In these cases the assumption was made of common ancestors at some time beyond the extent of traced pedigrees. Friedman *et al.* (1995), in sourcing patients from an isolated village of Bali, specifically targeted the children of parents who were not known to be related, to exclude excessively inbred individuals. They also preferred patients who had both parents unaffected, in the hope that informative recombinations would be detectable.

All of these considerations assume of course, that human matings are always consistent with the village marriage register; however any extra-marital matings occurring in the pedigree prior to the genotyped individuals would be very difficult to establish.

In some studies, outbred families were included alongside consanguineous families for HM (for example Kondo *et al.* 2004).

Where a disease was known or suspected to be genetically heterozygous, it was clearly an advantage to use a number of nuclear families from within an extended kindred or related kindreds — on the basis of their likelihood to be segregating for disease under the same genetic influence. Stockton *et al.* (1998) used this strategy but mindful of the heterogeneity also ensured that each pedigree had the statistical potential to surpass the LOD score threshold. Alternatively, if there was no reason to suspect genetic heterogeneity then use of unrelated or geographically diverse families would maximise experimental power.

In searching for homozygosity among affected individuals, reference to unaffected individuals was important to reveal, for example, whether homozygosity was due to a marker that was uninformative in the family. In particular studies, the selected unaffected individuals were closely related, distantly related or even unrelated within a small population.

2.10 Marker density

Marker densities for a genome-wide scan varied widely. The smallest numbers of microsatellite markers used to cover the human genome were 52 (LeGuern *et al.* 1996), 63 (Tsujikawa *et al.* 1998), 68 (Friedman *et al.* 1995) and 96 (Kant *et al.* 1998). All these searches were successful.

At the other end of the scale, Goldberg *et al.* (2007) used 763 conventional autosomal markers, McLean *et al.* (2003) used 800 markers, and a set of 811 markers was used by each of Kirby *et al.* (2004), Berkovic *et al.* (2005) and Malmer *et al.* (2005), the last without success. Kirby *et al.* (2004) cited the highly heterogeneous nature of the disease to explain their high marker density, but this justification was unusual among the reports. Goldberg *et al.* (2007) also used 48 markers on the X-chromosome, but similarly gave no explanation — there was no indication of sex-linkage or sex-limitation.

Single Nucleotide Polymorphism (SNP) markers largely superseded conventional markers in later studies, and most papers reported the use of a commercially available SNP chip typing around 10 000 SNPs. Even allowing for biallelic SNPs, the information density from a 10k SNP chip clearly exceeded even the densest set of conventional markers. Melin *et al.* (2007) failed to find note-worthy homozygosity following genotyping on a 10k SNP chip and proceeded to a 100k SNP chip, with success. Crisponi *et al.* (2007) used a 250k SNP chip, while Morrow *et al.* (2007) reported using a 500k SNP chip.

Aside from the notable exceptions mentioned above, the bulk of reports using conventional markers used 200 to 400 markers to scan the human genome. This resulted in an average marker spacing ranging from almost 20cM down to less than 10cM, broadly in line with the original recommendation of Lander and Botstein (1987) that a 10cM spacing of modestly polymorphic markers would be sufficient to detect linkage.

The average length of IBD segments which can be expected are determined by the level of consanguinity in the affected individuals, or more particularly the number of recombination events (n) between the affected individual and their common ancestor (Genin *et al.* 1998). Genin *et al.* (1998) provide a formula for determination of expected IBD fragment length around the causative locus, based on the number of meioses and the length of the chromosome. However their work includes some approximations and in some respects rest upon unstated assumptions particular to typical human pedigrees. In essence they calculate expected IBD fragment length equal to $200/n$ cM but with some reduction due to the chance of the locus being situated close to a telomere. Table 2.2 shows the expected IBD fragment lengths calculated for some typical parental relationships.

Table 2.2 *Expected IBD fragment length around the causative locus in the affected progeny of certain parental relationships. (An expansion of Table 1 from Genin et al. 1998). Assumed chromosome length is 213cM.*

Parental Relationship	Inbreeding coefficient (F)	Number of meioses (n)	Expected IBD fragment length cM
Full sibs	1/4	4	38
Uncle-neice	1/8	5	32
First-cousins	1/16	6	28
Second-cousins	1/64	8	22
Half sibs	1/8	4	38
Sire-daughter	1/4	3	46

Knowledge of expected IBD fragment length should guide the choice of marker density, along with the number of individuals to be genotyped. However authors rarely made any comment justifying their selection of marker density. It would appear that choice of marker density was more closely related to financial constraints than practical requirements.

2.11 Genotyping strategy

In most cases it appeared that, having decided upon a marker set, the genotyping was completed before any search for homozygosity was undertaken.

In a few cases genotyping appeared to have proceeded chromosome by chromosome, with homozygosity searches undertaken at regular intervals; any significant level of homozygosity was investigated while the remainder of the genome scan was suspended temporarily or permanently. Tammen *et al.* (1999) utilized an approach whereby they first genotyped in the vicinity of candidate genes or regions, in order of likelihood. They then proceeded to a whole genome scan in random order so that each successive batch of markers was selected to break the longest remaining intervals, and each marker was assessed for homozygosity as it was typed, with any significant homozygosity followed up straight away.

I have characterised these sequential approaches as "first cab off the rank" strategies. If the first sign of homozygosity looks convincing and is verified by additional closely linked markers, then large parts of the genome may remain totally untested for homozygosity. Clearly this saves on time and resources. However the possibility of homozygosity occurring independently of association with the disease locus should be borne in mind. This will be discussed further with other pitfalls in Section 2.16.

In cases when the initial genome scan did not reveal the homozygous region, additional markers were typed to replace uninformative markers and to cover the largest remaining intervals. In some of these cases homozygosity was eventually detected close to a telomere.

Regions highlighted by an initial whole genome scan were typically investigated by typing additional closely linked markers, to confirm that the region did indeed show homozygosity through identity by descent in the affected individuals and heterozygosity in unaffected individuals. If DNA pooling had been used for the initial scan then individual genotyping would now supplant that. Often a subset of affected individuals was used for the initial scan so the remainder of the available DNA specimens would be typed for the markers of interest. If additional families were available then these would be tested for further confirmation.

If no additional affected individuals were available (i.e. only those used in the initial genome-wide scan) then a potential pitfall arose in that authors might think that, by confirming the presence of homozygosity in affected individuals and absence of homozygosity in unaffected individuals in a short region with dense marker coverage, they were actually confirming linkage to the disease locus. This will be further discussed below.

2.12 DNA pooling

The fundamental precept of HM is detecting homozygous markers — markers that show only one allele — in common across affected individuals. In principle therefore, a pooled sample of DNA from two or more affected individuals which shows the presence of a single allele can establish homozygosity across all members of the pool. Genotyping pooled DNA samples has the potential to reduce significantly the genotyping costs.

DNA pooling along phenotypic lines appears to have been proposed independently by three diverse groups (Arnheim *et al.* 1985 in humans; Taylor and Rowe 1989 in a mouse study; Michelmore *et al.* 1991 in plant breeding), all of whom were also unaware of the homozygosity mapping concept of Lander and Botstein (1987). Arnheim *et al.* (1985) showed that changes in marker allele frequency distribution should occur when a marker is linked to a disease locus; they predicted the marker allele frequency changes under recessive, dominant and additive inheritance, while noting that genes for dominant or additive inheritance would be difficult to detect at intermediate-range disease allele frequencies. Darvasi and Soller (1994) drew on the work of Michelmore *et al.* (1991) and Lander and Botstein (1987) in suggesting refinements in pooling strategies for QTL detection.

DNA pooling in concert with homozygosity mapping was first demonstrated by Sheffield *et al.* (1994). The literature search revealed reports of 28 HM investigations published between 1994 and 2003 which utilised DNA pooling. Of these, 24 reports were published from 1997 to 2002, and only a single report after 2002. Only one of these reports of the use of DNA pooling failed to locate the causative locus to the authors' satisfaction. In addition Wang *et al.* (2001) retrospectively used individual genotype data to simulate DNA pooling, with a positive result. It may be concluded that the use of DNA pooling has been rather restricted, and the limited favour which it gained has now waned. The decline in use after 2000 is probably due to the increasing use of SNP markers; as SNP markers are generally dimorphic, each SNP from a pooled sample would give little information. However regions of common homozygosity could still be detected. Despite this, it appears that researchers prefer to proceed directly to individual genotyping with all its possibilities for haplotype determination, etc.

The literature lacks insight on the design of DNA pools, so several different approaches were followed. In all cases, the quantity of DNA contributed to the pool by each individual was carefully standardised. In some instances the genotyping seems to have been entirely qualitative. Thus the experimenters sought either the presence of a single marker allele in the affected pool/s or a significant reduction in the number of alleles present between unaffected pool/s and affected pool/s. In other cases quantitative assessment was made by gel band silver staining (e.g. Jamieson *et al.* 1999) or fluorescent tagging (e.g. Winick *et al.* 1999). Carmi *et al.* (1995) described visual comparison of gel band intensities, and favoured rapid visual

assessment over actual scoring of gels. Otherwise it was generally not clear whether the quantitative assessment was performed by eye or machine.

In its simplest form, DNA pooling involved two combined samples, being all of the affected individuals and all the unaffected individuals. The largest such pools consisted of 16 affected and 20 unaffected individuals from a highly complex pedigree (Ghiasvand *et al.* 2000). The smallest such paired pools were four affected and two unaffected (Jamieson *et al.* 1999) and three affected and five unaffected (Chen *et al.* 1997). El-Shanti *et al.* (2000) used paired affected and unaffected pools formed separately within each of two families. At the other extreme, Wang *et al.* (1997) formed a pool of 31 affected individuals spread across 19 families. Coucke *et al.* (2003) formed only one pool, of six affected members of one family (i.e. no unaffected pool was used).

A number of papers reported multiple pools of unaffected individuals, segregated along various lines. For example, Sheffield *et al.* (1994) formed a pool of the unaffected siblings of patients, and a second pool of unaffected parents of patients; they ultimately preferred the sibling pool as a control because it was expected to carry the disease haplotype at a lower frequency (0.33) compared to parents (0.50) — this distinction is relevant to a qualitative assessment of allele frequency distribution. El-Shanti *et al.* (1998) formed a pool of unaffected obligate disease carriers and a separate pool of other unaffected individuals. Others formed an unaffected pool from more distant relatives or even from unrelated controls. Notwithstanding consideration of missing individuals, a pool of unaffected siblings can at most contain all of the alleles present in the pool of unaffected parents, but the inverse is not true; for a qualitative assessment a parental pool would appear superior. Given the relatively meagre information yield available from unaffected individuals (Lander and Botstein 1987) the use of more than one unaffected pool would appear to be beyond necessity. Certainly genotyping one or two additional samples was not excessively taxing, and no authors expressed regret at their decision to use multiple unaffected pools. On the other hand, no authors indicated that multiple unaffected pools genuinely enhanced the detection of homozygosity in their initial genome scan.

Tammen *et al.* (1999) advocated a strategy of individually genotyping the affected individuals alongside up to three unaffected pools (sibs, parents, unrelated). A similar approach was reported by Haider *et al.* (1998). This apparent compromise offers a modest efficiency gain while maximising information from affected individuals. However neither paper raises a justification for this particular strategy and the observer might well wonder whether undue caution is standing in the way of a far greater efficiency gain from using a DNA pool of affected individuals. Perhaps the relatively small number of successful precedents in the literature has not inspired confidence in DNA pooling. Strong support for DNA pooling does come from many of the investigators who have used it, including Wang *et al.* (1997) who genotyped 20 affected individuals as well as an affected pool of 31 patients. In retrospect they declared the

individual genotyping unnecessary, a realisation that could have saved more than 80% of their genotyping effort.

The uptake of DNA pooling may in part have been held back by a lack of analytical software suitable for genotype data from pooled DNA samples. For their initial genome scan, investigators sometimes applied χ^2 statistics, and in other cases used very approximate assessments to detect a shift towards homozygosity. The identified regions were typically then individually genotyped so that their accustomed linkage software could be used to calculate LOD scores. Of course, individual genotyping in regions of interest (often with a high marker density) can also give much useful information in terms of delimiting the homozygous fragments.

2.13 Statistical methods

The usual descriptions of statistical methodology were by reference to the software and version used. The most common of these were HOMOZ from the MAPMAKER software suite (Kruglyak *et al.* 1995) and various routines from the LINKAGE software suite (Lathrop and Lalouel 1984; Lathrop *et al.* 1984, 1986): FASTLINK (Cottingham *et al.* 1993; Schaffer *et al.* 1994), LINKMAP and MLINK. The key methodological innovations of these routines have been described above. Other software mentioned included GENEHUNTER, SLINK, RHMAP, Merlin, LODSCORE and CRIMAP. Some papers reported the use of χ^2 statistics to evaluate homozygosity of their initial scan. Other statistical approaches were also used for matters unrelated to HM.

None of the papers offered comparison or critique of the various packages that would allow for their objective evaluation or comparison. Additionally there was no evidence to suggest any systematic difference in the statistical outcome. A systematic comparison of statistical packages might be of some use, but would need to consider a wide range of data/pedigree constructs. Of course, choice of software will also be influenced by practical issues such as user-friendliness and ease of data handling, as well as processing time.

Many authors commented that the chosen software was incapable of performing computations based on a complex pedigree (anything beyond a single inbreeding loop) within a practical time limit — particularly when using an early software version. Therefore pedigrees were truncated and, for example, nuclear families within a complex pedigree were analysed separately. FASTLINK was capable of calculating linkage probabilities more rapidly but even so many pedigrees were beyond its ability. Authors widely recognised that truncating pedigrees would lead to a loss of experimental power. However many did not seem to be aware that ignoring the ancestral contribution to inbreeding would increase the apparent significance of linkage as shown by Miano *et al.* (2000).

2.14 Homozygosity criteria

From a whole genome scan, some criteria were required to select those markers which showed a sufficient degree of homozygosity to be worthy of closer examination. The suitable criteria would depend upon the marker spacing, the number of subjects and any DNA pooling. Only a small number of authors described the criteria used, and in most of those cases it was unclear whether the criteria had been set prior to genotyping, or were established pragmatically in order that a certain number of markers would be further tested. Some authors admitted that their initial criteria excluded all markers and had to be relaxed. Quite commonly, the criterion was complete homozygosity across all affected individuals and zero homozygosity among unaffected individuals. Depending on the number genotyped, it was rare for more than one marker to meet this condition; if this criterion was not met then often the marker closest to satisfying it was selected.

I have characterised this as the "last man standing" approach; if all but one of the markers spread across the genome have failed to show significant homozygosity, then it was assumed that the disease must be linked to this last marker. This approach may be ill-advised if it ignores other possibilities — for example that a second region of homozygosity may fall between two other markers, or that complete homozygosity across affected individuals at the disease locus may not occur (because of a misdiagnosis of one or more patients, because of genetic heterogeneity of the disease, because of a recombination between locus and closest marker or because of a genotyping error). If the LOD score is only in the vicinity of the significance threshold then confirmation should be sought through the genotyping of additional affected individuals; clearly a problem if such individuals have not been ascertained. This issue is discussed further with other pitfalls in Section 2.16.

Genin *et al.* (1998) used a theoretical argument to caution against the use of overly-stringent homozygosity criteria because of the risk that the true locus could be excluded.

Friedman *et al.* (1995) first genotyped 13 affected individuals from six kindreds within an isolated population, for 68 microsatellite markers. Their homozygosity criterion was that at least four of the thirteen were homozygous for the marker. This then triggered genotyping of unaffected individuals and a χ^2 test of marker allele frequencies to determine regions worthy of additional markers.

Bolino *et al.* (1996) genotyped five patients from an extended pedigree for 378 microsatellite markers. Their homozygosity criterion was met if at least seven of the ten marker alleles across this group were identical. Thirty markers satisfied this criterion but 29 were excluded from consideration after unaffected individuals were genotyped.

Wijmenga *et al.* (1998) included two affected individuals in their initial screen of 338 microsatellite markers. Their criteria demanded that both patients were homozygous for at least

two consecutive markers. In this way six regions of interest were identified for further testing by genotyping unaffected members of the pedigree and affected individuals from other pedigrees. It was interesting to note that the authors seem to have been unaware that this strategy resulted in the redundancy of up to half of their genotyping; if they had initially typed every second marker then they needed to type the intervening marker only when there was a chance of their criteria being met.

Arbour *et al.* (1997) and El-Shanti *et al.* (1998), both using DNA pooling, observed the reduction in number of alleles between pooled samples as the homozygosity criterion. No further details were given. Asaka *et al.* (2001) genotyped individuals but assessed the ratio of homozygosity between affected and unaffected groups by χ^2 test.

Coucke *et al.* (2003) screened a pool of six affected individuals from one nuclear family with first cousin parents. A region was flagged for further investigation if a marker was represented by only one marker allele and both flanking markers were represented by no more than two marker alleles. This resulted in ten regions being selected for individual genotyping.

Laurier *et al.* (2006) individually genotyped eight affected and five unaffected individuals for a 10 000 SNP chip. Homozygosity in a single subject was noted if it covered more than 25 adjacent SNPs, and the regions of aligned homozygosity across patients were further investigated.

Wu *et al.* (2006) having genotyped five patients from one extended family by 10k SNP chip, simply selected the longest region showing homozygosity across all five. This case and other examples of this approach were successful, but the system could fail if recombinations had occurred close to the locus in affected individuals; also, where the affected individuals are progeny of closely-related parents and are relatively few in number, there is a finite chance of a lengthy segment of common homozygosity that is unlinked to the disease locus.

Melin *et al.* (2007), using both 10k and 100k SNP chips genotyped four patients from an extended pedigree. One patient was the offspring of second cousins but the other patients were presumed to have ancestral inbreeding. Three homozygosity criteria were set. First, regarding the number of consecutive SNPs homozygous across the patients. Second, regarding the map length of these homozygous regions. Third, regarding the number of consecutive SNPs homozygous in at least three of the four patients. This last criterion was included to reduce the risk of a false negative due to either a recombination close to the mutation, or the existence of a phenocopy among the cohort. Genotyping by 10k SNP chip revealed candidate regions but none were confirmed. After genotyping by 100k SNP chip, 30 regions satisfied the criteria and one was confirmed by microsatellite markers. The authors noted in retrospect the 10k results in the vicinity of the locus were quite unremarkable.

2.15 Success

Almost all of the homozygosity mapping papers reported success in locating a significant or near-significant linkage to the disease locus, and only seven papers were unable to report any putative locus. This may in part reflect a trend not to report negative results — despite the fact that these could be scientifically interesting if, for example, it were to challenge the assumed mode of inheritance.

Most authors set themselves a LOD score threshold of 3.0 or 3.3, as are generally accepted for genome-wide searches. A lower threshold was sometimes used if a reasonably small part of the genome had been scanned. On the other hand, there were a number of cases where the LOD score exceeded 10 once supplementary individuals had been genotyped for the region of interest. Defining of haplotypes and the detection of recombination events were used to limit the critical region including the locus. These ranged in size from around 30cM down to around 2cM, depending upon the density and informativeness of markers, the number of individuals genotyped and, to a degree, good fortune in the occurrence of recombination.

In two cases authors were courageous enough to admit to initial errors in their work and to outline the misapprehensions that had led to them (Miano *et al.* 2000; Laurier *et al.* 2006). These reports are of course enormously instructive. These matters will be discussed below with other pitfalls in Section 2.16.

Many of the papers reported that the work had proceeded beyond HM — usually through candidate gene analysis to detection of causative mutation/s. Recently, some investigators were able to proceed to identifying candidate genes even before they had narrowed their interest to a single chromosomal region (Hasselbacher *et al.* 2006, Ostergaard *et al.* 2007). It was noticeable that over time the typical end point moved from reporting a statistically confirmed locus, through to describing the actual mutation. This was probably due to the declining workload associated with HM and the other laboratory techniques, along with the increasingly powerful resources available in terms of genetic maps and catalogues of gene function, etc.

A number of papers reporting linkage also revealed that a search of region/s of interest for the pathological mutation had not been successful (for example Weigell-Weber *et al.* 2003). While this may have brought the linkage into question, it was also possible that the mutation had been missed through genotyping errors or through a focus on candidate gene/s which were not in fact causative.

For a small number of reports where I considered there was a chance that the finding of linkage was unsafe, I had planned to use citation tracking to see whether the result was confirmed or debunked in subsequent work. This was attempted for four cases. In one instance there was clear-cut subsequent confirmation of the locus. In the other three cases there was neither clear confirmation nor any hint of a discrediting of the locus.

For at least four reasons, such an analysis of the forward literature may not deliver a conclusive result. First, the laboratory undertaking the initial work may have exclusive access to the patient details and samples, meaning that no independent laboratory can replicate the work on the same materials. Any authoritative debunking could only realistically occur through further evaluation of the original work (perhaps with additional data) in the original laboratory, and due to all of the facts of scientific life, such as project funding cycles and contract appointments, this may be entirely impractical. Second, if other workers investigating the same condition fail to find linkage to the earlier reported locus then this would often be attributed to genetic heterogeneity of the disease rather than earlier experimental error. Third, if other workers find a conclusive locus for the disease elsewhere, they are still reliant upon the first laboratory to test for linkage to that locus in the initial data set. Fourth, it would require the passage of some time and the attention of an astute observer to bring to light a prolonged absence of confirmation of a claimed locus — which may even then never reach the published literature. Thus it may be that an erroneously claimed locus cannot ever be positively refuted but is simply never confirmed.

2.16 Pitfalls

The literature reveals a small number of reports which specify problems that researchers have encountered in using homozygosity mapping. While these cases are certainly in the minority, they may point to additional investigations which have not been reported. The problems encountered and potential problems are categorised below but the boundaries are often blurred. As in many situations, the presence of two or more pitfalls can multiply the consequences.

False negative

Miano *et al.* (2000) conducted a genome-wide scan with 386 microsatellite markers at an average spacing of about 10cM using pooled DNA samples from one extended family: four affected individuals; unaffected siblings; and unaffected parents. Their genotyping results appear to have been qualitatively assessed only — recording the number of marker alleles without any rating for allele frequency. To pass the initial screening, markers had to show homozygosity in the affected pool and heterozygosity in both unaffected pools. These markers were further tested by individual genotyping and typing of two additional families.

Miano *et al.* (2000) went on to report that as their work continued the report of a gene for the same condition went to press (Haider *et al.* 2000). Markers close to this gene had been excluded by Miano *et al.* (2000) at the initial screening stage, but upon closer examination the gene was confirmed as causative. It transpired that there were two distinct pathogenic mutations segregating in the same pedigree; among the four patients seven haplotypes spanning this gene were identical but one was not. The three nearby markers each showed two or three alleles among the affected pool, thus failing the qualitative homozygosity test.

An alternative approach to genotype assessment may have avoided this result. Qualitative analysis, even approximate, would have revealed markers where the majority allele was up to seven times more frequent than the minority allele — a result very close to homozygosity. In addition, a contrast between this allele frequency distribution and that of the unaffected pools may have aroused interest. The wise caution of Genin *et al.* (1998) to apply more relaxed homozygosity criteria is relevant; the definition of "relaxed" should take into account the effect of a single recombination. To generalise this principle, homozygosity criteria should not be too stringent or absolute; increasing the number of regions screened at the initial stage will not greatly increase the confirmatory effort but may avoid the need to backtrack at a later stage.

Genetic and/or allelic heterogeneity

While no other report was quite as candid as Miano *et al.* (2000), others have reported similar problems related to two or more causative genes or alleles segregating within a family. This outcome is clearly more likely for a condition that is already known to be heterogeneous; and where an extended pedigree is used; and again when the condition is more common. Laurier *et al.* (2006) investigated patients from a large pedigree for a highly heterogeneous condition. They found that some patients were homozygous-by-descent at previously documented loci but went on to map a novel locus for which three causative mutations were segregating. Ducroq *et al.* (2006) could not find a common region of homozygosity across nine patients from a large and complex pedigree. However by examining each nuclear family separately they were able to confirm that some previously known disease loci were segregating, and eventually that one patient was misdiagnosed. Frishberg *et al.* (2007) discovered three separate mutations to the same gene segregating within two large pedigrees from an isolated population. Some patients were homozygous for a mutation, with a surrounding region of homozygosity but others were compound heterozygotes (i.e. each of their copies of the key gene was afflicted by a separate mutation) with no region of homozygosity.

In the absence of congruent homozygosity in all of the affected individuals, the examination of subsets of the affected cohort may reveal homozygosity within certain pedigrees or in certain nuclear families within an extended pedigree, or segregated along alternative lines.

False positive

In addition to excluding the causative locus (above) Miano *et al.* (2000) believed they had found the disease locus on another chromosome. After the initial screening, potential linkage regions were further investigated by individual genotyping and genotyping of two additional families, leading to a focus of attention on one region of interest. Additional markers were typed in this vicinity, and a long region of homozygosity was revealed in three patients from one family. The other two families showed quite short homozygous segments, which might have been suspicious but was attributed to reportedly high recombination rates in the neighbourhood.

Miano *et al.* (2000) believed that since homozygosity was confirmed across ten patients from three unrelated families, and the peak LOD score of 3.69 exceeded the prior threshold of 3.30, they had identified the causative region. The work progressed to physical mapping and assessment of candidate genes.

Miano *et al.* (2000) attributed this misapprehension to two distinct but related effects. First, the finite probability of identity-by-descent in inbred individuals in parts of the genome unrelated to disease genes. While the probability of finding an association of equal or greater strength by chance was small enough to satisfy a statistical finding of significance, that did not mean that chance could be ruled out. But a second effect was also significant — the possible overestimation of LOD scores if undocumented ancestral inbreeding was present. The LOD scores were recalculated assuming that each set of founder parents were first cousins, thus doubling the inbreeding coefficients of the patient generation. The LOD scores fell below the significance threshold. An arbitrary exaggeration of known inbreeding seems an unsatisfactory approach. Perhaps a more reasonable alternative would be a sensitivity test — what level of relationship between founders would cast doubt on the statistical significance, the likelihood of which could then be assessed against what is known about the population history.

Deletion mutation

Garshasbi *et al.* (2006) reported a study where the causative mutation was eventually found to be a substantial deletion (150–200kb). Their initial scan revealed a region of homozygosity spanning this locus. However the SNPs within the deletion gave a null genotype in the affected individuals while being apparently homozygous in carrier relatives, therefore being classified as uninformative markers. Fortunately the LOD score threshold was exceeded at a nearby SNP so that the region was investigated in more detail and the mutation uncovered.

Phenocopy or other misdiagnosis

There were incidences where one of the patients was discovered to be a phenocopy (Melin *et al.* 2007) or to have been a misdiagnosed case of a similar condition (Ducroq *et al.* 2006). When multiple family members have been diagnosed with a genetic condition, it is possible that subsequent patients are not examined with the same thoroughness or open-mindedness as to diagnosis. The challenges of such problems are of course not limited to homozygosity mapping studies. However the risks may be increased when the patients are drawn from inbred families and/or from isolated communities and/or from developing countries remote from the project leaders. Clearly there is a need for accurate communication between diagnostic practitioners and the other project scientists, particularly when language, cultural and geographic boundaries are crossed.

Incorrect placement of the critical region

Macgregor *et al.* (2006) pointed out that in some cases phenocopies may not prevent the discovery of significant linkage to a disease gene, if there is an adequate number of subjects. However if a phenocopy is not detected as such because by chance it shares a region of homozygosity with the other patients, then the boundaries of the critical region may be incorrectly set. This could lead to fine mapping, candidate gene analysis, mutation analysis, etc., in a region which is close to but excludes the true causative gene. Similarly, Macgregor *et al.* (2006) showed that incomplete penetrance could result in an unaffected individual that is homozygous for the disease mutation. If the possibility of incomplete penetrance was not considered then once again the bounds of the critical region could be incorrectly placed.

Mizrachi-Koren *et al.* (2005) reported a novel locus for congenital recessive ichthyoses (CRI). From a total of five patients and nine unaffected subjects across two families, one affected individual and one unaffected individual each showed a recombination event which significantly narrowed the critical region to less than 6.5cM. This region was only 5cM distant from a previously-reported locus for CRI (Hatsell *et al.* 2003). The authors expressed some confidence that their locus was indeed novel, however there was no indication that they had considered the risks of a phenocopy or of incomplete penetrance.

Inadequate confirmation

There were instances noted where a region of homozygosity identified in an initial genome scan was not subjected to rigorous confirmation before being claimed as a disease locus. Exclusion of other potentially homozygous regions was open to misinterpretation as positive confirmation. Genotyping of additional markers in the region of interest was capable of showing that homozygosity was due to descent rather than due to state. Genotyping of unaffected relatives for the region of interest could establish that these individuals were not homozygous but this added little statistical weight because of the high probability that any random marker was heterozygous.

Clearly, the strongest confirmation of a putative locus comes from additional affected individuals. However this can cause difficulties. If there are no more affected individuals in the first pedigree/s then specimens from other families must be sought. If the condition is known or suspected to be heterogeneous then this involves the risk that other genes will be involved. If the patients have been ascertained from a remote region then additional assistance will be required from the co-operating agencies with access to the particular community. Project funding and time-line constraints will often come into play.

All of these issues place the primary emphasis on appropriate experimental design: ensuring from the start that the experiment will have sufficient genetic resources to make valid statistical claims of linkage. Ideally the subjects available for genotyping will be sufficient to allow for a

statistically significant preliminary finding of linkage, plus some additional affected individuals to be available for confirmatory genotyping.

Cases from the literature where the claiming of a locus may have been suspect were separated into two main categories, and an example of each analysed.

The *"last man standing"* approach involved tending to regard a marker as disease-linked because it gave the highest LOD score or was the only marker to surpass the homozygosity criteria. This was particularly suspect if the homozygosity criteria had been set pragmatically after the event; there seemed to be a high risk that the result was due to an artefact. Borglum *et al.* (2001) investigated a family of four affected and one unaffected full-sibs with the known consanguinity giving inbreeding coefficient $F = 5/64 = 0.078$. A total of 424 microsatellite markers had an average spacing of less than 8cM. They found one region of homozygosity across all four patients with corresponding heterozygosity in the unaffected sibling and both parents. An additional 14 markers in this region were genotyped, limiting the homozygosity to a span of 30cM. However the maximum LOD score was only 3.1 — that is, close to the significance threshold. Importantly, the authors reported that the highest LOD score from a marker outside this region was well below the significance threshold at 2.0. Four points emerge. First, that the experiment, from the outset, lacked the power to lead to a result of clear-cut statistical significance. Second, consideration should have been given to the possibility that another part of the genome may harbour the causative locus — possibly because of a wider than average marker spacing, or because one or more consecutive markers were uninformative, or because of a recombination between the disease locus and the nearest marker. Third, if the caution expressed by Genin *et al.* (1998) to use relaxed homozygosity criteria had been heeded, then the markers showing sub-significant LOD scores would be further examined; it is likely that the difference of LOD score was due to only one recombination in an affected individual. Fourth, to provide adequate confirmation of the locus additional patients should be genotyped.

The *"first cab off the rank"* approach was typically used in conjunction with the testing of candidate genes and/or a progressive genome scan. A significant or near-significant LOD score was highly regarded and resulted in cessation of genotyping in unexplored regions, thus ignoring the possibility of an alternative region of homozygosity. El-Shanti *et al.* (1998) used a DNA pool from 5 patients and two unaffected pools to commence typing of 350 microsatellite markers. After typing 140 markers across six chromosomes, a region of interest was identified where three markers showed a reduction in the number of alleles in the affected pool compared to the controls. Individual genotyping and the inclusion of additional markers produced a maximum LOD score of 3.58, with the critical region restricted to about 12.9cM. A candidate gene was examined but no mutations were found. While the statistical strength in favour of linkage was reasonable, it could be judged to fall short of compelling. Importantly, the work performed in order to confirm the initial suspicion of linkage added very little statistical power.

Meanwhile, more than half the genome remained completely unexplored. It could be noted however, that widespread use of SNP chips for genotyping has obviated this approach in humans at least.

Genome-wide LOD score thresholds are well-established and serve a useful purpose in the indication of linkage. However LOD scores can exceed the threshold by chance and also because of undocumented ancestral inbreeding (see above). Efforts should be made to ensure that confirmatory genotyping is statistically independent and adds to the experimental power; this means genotyping additional affected individuals. Furthermore, for this to be both feasible and timely, experimenters must prepare such specimens in advance.

2.17 Non-human species

Reports of homozygosity mapping were found covering the bovine (Tammen *et al.* 1999, 2002; *Cavanagh et al.* 2000, 2002; Ohba *et al.* 2000; Kuhn *et al.* 2002), canine (van de Sluis *et al.* 2000; Moody *et al.* 2005), equine (Tryon *et al.* 2007), murine (Kao *et al.* 2006), ovine (Tammen *et al.* 1999; Cook *et al.* 2002) and porcine (Sironen *et al.* 2002, 2006). Only three papers reported on whole genome scans, all successful, plus Tammen *et al.* (1999) listed a genome scan in progress. Two of these papers also used DNA pooling. The remainder were focussed upon candidate genes — probably a frugal approach due to limited resources for genotyping.

The sampling strategies are of interest. In the mouse study, an F2 cross was created and 8 affected individuals genotyped for only 262 SNPs (Kao *et al.* 2006) — indicating the potential to use sparse markers when inbreeding is high and all individuals are closely related. For the study of neuronal ceroid lipofuscinosis reported by Tammen *et al.* (1999) and Cook *et al.* (2002), affected sheep from three industry flocks were combined and bred in a small experimental flock. In the other studies, animals and, importantly, pedigree information were apparently all obtained from normal private breeding programs, without resort to planned experimental matings. Cavanagh *et al.* (2002) studied bovine chondrodysplasia in seven affected and eleven unaffected carriers drawn from a single extended pedigree, plus three unrelated controls. Similarly van de Sluis *et al.* (2000) sampled nine affected, twelve unaffected carriers and two other unaffected dogs from an extended pedigree, plus eleven unrelated controls. Moody *et al.* (2005) and Tryon *et al.* (2007) apparently drew their samples from the general population. Ohba *et al.* (2000) studying bovine renal tubular dysplasia took advantage of two widely-used AI sires being father and son. They were able to locate many instances of the son being mated to a half-sister, resulting in 38 affected and 37 unaffected progeny being sampled.

Sironen *et al.* (2002) sampled 28 affected boars which were all descendants of one sire — a founder presumed to be the source of the mutation. Sheffield *et al.* (1995) commented on the

usefulness of recently-founded populations for HM studies. Where such a population is not otherwise available to livestock researchers, it may be reasonable to synthesise one.

2.18 Discussion

Homozygosity mapping has been an important technique for the mapping of genes associated with recessive Mendelian characters. Lander and Botstein (1987) quoted the then-current estimate of 1420 suspected recessive disorders known in humans. Many of these have been mapped, including some hundreds by homozygosity mapping, based on the papers examined for this review. Many conditions have also been identified as heterogeneous, meaning that the number of genes associated with recessive disorders, if known, would now greatly exceed the 1987 estimate.

The advantages of homozygosity mapping are based on the relative flexibility of requirements as to pedigree structure and the modest requirements as to number of patients. Moreover the statistical power available for inference in relation to the gross genotyping effort has been a highly attractive prospect for many investigators. The ability to set definite limits on the critical region can also have advantages.

Perhaps the greatest impediment to earlier or more widespread use of HM has been the availability of a reliable and adequately dense marker map for the organism in question. Ascertaining suitable pedigrees has not always been easy, although the range of pedigrees considered suitable has extended over time as investigators have experimented with more distant and even unspecified consanguinity. Firm knowledge of the mode of inheritance is an advantage but recessivity is not essential. Complications can arise if the condition is not sufficiently rare.

Practical application of homozygosity mapping was assisted by Lander and Botstein (1987) setting out the technique in a clear and comprehensive fashion including the statistical framework. Ease-of-use was further enhanced by the development of appropriate analytical off-the-shelf software packages. Early use was probably inhibited by the relative sparseness of human genetic maps. I did not find any reports of HM by RFLP markers as originally proposed, but the more convenient microsatellite markers were readily adopted, followed in time by SNP markers, each available at progressively higher densities.

The basic theory of homozygosity mapping was so well described by Lander and Botstein (1987) and possessed of such simple elegance that later developments have largely been restricted to analytical considerations: more rapid determination of linkage likelihoods; more sophisticated calculation of IBD probabilities and IBD fragment lengths; and better handling of ancestral inbreeding. Perhaps the one part of homozygosity mapping subject to inconsistent practice relates to establishing the homozygosity criteria for an initial genome scan. Some criteria were somewhat one-dimensional, while setting the criteria too strictly carried the risk of

excluding the disease locus; efforts at theoretical guidance in this area (e.g. Genin *et al.* 1998) appear not to have influenced practice, perhaps due to the complexity caused by a multiplicity of experimental design factors.

DNA pooling gives the potential to assess the genotypes of many individuals rapidly and cheaply and scan for association to a genetic condition. This was particularly important in the 1980s and 1990s when genotyping was a more laborious and costly exercise, and not always reliable. Despite the great potential, and the publication of several reports of successful use of DNA pooling, there appears to have been an undue measure of caution steering many potential users toward the perceived safety of individual genotyping. Perhaps DNA pooling suffered (maybe still suffers) the perception that it is outside of the mainstream of genetic techniques. Such a view may have been reinforced by an absence of literature covering theoretical considerations of DNA pool composition, and also the absence of theoretical approaches to assessing the results of pooled DNA genotyping, including relevant analytical software. As a result, many differing standards have been applied to the composition of DNA pools and to criteria in homozygosity screening. While the economic imperative for efficient genotyping has diminished in some spheres, in general investigators working in non-human species continue to be constrained by genotyping costs.

A major attraction of HM has been its efficiency in terms of genotyping effort for a genome-wide search. I have not attempted to identify from the literature the record-holder for minimal genotyping for a genome-wide search leading to the mapping of a disease locus. However the prime contenders would be those who pooled DNA across a relatively large number of subjects, used a single unaffected pool, quantitatively compared allele frequency in the pools, and used widely-spaced markers for their initial scan (see for example Kant *et al.* 1998).

The advent of high-throughput SNP chip genotyping presents many new opportunities for genetics but also creates a dilemma for homozygosity mapping. Certainly a SNP chip genotype is far more informative than any reasonable density of microsatellite markers. As the cost of SNP chips has declined, SNP genotyping has rightly come to be regarded as the most efficient available in terms of information per dollar spent. However in many cases the information gained from SNP genotyping would be orders of magnitude greater than is required for homozygosity mapping. In addition, SNP genotyping has not been reported in conjunction with pooled DNA samples. In consequence, the early stages of gene discovery work –- the whole-genome sweep — may be more cost-effective through the use of highly polymorphic microsatellite markers at a relatively low density.

Homozygosity mapping has enjoyed a high rate of success according to the published reports. Although a range of potential pitfalls have been identified, the combined incidence of such problems has been small. Disease heterogeneity was feared in some quarters to be a problem beyond the power of HM. However as Lander and Botstein (1987) boldly predicted, the power

of HM in such cases greatly exceeds that of traditional family studies. While requiring a greater number of subjects and a higher marker density compared to homogeneous conditions, HM has surely satisfied its critics through the complexity of heterogeneous conditions which have been progressively unravelled (for example Laurier *et al.* 2006).

It would be appealing to create a set of guidelines for HM which would ensure that all of the pitfalls were avoided. This does not seem possible and, even if it were, may prove to be overly cautious for the majority of situations. However a series of strategies can be adopted which will at least alert the experimenter to complications and risks:

1. The criteria for homozygosity at the initial scan should be somewhat flexible to allow for cases where perfect homozygosity is not achieved for various reasons.

2. If homozygosity is not found across the cohort of affected subjects, then subsets of the cohort could be considered, perhaps by breaking up a complex pedigree into nuclear families.

3. Consideration should be given to the possibility of ancestral inbreeding and the influence that would have on LOD scores.

4. Being alert to genotyping anomalies may point to a rare event such as a large deletion.

5. Consideration should be given to the possibility of a phenocopy among the patients, or of incomplete penetrance of the condition, and how either occurrence might have affected the boundaries of the critical region.

6. Confirmation of a suspected locus should always include the genotyping of additional affected individuals.

7. The experimental power should ideally be sufficient to reach the appropriate statistical threshold while keeping in reserve some affected individuals for subsequent confirmation.

The use of homozygosity mapping in humans appears to have been in slight decline in recent years. This is unlikely to be due to a diminishing supply of unmapped recessive conditions, but it could be because the more common and well-documented conditions have largely been mapped. If true this would mean that researchers are likely to face conditions which are less clearly defined in medical terms, are poorly understood in terms of inheritance, and for which suitable patients are more difficult to find. However to counter these problems researchers have the option to use high density SNP genotyping at a declining unit cost, and also techniques such as that described by Leutenegger *et al.* (2006) for HM without knowledge of or access to other members of the pedigree — obviously increasing the population of suitable patients.

2.19 Conclusion — application to livestock

Homozygosity mapping may now be of declining relevance to human genetics due to sequencing of the entire genome, haplotype maps in progress, map-based information on gene function, and the decreasing cost of genotyping. With possible exceptions for laboratory species, all other species lag to varying degrees behind the status of the human genome. HM is most likely to be relevant in species where an accurate marker map exists with a maximum spacing of about 20cM.

HM has enjoyed limited use to date for the discovery of genes behind Mendelian characters in livestock. However recent rapid increases in marker density and the development of SNP chips for some species might serve to improve the prospects. It may be generally observed that research laboratories dealing with livestock are often less-well funded than their human equivalents. Therefore the attraction to efficiency of genotyping effort offered by HM will be strong.

Further efficiency gains are likely if a less cautious attitude to DNA pooling can be encouraged. A boost might come from supportive theoretical examination of the topic and the establishment of relevant analytical/statistical tools.

In the cases where HM has been used for non-human species, research material has generally been obtained from the population at large, rather than from experimental matings. Clearly, however, pedigree records were also available, facilitating sampling within a family as well as providing vital information for the HM method. In instances where appropriate animals and their pedigrees cannot be obtained from the general population then experimental matings may be the best option — for example, comprehensive pedigree records are a rarity in Australian Merinos.

Predictions as to the power of HM and therefore the number of affected individuals required can be readily made according to the literature. These will vary significantly from case to case and there is little to be offered in the way of general guidance. Minimisation of genotyping effort through optimising the number of affected animals and marker spacing will be beneficial. Design variations to HM, for example a mating design to better suit livestock, will affect the experimental power and the impact should be tested before such variations are implemented.

The HM pitfalls catalogued in this review and the strategies recommended to combat them are all relevant to livestock studies. However it should not be concluded that HM is generally prone to hazards — in fact it has been shown to be a robust technique in a great majority of cases.

Many genetic diseases of livestock can severely compromise the productive capacity of an animal, so the economic value of gene mapping can be quite high. In addition, livestock breeders stand to benefit from the mapping of many morphological characters with less dramatic impact on production: hornedness in many species; coat colour and pattern; eye

pigmentation as protection from cancer; and of course major genes affecting a host of quantitative traits. Livestock matings can obviously be managed to specifically provide animals suitable for gene discovery, however this can be a logistical challenge as well as expensive and time-consuming in large animals. For this reason the potential to use unpedigreed individuals for homozygosity mapping, as proposed by Leutenegger *et al.* (2006), is particularly exciting, and points to an important role for homozygosity mapping in livestock genetics over the coming years.

2.20 References

Abney, M., Ober, C. and McPeek, M.S. (2002). Quantitative-trait homozygosity and association mapping and empirical genomewide significance in large, complex pedigrees: Fasting serum-insulin level in the Hutterites. *American Journal of Human Genetics* **70**: 920–934.

Agarwala, R., Biesecker, L.G. and Schaffer, A.A. (2000). Inverse inbreeding coefficient problems with an application to linkage analysis of recessive diseases in inbred populations. *Discrete Applied Mathematics* **104**: 3–44.

Arbour, N.C., Zlotogora, J., Knowlton, R.G., Merin, S., Rosenmann, A., Kanis, A.B., Rokhlina, T., Stone, E.M. and Sheffield, V.C. (1997). Homozygosity mapping of achromatopsia to chromosome 2 using DNA pooling. *Human Molecular Genetics* **6**: 689–694.

Arnheim, N., Strange, C. and Erlich, H. (1985). Use of pooled DNA samples to detect linkage disequilibrium of polymorphic restriction fragments and human disease: Studies of the *HLA* class II loci. *Proceedings of the National Academy of Sciences of the United States of America – Biological Sciences* **82**: 6970–6974.

Asaka, T., Ikeuchi, K., Okino, S., Takizawa, Y., Satake, R., Nitta, E., Komai, K., Endo, K., Higuchi, S., Oyake, T., Yoshimura, T., Suenaga, A., Uyama, E., Saito, T., Konagaya, M., Sunohara, N., Namba, R., Takada, H., Honke, K., Nishina, M., Tanaka, H., Shinagawa, M., Tanaka, K., Matsushima, A., Tsuji, S. and Takamori, M. (2001). Homozygosity and linkage disequilibrium mapping of autosomal recessive distal myopathy (Nonaka distal myopathy). *Journal of Human Genetics* **46**: 649–655.

Berkovic, S.F., Mazarib, A., Walid, S., Neufeld, M.Y., Manelis, J., Nevo, Y., Korczyn, A.D., Yin, J.G., Xiong, L., Pandolfo, M., Mulley, J.C. and Wallace, R.H. (2005). A new clinical and molecular form of Unverricht-Lundborg disease localized by homozygosity mapping. *Brain* **128**: 652–658.

Bolino, A., Brancolini, V., Bono, F., Bruni, A., Gambardella, A., Romeo, G., Quattrone, A. and Devoto, M. (1996). Localization of a gene responsible for autosomal recessive demyelinating neuropathy with focally folded myelin sheaths to chromosome 11q23 by homozygosity mapping and haplotype sharing. *Human Molecular Genetics* **5**: 1051–1054.

Borglum, A.D., Balslev, T., Haagerup, A., Birkebaek, N., Binderup, H., Kruse, T.A. and Hertz, J.M. (2001). A new locus for Seckel syndrome on chromosome 18p11.31–q11.2. *European Journal of Human Genetics* **9**: 753–757.

Bouchlaka, C., Abdelhak, S., Amouri, A., Ben Abid, H., Hadiji, S., Frikha, M., Ben Othman, T., Amri, F., Ayadi, H., Hachicha, M., Rebai, A., Saad, A. and Dellagi, K. (2003). Fanconi anemia in Tunisia: high prevalence of group A and identification of new FANCA mutations. *Journal of Human Genetics* **48**: 352–361.

Carmi, R., Rokhlina, T., Kwitekblack, A.E., Elbedour, K., Nishimura, D., Stone, E.M. and Sheffield, V.C. (1995). Use of a DNA pooling strategy to identify a human obesity syndrome locus on chromosome-15. *Human Molecular Genetics* **4**: 9–13.

Carr, I.M., Flintoff, K.J., Taylor, G.R., Markham, A.F. and Bonthron, D.T. (2006). Interactive visual analysis of SNP data for rapid autozygosity mapping in consanguineous families. *Human Mutation* **27**: 1041–1046.

Cavanagh, J.A.L., Tammen, I., Harper, P.A.W., Raadsma, H. and Nicholas, F.W. (2000). Homozygosity mapping approach for the chondrodysplasia gene in dexter cattle.. *Asian-Australasian Journal of Animal Sciences* **13** (Suppl. B): 215.

Cavanagh, J.A.L., Tammen, I., Windsor, P.A., Nicholas, F.W. and Raadsma, H.W. (2002). Identification of the gene causing chondrodysplasia in Dexter cattle.. *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production.* Paper 22–26.

Chen, A., Wayne, S., Bell, A., Ramesh, A., Srisailapathy, C.R.S., Scott, D.A., Sheffield, V.C., VanHauwe, P., Zbar, R.I.S., Ashley, J., Lovett, M., VanCamp, G. and Smith, R.J.H. (1997). New gene for autosomal recessive non-syndromic hearing loss maps to either chromosome 3q or 19p. *American Journal of Medical Genetics* **71**: 467–471.

Clark, A.G. (1999). The size distribution of homozygous segments in the human genome. *American Journal of Human Genetics* **65**: 1489–1492.

Cook, R.W., Jolly, R.D., Palmer, D.N., Tammen, I., Broom, M.F. and McKinnon, R. (2002). Neuronal ceroid lipofuscinosis in Merino sheep. *Australian Veterinary Journal* **80**: 292–297.

Cottingham, R.W., Idury, R.M. and Schaffer, A.A. (1993). Faster sequential genetic-linkage computations. *American Journal of Human Genetics* **53**: 252–263.

Coucke, P.J., Wessels, M.W., Van Acker, P., Gardella, R., Barlati, S., Willems, P.J., Colombi, M. and De Paepe, A. (2003). Homozygosity mapping of a gene for arterial tortuosity syndrome to chromosome 20q13. *Journal of Medical Genetics* **40**: 747–751.

Crisponi, L., Crisponi, G., Meloni, A., Toliat, M.R., Nurnberg, G., Usala, G., Uda, M., Masala, M., Hohne, W., Becker, C., Marongiu, M., Chiappe, F., Kleta, R., Rauch, A., Wollnik, B., Strasser, F., Reese, T., Jakobs, C., Kurlemann, G., Cao, A., Nurnberg, P. and Rutsch, F. (2007). Crisponi syndrome is caused by mutations in the CRLF1 gene and is allelic to cold-induced sweating syndrome type 1. *American Journal of Human Genetics* **80**: 971–981.

Darvasi, A. and Soller, M. (1994). Selective DNA pooling for determination of linkage between a molecular marker and a quantitative trait locus. *Genetics* **138**: 1365–1373.

Ducroq, D., Shalev, S., Habib, A., Munnich, A., Kaplan, J. and Rozet, J.M. (2006). Three different ABCA4 mutations in the same large family with several consanguineous loops affected with autosomal recessive cone-rod dystrophy. *European Journal of Human Genetics* **14**: 1269–1273.

El-Shanti, H., Murray, J.C., Semina, E.V., Beutow, K.H., Scherpbier, T. and Al-Alami, J. (1998). Assignment of gene responsible for progressive pseudorheumatoid dysplasia to chromosome 6 and examination of COL10A1 as candidate gene. *European Journal of Human Genetics* **6**: 251–256.

El-Shanti, H., Lidral, A.C., Jarrah, N., Druhan, L. and Ajlouni, K. (2000). Homozygosity mapping identifies an additional locus for Wolfram syndrome on chromosome 4q. *American Journal of Human Genetics* **66**: 1229–1236.

Ewald, H., Kruse, T.A. and Mors, O. (2003). Genome wide scan using homozygosity mapping and linkage analyses of a single pedigree with affective disorder suggests oligogenic inheritance. *American Journal of Medical Genetics Part B — Neuropsychiatric Genetics* **120B**: 63–71.

Ewald, H., Wikman, F.P., Teruel, B.M., Buttenschon, H.N., Torralba, M., Als, T.D., El Daoud, A., Flint, T.J., Jorgensen, T.H., Blanco, L., Kruse, T.A., Orntoft, T.F. and Mors, O. (2005). A genome-wide search for risk genes using homozygosity mapping and microarrays with 1,494 single-nucleotide polymorphisms in 22 eastern Cuban families with bipolar disorder. *American Journal of Medical Genetics Part B — Neuropsychiatric Genetics* **133B**: 25–30.

Farrall, M. (1993). Homozygosity mapping — familiarity breeds debility. *Nature Genetics* **5**: 107–108.

Forshew, T. and Johnson, C.A. (2004). SCAMP: a spreadsheet to collate autozygosity mapping projects. *Journal of Medical Genetics* **41**: e125.

Friedman, T.B., Liang, Y., Weber, J.L., Hinnant, J.T., Barber, T.D., Winata, S., Arhya, I.N. and Asher, J.H. (1995). A gene for congenital, recessive deafness DFNB3 maps to the pericentromeric region of chromosome-17. *Nature Genetics* **9**: 86–91.

Frishberg, Y., Ben-Neriah, Z., Suvanto, M., Rinat, C., Mannikko, M., Feinstein, S., Becker-Cohen, R., Jalanko, H., Zlotogora, J. and Kestila, M. (2007). Misleading findings of homozygosity mapping resulting from three novel mutations in NPHS1 encoding nephrin in a highly inbred community. *Genetics in Medicine* **9**: 180–184.

Garshasbi, M., Motazacker, M.M., Kahrizi, K., Behjati, F., Abedini, S.S., Nieh, S.E., Firouzabadi, S.G., Becker, C., Ruschendorf, F., Nurnberg, P., Tzschach, A., Vazifehmand, R., Erdogan, F., Ullmann, R., Lenzner, S., Kuss, A.W., Ropers, H.H. and Najmabadi, H. (2006). SNP array-based homozygosity mapping reveals MCPH1 deletion in family with autosomal recessive mental retardation and mild microcephaly. *Human Genetics* **118**: 708–715.

Genin, E., Todorov, A.A. and Clerget-Darpoux, F. (1998). Optimization of genome search strategies for homozygosity mapping: influence of marker spacing on power and threshold criteria for identification of candidate regions. *Annals of Human Genetics* **62**: 419–429.

Ghiasvand, N.M., Kanis, A.B., Helms, C., Sheffield, V.C., Stone, E.M. and Donis-Keller, H. (2000). Nonsyndromic congenital retinal nonattachment gene maps to human chromosome band 10q21. *American Journal of Medical Genetics* **90**: 165–168.

Gibbs, J.R. and Singleton, A. (2006). Application of genome-wide single nucleotide polymorphism typing: Simple association and beyond. PLOS Genetics 2: 1511–1517.

Goldberg, Y.P., MacFarlane, J., MacDonald, M.L., Thompson, J., Dube, M.P., Mattice, M., Fraser, R., Young, C., Hossain, S., Pape, T., Payne, B., Radomski, C., Donaldson, G., Ives, E., Cox, J., Younghusband, H.B., Green, R., Duff, A., Boltshauser, E., Grinspan, G.A., Dimon, J.H., Sibley, B.G., Andria, G., Toscano, E., Kerdraon, J., Bowsher, D., Pimstone, S.N., Samuels, M.E., Sherrington, R. and Hayden, M.R. (2007). Loss-of-function mutations in the Na(v)1.7 gene underlie congenital indifference to pain in multiple human populations. *Clinical Genetics* **71**: 311–319.

Guo, S.W. (1997). Computation of multilocus prior probability of autozygosity for complex inbred pedigrees. *Genetic Epidemiology* **14**: 1–15.

Haider, N.B., Carmi, R., Shalev, H., Sheffield, V.C. and Landau, D. (1998). A Bedouin kindred with infantile nephronophthisis demonstrates linkage to chromosome 9 by homozygosity mapping. *American Journal of Human Genetics* **63**: 1404–1410.

Haider, N.B., Jacobson, S.G., Cideciyan, A.V., Swiderski, R., Streb, L.M., Searby, C., Beck, G., Hockey, R., Hanna, D.B., Gorman, S., Duhl, D., Carmi, R., Bennett, J., Weleber, R.G., Fishman, G.A., Wright, A.F., Stone, E.M. and Sheffield, V.C. (2000). Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. *Nature Genetics* **24**: 127–131.

Hasselbacher, K., Wiggins, R.C., Matejas, V., Hinkes, B.G., Mucha, B., Hoskins, B.E., Ozaltin, F., Nurnberg, G., Becker, C., Hangan, D., Pohl, M., Kuwertz-Broking, E., Griebel, M., Schumacher, V., Royer-Pokora, B., Bakkaloglu, A., Nurnberg, P., Zenker, M. and Hildebrandt, F. (2006). Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. *Kidney International* **70**: 1008–1012.

Hatsell, S.J., Stevens, H., Jackson, A.P., Kelsell, D.P and Zvulunov, A. (2003). An autosomal recessive exfoliative ichthyosis with linkage to chromosome 12q13. *British Journal of Dermatology* **149**: 174–180.

Houwen, R.H.J., Baharloo, S., Blankenship, K., Raeymaekers, P., Juyn, J., Sandkuijl, L.A. and Freimer, N.B. (1994). Genome screening by searching for shared segments -- mapping a gene for benign recurrent intrahepatic cholestasis. *Nature Genetics* **8**: 380–386.

Jamieson, C.R., Govaerts, C. and Abramowicz, M.J. (1999). Primary autosomal recessive microcephaly: Homozygosity mapping of MCPH4 to chromosome 15. *American Journal of Human Genetics* **65**: 1465–1469.

Kant, S.G., Polinkovsky, A., Mundlos, S., Zabel, B., Thomeer, R.T.W.M., Zonderland, H.M., Shih, L.Y., van Haeringen, A. and Warman, M.L. (1998). Acromesomelic dysplasia Maroteaux type maps to human chromosome 9. *American Journal of Human Genetics* **63**: 155–162.

Kao, H.J., Cheng, C.F., Chen, Y.H., Hung, S.L., Huang, C.C., Millington, D., Kikuchi, T., Wu, J.Y. and Chen, Y.T. (2006). ENU mutagenesis identifies mice with cardiac fibrosis and hepatic steatosis caused by a mutation in the mitochondrial trifunctional protein beta-subunit. *Human Molecular Genetics* **15**: 3569–3577.

Kirby, D.M., Salemi, R., Sugiana, C., Ohtake, A., Parry, L., Bell, K.M., Kirk, E.P., Boneh, A., Taylor, R.W., Dahl, H.H.M., Ryan, M.T. and Thorburn, D.R (2004). NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency. *Journal of Clinical Investigation* **114**: 837–845.

Kondo, H., Qin, M.H., Mizota, A., Kondo, M., Hayashi, H., Hayashi, K., Oshima, K., Tahira, T. and Hayashi, K. (2004). A homozygosity-based search for mutations in patients with autosomal recessive retinitis pigmentosa, using microsatellite markers. *Investigative Ophthalmology & Visual Science* **45**: 4433–4439.

Kong, A. (1991). Efficient methods for computing linkage likelihoods of recessive diseases in inbred pedigrees. *Genetic Epidemiology* **8**: 81–103.

Kruglyak, L., Daly, M.J. and Lander, E.S. (1995). Rapid multipoint linkage analysis of recessive traits in nuclear families, including homozygosity mapping. *American Journal of Human Genetics* **56**: 519–527.

Kuhn, C., Weikard, R., Panicke, L. and Schwerin, M. (2002). Fibroblast growth factor 10: a potential candidate gene for bovine developmental defect Tetradysmelia?. *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production*. Paper 01-82.

Lander, E.S. and Botstein, D. (1987). Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* **236**: 1567–1570.

Lathrop, G.M. and Lalouel, J.M. (1984). Easy calculations of LOD scores and genetic risks on small computers. *American Journal of Human Genetics* **36**: 460–465.

Lathrop, G.M., Lalouel, J.M., Julier, C. and Orr, J. (1984). Strategies for multilocus linkage analysis in humans. *Proceedings of the National Academy of Sciences of the United States of America – Biological Sciences* **81**: 3443–3446.

Lathrop, G.M., Lalouel, J.M. and White, R.L. (1986). Construction of human linkage maps – likelihood calculations for multilocus linkage analysis. *Genetic Epidemiology* **3**: 39–52.

Laurier, V., Stoetzel, C., Muller, J., Thibault, C., Corbani, S., Jalkh, N., Salem, N., Chouery, E., Poch, O., Licaire, S., Danse, J.M., Amati-Bonneau, P., Bonneau, D., Megarbane, A., Mandel, J.L. and Dollfus, H. (2006). Pitfalls of homozygosity mapping: an extended consanguineous Bardet-Biedl syndrome family with two mutant genes (BBS2, BBS10), three mutations, but no triallelism. *European Journal of Human Genetics* **14**: 1195–1203.

LeGuern, E., Guilbot, A., Kessali, M., Ravise, N., Tassin, J., Maisonobe, T., Grid, D. and Brice, A. (1996). Homozygosity mapping of an autosomal recessive form of demyelinating Charcot-Marie-Tooth disease to chromosome 5q23–q33. *Human Molecular Genetics* **5**: 1685–1688.

Leutenegger, A.L., Prum, B., Genin, E., Verny, C., Lemainque, A., Clerget-Darpoux, F. and Thompson, E.A. (2003). Estimation of the inbreeding coefficient through use of genomic data. *American Journal of Human Genetics* **73**: 516–523.

Leutenegger, A.L., Labalme, A., Genin, E., Toutain, A., Steichen, E., Clerget-Darpoux, F. and Edery, P. (2006). Using genomic inbreeding coefficient estimates for homozygosity mapping of rare recessive traits: Application to Taybi-Linder syndrome. *American Journal of Human Genetics* **79**: 62–66.

Libiger, O. and Schork, N.J. (2005). Simulation-based homozygosity mapping with the GAW14 COGA dataset on alcoholism. *BMC Genetics* **6** (Suppl. 1).

Liu, F., Elefante, S., van Duijn, C.M. and Aulchenko, Y.S. (2006). Ignoring distant genealogic loops leads to false-positives in homozygosity mapping. *Annals of Human Genetics* **70**: 965–970.

Macgregor, S., Knott, S.A. and Visscher, P.M. (2006). False disease region identification from identity-by-descent haplotype sharing in the presence of phenocopies. *Twin Research and Human Genetics* **9**: 9–16.

Malmer, B., Haraldsson, S., Einarsdottir, E., Lindgren, P. and Holmberg, D. (2005). Homozygosity mapping of familial glioma in Northern Sweden. *Acta Oncologica* **44**: 114–119.

McLean, W.H.I., Irvine, A.D., Hamill, K.J., Whittock, N.V., Coleman-Campbell, C.M., Mellerio, J.E., Ashton, G.S., Dopping-Hepenstal, P.J.H., Eady, R.A.J., Jamil, T., Phillips, R.J., Shabbir, S.G., Haroon, T.S., Khurshid, K., Moore, J.E., Page, B., Darling, J., Atherton, D.J., van Steensel, M.A.M., Munro, C.S., Smith, F.J.D. and McGrath, J.A. (2003). An unusual N-terminal deletion of the laminin alpha 3a isoform leads to the chronic granulation tissue disorder laryngo-onycho-cutaneous syndrome. *Human Molecular Genetics* **12**: 2395–2409.

Melin, M., Entesarian, M., Carlsson, G., Garwicz, D., Klein, C., Fadeel, B., Nordenskjold, M., Palmblad, J., Henter, J.I. and Dahl, N. (2007). Assignment of the gene locus for severe congenital neutropenia to chromosome 1q22 in the original Kostmann family from

Northern Sweden. *Biochemical and Biophysical Research Communications* **353**: 571–575.

Miano, M.G., Jacobson, S.G., Carothers, A., Hanson, I., Teague, P., Lovell, J., Cideciyan, A.V., Haider, N., Stone, E.M., Sheffield, V.C. and Wright, A.F. (2000). Pitfalls in homozygosity mapping. *American Journal of Human Genetics* **67**: 1348–1351.

Michelmore, R.W., Paran, I. and Kesseli, R.V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis — a rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 9828–9832.

Mizrachi-Koren, M., Geiger, D., Indelman, M., Bitterman-Deutsch, O., Bergman, R. and Sprecher, E. (2005). Identification of a novel locus associated with congenital recessive ichthyosis on 12p11.2–q13. *Journal of Investigative Dermatology* **125**: 456–462.

Mizrachi-Koren, M., Shemer, S., Morgan, M., Indelman, M., Khamaysi, Z., Petronius, D., Bitterman-Deutsch, O., Hennies, H.C., Bergman, R. and Sprecher, E. (2006). Homozygosity mapping as a screening tool for the molecular diagnosis of hereditary skin diseases in consanguineous populations. *Journal of the American Academy of Dermatology* **55**: 393–401.

Moody, J.A., Famula, T.R., Sampson, R.C. and Murphy, K.E. (2005). Identification of microsatellite markers linked to progressive retinal atrophy in American Eskimo Dogs. *American Journal of Veterinary Research* **66**: 1900–1902.

Morrow, E.M., Yoo, S.Y., Hill, R.S., Bodell, A., Apse, K.A., Balkhy, S., Gascon, G., Herguner, S., Mukaddes, N.M. and Walsh, C.A. (2007). Autosomal recessive loci in familial autism using a systematic homozygosity mapping strategy. *Biological Psychiatry* **61** (Suppl.): 10S.

Newman, D.L., Abney, M., Dytch, H., Parry, R., McPeek, M.S. and Ober, C. (2003). Major loci influencing serum triglyceride levels on 2q14 and 9p21 localized by homozygosity-by-descent mapping in a large Hutterite pedigree. *Human Molecular Genetics* **12**: 137–144.

Ohba, Y., Kitagawa, H., Kitoh, K., Asahina, S., Nishimori, K., Yoneda, K., Kunieda, T. and Sasaki, Y. (2000). Homozygosity mapping of the locus responsible for renal tubular dysplasia of cattle on bovine Chromosome 1. *Mammalian Genome* **11**: 316–319.

Ostergaard E., Christensen E., Kristensen E., Mogensen B., Duno M., Shoubridge E.A. and Wibrand F. (2007). Deficiency of the a subunit of succinate-coenzyme A ligase causes fatal infantile lactic acidosis with mitochondrial DNA depletion. *American Journal of Human Genetics* **81**: 383–387.

Peretz, H., Naamati, M.S., Levartovsky, D., Lagziel, A., Shani, E., Horn, I., Shalev, H. and Landau, D. (2007). Identification and characterization of the first mutation (Arg776Cys) in the C-terminal domain of the Human Molybdenum Cofactor Sulfurase (HMCS) associated with type II classical xanthinuria. *Molecular Genetics and Metabolism* **91**: 23–29.

Puffenberger, E.G., Kauffman, E.R., Bolk, S., Matise, T.C., Washington, S.S., Angrist, M., Weissenbach, J., Garver, K.L., Mascari, M., Ladda, R., Slaugenhaupt, S.A. and Chakravarti, A. (1994). Identity-by-descent and association mapping of a recessive gene for Hirschsprung disease on human-chromosome 13q22. *Human Molecular Genetics* **3**: 1217–1225.

Roscioli, T., Buckley, M.F., Taylor, P.J., Kirk, E., Ziegler, J., Wong, M., Donald, J.A. and Lindeman, R. (2003). Familial veno-occlusive disease of the liver with immunodeficiency: Localization to a 1Mb region of 2q36.3–37.1 via homozygosity mapping. *American Journal of Human Genetics* **73**: 467.

Saar, K., Al-Gazali, L., Sztriha, L., Rueschendorf, F., Nur-E-Kamal, M., Reis, A. and Bayoumi, R. (1999). Homozygosity mapping in families with Joubert syndrome identifies a locus on chromosome 9q34.3 and evidence for genetic heterogeneity. *American Journal of Human Genetics* **65**: 1666–1671.

Schaffer, A.A. (1999). Computing probabilities of homozygosity by descent. *Genetic Epidemiology* **16**: 135–149.

Schaffer, A.A., Gupta, S.K., Shriram, K. and Cottingham, R.W. (1994). Avoiding recomputation in linkage analysis. *Human Heredity* **44**: 225–237.

Shalata, A., Mandel, H., Reiss, J., Szargel, R., Cohen-Akenine, A., Dorche, C., Zabot, M.T., Van Gennip, A., Abeling, N., Berant, M. and Cohen, N. (1998). Localization of a gene for molybdenum cofactor deficiency, on the short arm of chromosome 6, by homozygosity mapping. *American Journal of Human Genetics* **63**: 148–154.

Sheffield, V.C., Carmi, R., Kwitekblack, A., Rokhlina, T., Nishimura, D., Duyk, G.M., Elbedour, K., Sunden, S.L. and Stone, E.M. (1994). Identification of a Bardet-Biedl-syndrome locus on chromosome-3 and evaluation of an efficient approach to homozygosity mapping. *Human Molecular Genetics* **3**: 1331–1335.

Sheffield, V.C., Nishimura, D.Y. and Stone, E.M. (1995). Novel approaches to linkage mapping. *Current Opinion in Genetics & Development* **5**: 335–341.

Sironen, A.I., Andersson, M., Uimari, P. and Vilkki, J. (2002). Mapping of an immotile short tail sperm defect in the Finnish Yorkshire on porcine Chromosome 16. *Mammalian Genome* **13**: 45–49.

Sironen, A., Thomsen, B., Karttunen, P., Andersson, M., Ahola, V. and Vilkki, J. (2006). Identification of the genomic mutation causing the immotile short tail sperm defect and marker assisted selection in Finnish Yorkshire population.. *Proceedings of the 8th World Congress on Genetics Applied to Livestock Production*. Paper 22-04.

Smith, C.A.B. (1953). The detection of linkage in human genetics. *Journal of the Royal Statistical Society B* **15**: 153–184.

Stockton, D.W., Lewis, R.A., Abboud, E.B., Al-Rajhi, A., Jabak, M., Anderson, K.L. and Lupski, J.R. (1998). A novel locus for Leber congenital amaurosis on chromosome 14q24. *Human Genetics* **103**: 328–333.

Strauss, K.A., Puffenberger, E.G., Craig, D.W., Panganiban, C.B., Lee, A.M., Hu-Lince, D., Stephan, D.A. and Morton, D.H. (2005). Genome-wide SNP arrays as a diagnostic tool: Clinical description, genetic mapping, and molecular characterization of Salla disease in an old order Mennonite population. *American Journal of Medical Genetics Part A* **138A**: 262–267.

Tammen, I., Cavanagh, J.A.L., Cook, R.W., Harper, P.A.W., Raadsma, H.W. and Nicholas, F.W. (1999). Combined homozygosity mapping and candidate gene approach for the identification of disease genes in cattle and sheep. *Archiv Fur Tierzucht-Archives Of Animal Breeding* **42** (Sp. Iss.): 163–166.

Tammen, I., Kurtz, E., Gibson, A. J., Tier, B. and Raadsma, H. W. (2002). Neuronal ceroid lipofuscinosis in Devon cattle — pedigree analysis and preliminary molecular genetic investigation.. *Proceedings of the 7th World Congress on Genetics Applied to Livestock Production*. Paper 13-42.

Taylor, B.A. and Rowe, L. (1989). A mouse linkage testing stock possessing multiple copies of the endogenous ecotropic murine leukemia-virus genome. *Genomics* **5**: 221–232.

Thompson, E.A. (1994). Monte-Carlo likelihood in genetic-mapping. *Statistical Science* **9**: 355–366.

Tryon, R.C., White, S.D. and Bannasch, D.L. (2007). Homozygosity mapping approach identifies a missense mutation in equine cyclophilin B (PPIB) associated with HERDA in the American Quarter Horse. *Genomics* **90**: 93–102.

Tsujikawa, M., Kurahashi, H., Tanaka, T., Okada, M., Yamamoto, S., Maeda, N., Watanabe, H., Inoue, Y., Kiridoshi, A., Matsumoto, K., Ohashi, Y., Kinoshita, S., Shimomura, Y., Nakamura, Y. and Tano, Y. (1998). Homozygosity mapping of a gene responsible for gelatinous drop-like corneal dystrophy to chromosome 1p. *American Journal of Human Genetics* **63**: 1073–1077.

van de Sluis, B., Kole, S., van Wolferen, M., Holmes, N.G., Pearson, P.L., Rothuizen, J., van Oost, B.A. and Wijmenga, C. (2000). Refined genetic and comparative physical mapping of the canine copper toxicosis locus. *Mammalian Genome* **11**: 455–460.

Wang, C.Y., HawkinsLee, B., Ochoa, B., Walker, R.D. and She, J.X. (1997). Homozygosity and linkage-disequilibrium mapping of the urofacial (Ochoa) syndrome gene to a 1-cM interval on chromosome 10q23–q24. *American Journal of Human Genetics* **60**: 1461–1467.

Wang, K., Pugh, E.W., Griffen, S., Doheny, K.F., Mostafa, W.Z., al-Aboosi, M.M., El-Shanti, H. and Gitschier, J. (2001). Homozygosity mapping places the acrodermatitis enteropathica gene on chromosomal region 8q24.3. *American Journal of Human Genetics* **68**: 1055–1060.

Weigell-Weber, M., Sarra, G.M., Kotzot, D., Sandkuijl, L., Messmer, E. and Hergersberg, M. (2003). Genomewide homozygosity mapping and molecular analysis of a candidate gene located on 22q13 (fibulin-1) in a previously undescribed vitreoretinal dystrophy. *Archives of Ophthalmology* **121**: 1184–1188.

Wijmenga, C., van den Heuvel, L.P.W.J., Strengman, E., Luyten, J.A.F.M., van der Burgt, I.J.A.M., de Groot, R., Smeets, D.F.C.M., Draaisma, J.M.T., van Dongen, J.J., De Abreu, R.A., Pearson, P.L., Sandkuijl, L.A. and Weemaes, C.M.R. (1998). Localization of the ICF syndrome to chromosome 20 by homozygosity mapping. *American Journal of Human Genetics* **63**: 803–809.

Winick, J.D., Blundell, M.L., Galke, B.L., Salam, A.A., Leal, S.M. and Karayiorgou, M. (1999). Homozygosity mapping of the achromatopsia locus in the pingelapese. *American Journal of Human Genetics* **64**: 1679–1685.

Woods, C.G., Valente, E.M., Bond, J. and Roberts, E. (2004). A new method for autozygosity mapping using single nucleotide polymorphisms (SNPs) and EXCLUDEAR. *Journal of Medical Genetics* **41**: e101.

Wu, H.M., Cowing, J.A., Michaelides, M., Wilkie, S.E., Jeffery, G., Jenkins, S.A., Mester, V., Bird, A.C., Robson, A.G., Holder, G.E., Moore, A.T., Hunt, D.M. and Webster, A.R. (2006). Mutations in the gene KCNV2 encoding a voltage-gated potassium channel subunit cause "cone dystrophy with supernormal rod electroretinogram" in humans. *American Journal of Human Genetics* **79**: 574–579.

3. Inheritance of *Australian Piebald*

3.1 Introduction

The *Australian Piebald* locus in Merino sheep is held responsible for a form of pigmented fleece spots. In affected individuals, one to several rounded spots containing fibres of black or chocolate brown cover from 0.0003 to greater than 0.5 of the fleece area (Brooker and Dolling 1969) and location is apparently random but with a degree of familial resemblance. The spots are present from birth and each spot may consist solely of pigmented fibres, or a mixture of white and pigmented fibres. Australian Piebald phenotypes occur throughout Australian Merino flocks at a low frequency, and occurrence persists despite long-term phenotypic culling to avoid the risk of costly dark fibre contamination of white fleece.

Understanding the inheritance of *Australian Piebald* is critical to assessing the frequency of causative allele/s in Merino flocks and to designing practical and cost-effective strategies for its elimination, whether by conventional breeding or incorporating molecular tools. Better knowledge may also assist in efforts to locate the causative locus or loci.

Brooker and Dolling (1969) reported that in many respects the occurrence of piebald phenotypes conformed to the existence of a recessive allele at a single locus: piebald phenotypes occurred at a low frequency from unpigmented parents; culling pigmented animals prior to breeding had little effect on occurrence; and by showing that complex combinations did occur, that *Australian Piebald* alleles were segregating independently to symmetrical pigmentation patterns — believed to be controlled by variant alleles at the *Agouti* locus (Parsons *et al.* 1999).

However matings among piebald phenotypes resulted in both white and piebald offspring which did not conform with the expectation of a simple recessive trait, that all such offspring must display the recessive character. Brooker and Dolling (1969) recorded 56 progeny of piebald x piebald matings, 40 (0.71) white and 16 (0.29) piebald. These results led to a conclusion that piebald spotting is controlled by a recessive gene with incomplete penetrance or variable penetrance (Brooker and Dolling 1969).

A major project within the CRC for Sheep Industry Innovation (Sheep CRC) has worked towards eliminating genetic causes of pigmentation. As part of this project a Merino pigmentation flock was established to produce fully pedigreed half-sibs including piebald phenotypes, so that these animals would be available for locating gene/s involved in piebald inheritance.

Over the last thirty years, empirical observations of incomplete penetrance, variable expression and other forms of non-Mendelian inheritance have in many cases been resolved, and there are precedents for a wide range of explanations. Table 3.1 is a summary from van Heyningen and Yeyati (2004) with additional mechanisms from Zlotogora (2003). This list is by no means exhaustive, even of the mechanisms reported in the two sources — a single mechanism can result in several distinct departures from Mendelian inheritance, not reflected in this summary.

Table 3.1 *Fifteen examples of known mechanisms of non-Mendelian inheritance patterns.*

Inheritance pattern	**Mechanism**
van Heyningen and Yeyati (2004):	
1. Apparently sporadic occurrence, no vertical transmission.	Recurrent *de novo* mutation, unable to reproduce
2. Non-Mendelian patterns of inheritance incomplete penetrance	a. Identified gene-gene interactions required: incompletely penetrant digenic/oligogenic disease b. Interaction with identified alleles/variants at same locus c. Reduced penetrance alleles at tumour suppressor loci, requiring second hit d. Environmental trigger required: infections, chemical/drug-induced triggers, stress
3. Complex multi-component phenotypes	Some fully penetrant, others incompletely — confusing segregation patterns
4. Variable expressivity	Variable expressivity so extreme that affected status may be missed
5. Imprinting diseases	a. Rare inherited variants: only give rise to phenotype when inherited from the appropriate parent; alteration in epigenetic organization b. Frequent occurrence of discordant monozygotic twins
6. Paradoxical inheritance	Heterozygotes most severely affected
7. Excess affected cases	Segregation distortion
8. Anticipation through generations	Triplet repeat expansion; telomere shortening
9. Non-Mendelian segregation of telomeric microsatellites	Inheritance patterns of cryptic telomeric alleles
10. Mitochondrial disease	Cytoplasmic maternal inheritance, heteroplasmy leads to unpredictable severity and tissue involvement
Zlotogora (2003):	
11. Pseudo-incomplete penetrance	Germ line mosaicism
12. Sex limitation	Organ or physiology restricted to one sex
13. Incomplete penetrance	a. Disease allele interaction with variant wild-type alleles b. Environmental factors
14. Pseudo-variable expressivity	Additive effect
15. Variable expressivity	Somatic mosaicism

With new field data available it is timely to reconsider the inheritance of *Australian Piebald* and judge whether the conclusion of Brooker and Dolling (1969) is still the most adequate explanation of the data. By testing a range of inheritance models, this chapter will test the hypothesis that *Australian Piebald* is inherited recessively with incomplete penetrance.

3.2 Materials and methods

Brooker and Dolling (1969) reported on a large number of matings involving piebald phenotypes, in the Pigment Study Flock and other flocks at the National Field Station, Cunnamulla, Queensland. In total, they summarise 56 progeny of piebald x piebald (male x female) matings, 266 progeny of piebald x non-piebald matings, 19 progeny of non-piebald x piebald matings, and 18 227 progeny of non-piebald x non-piebald matings. Additional information on the sires and mating structures was obtained from Brooker (1968).

Within the Sheep CRC a Pigmentation Resource Flock was established in autumn 2004. Two groups of Merino ewes were obtained from industry flocks, the larger group of full-mouth adults and the second group of maiden ewes around 18 months of age. An unrelated piebald ram was mated by artificial insemination. This mating resulted in one male piebald lamb (tag 222), and 271 white lambs. In autumn 2006, the F_1 piebald ram 222 was mated naturally to his white half-sisters, at approximately 16 months of age, resulting in one piebald lamb and 67 white lambs. The three piebald individuals are illustrated in Figure 3.1.

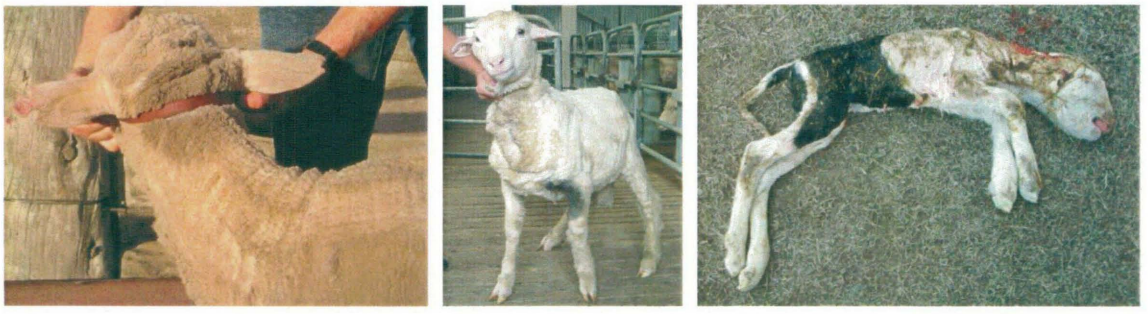


Figure 3.1 *The three piebald individuals from the Sheep CRC Pigmentation Resource Flock. a. The F_0 sire showing pigmented patch at the back of head and neck; b. The F_1 ram 222 aged approximately 12 months, showing pigmented patch on near foreleg; c. The F_2 piebald lamb. A single pigmented patch extended across the backline and right down the off side, covering most of the groin and extending from there to the underside of the tail.*

Unpublished data was made available to this study from a survey of Merino breeders conducted by the Australian Sheep Industry Co-operative Research Centre. A total of 101 breeders, encompassing stud and commercial sectors of the industry, responded to questions on the incidence of *Australian Piebald* and other forms of inherited pigmentation at their latest lambing, their attitudes to the funding of research into inherited pigmentation, and their attitude to adoption of any future commercialised gene tests.

Various modes of Mendelian inheritance were proposed to explain the inheritance of piebald in Merinos. To avoid duplication these models are described in detail in the Results section (see also Table 3.3). Models were evaluated by comparing the model predictions to field data across four classes of observation:

1. White x white matings (can result in white and piebald offspring).
2. Piebald x piebald matings (can result in white and piebald offspring).

3. The observed phenotypic incidence rates (details of observations are below) for piebald resulting from matings of:

   a. Piebald x piebald

   b. Piebald x non-piebald

   c. F_2 *inter se* mating of piebald x non-piebald.

4. Allele frequency decline under phenotypic culling (is slow).

The outcomes for each model were predicted by establishing the allele frequency which under Hardy-Weinberg Equilibrium would give an expected phenotypic incidence of 0.005. Where several allele frequencies could give this outcome, all were considered. The outcome of various *inter se* matings were then assessed via representative sampling from the possible genotypes. Similarly, the effect of phenotypic culling was predicted through its effects on allele frequency with random mating.

3.3 Results

Field observations

Piebald x piebald matings

Brooker and Dolling (1969) reported on 56 progeny of piebald x piebald matings, yielding 16 piebald lambs and 40 white lambs, an incidence of 0.286. (See Table 3.2 for a summary of the field data).

Piebald x non-piebald matings

Brooker and Dolling (1969) reported on 266 progeny of piebald x unselected non-piebald and reciprocal matings, of which 31 were piebald. However, one apparently pre-potent piebald ram (Sire 16) was responsible for 26 of these piebald lambs from his total of 88 offspring, an incidence of 0.295. The remaining matings produced 5 piebald from 178 lambs, or an incidence of 0.028. According to a χ^2 test of heterogeneity, this difference is extremely significant ($p < 10^{-9}$), indicating that there is some unobserved difference between the sire genotypes or the transmission of their genes. The F_0 piebald x non-piebald matings in the Pigmentation Resource Flock yielded 1 piebald lamb of 272, an incidence of 0.004. A χ^2 test of heterogeneity showed this incidence to be significantly different from the Brooker and Dolling (1969) incidence, even after Sire 16 was excluded ($p < 0.05$).

In both flocks, matings were made between one piebald ram and white ewes, all progeny of piebald sire/s. In the Pigmentation Resource Flock, these animals were all half-sibs, but Brooker and Dolling (1969) report that only some of the 14 white F_1 ewes were half-sibs to the F_1 sire. For Brooker and Dolling (1969) this mating resulted in 9 piebald lambs in a total of 16,

an incidence of 0.563. In the Pigmentation Resource Flock, 1 piebald lamb from 68 was an incidence of 0.015. A X^2 test showed there was significant heterogeneity between these two independent results ($p < 10^{-8}$).

Non-piebald x non-piebald matings

Brooker and Dolling (1969) reported on 18 227 progeny where neither parent is piebald, across three research flocks. A total of 80 piebald lambs were detected, an overall incidence of 0.004. The incidence rate varied between the three flocks but the difference tested by X^2 was not significant. In the survey of Merino breeders, respondents reported on a total of 201 731 lambs at their latest lambing, of which 550 were piebald, an incidence of 0.003. However incidence within flocks ranged from 0.000 to 0.042, and the distribution of incidence was heavily skewed: the average incidence in the most affected decile of flocks was 0.015, compared to the second-most affected decile at 0.004. Figure 3.2 presents a visual representation of the phenotypic frequency distribution for piebald and symmetrical (agouti) pigmentation. The distributions appear similar which would tend to suggest similar modes of inheritance. The incidence of piebald was correlated to the incidence of symmetrical pigmentation at 0.53 pointing to the impact of management upon both conditions — for example attitudes to thorough detection and early culling of pigmented lambs, and also the rate of introductions from other flocks. Interestingly, both data sources reported the incidence of piebald in white flocks to be about 40% greater than the incidence of symmetrical pigmentation.

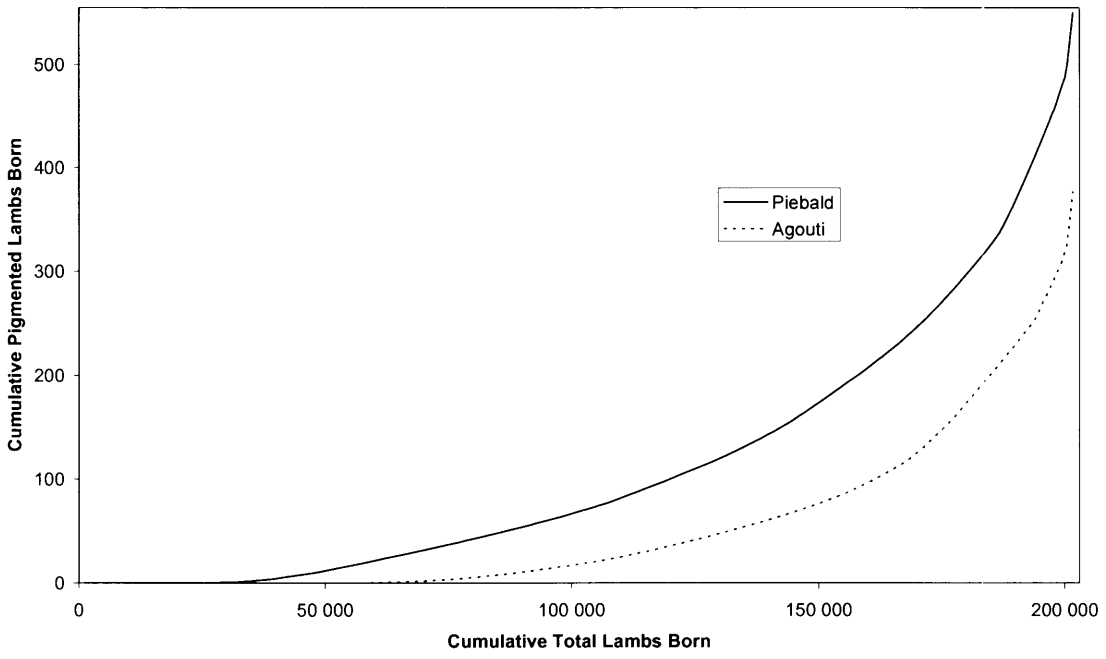


Figure 3.2 *The frequency distribution of pigmentation patterns across 101 Merino flocks, charted as the cumulative total pigmented lambs after sorting flocks by pigmentation frequency separately for each condition.*

Table 3.2 *Summary of field data, showing the occurrence of piebald (P) and non-piebald (W) phenotypes from different mating structures. Confidence intervals determined by iterative* χ^2.

	Offspring phenotypes			Freq(P) confidence interval	
Mating	**P**	**W**	**Freq(P)**	**95%**	**99%**
Piebald x piebald [A]	16	40	0.286	0.184–0.415	0.160–0.457
Piebald x non-piebald [A]	31	235	0.117	0.083–0.161	0.075–0.177
Sire 16 [A]	26	62	0.295	0.210–0.398	0.188–0.431
Other sires [A]	5	173	0.028	0.014–0.064	0.009–0.081
Piebald x non-piebald [B]	1	271	0.004	0.001–0.021	0.000–0.031
F_1 Piebald x non-piebald [A]	9	7	0.563	0.332–0.769	0.275–0.813
F_1 Piebald x non-piebald [B]	1	67	0.015	0.003–0.079	0.002–0.114
Non-piebald x non-piebald [A]	80	18 147	0.004	0.004–0.005	0.003–0.006
Non-piebald x non-piebald [C]	550	201 181	0.003	0.003–0.003	0.002–0.003

[A] Data from Brooker and Dolling (1969).
[B] Data from Sheep CRC Pigmentation Resource Flock.
[C] Data from Sheep CRC breeder survey.

Putative inheritance models

The models tested are described below and additionally the allelic-phenotypic relationship for models 1–16 are given in Table 3.3. The alleles are denoted w, x for a single locus with two alleles, w, x, y for a single locus with three alleles, and w, x and y, z for two loci models, and dominance declines in alphabetical order.

Models — Single locus with two alleles

1. Simple dominant.
2. Simple recessive
3. Recessive with incomplete (0.29) penetrance
4. Dominant with incomplete (0.07) penetrance in heterozygotes
   (penetrance of 0.07 allows for a 0.29 piebald incidence from piebald x piebald matings, assuming that most piebald parents were heterozygous)
5. Overdominance — trait is expressed only in heterozygotes
6. Overdominance with imprinting — trait is expressed only in the heterozygote and only when the allele is maternally (or paternally) inherited

Models — Single locus with three alleles

7. Two recessive alleles, homozygosity for either produces Piebald phenotype
8. Two recessive alleles, heterozygosity between them produces Piebald phenotype
9. As for 8 but with imprinting — Piebald expressed only when the allele is maternally (or paternally) inherited
10. White dominant to Piebald allele in turn dominant to a third allele which results in white phenotype when homozygous
11. As for 10 but with imprinting — Piebald expression in xx and half of xy individuals
12. As for 7, plus piebald displayed in heterozygotes with imprinting — xx, yy and half of xy individuals are Piebald.

Models — Two loci each with two alleles (no linkage between loci)

13. At least one dominant allele is required at each locus for white, all other combinations are Piebald. In other words, Piebald is caused by recessive alleles at either or both of two independent loci. Dominance within locus only. (The expected F_2 ratio following a cross of two homozygous lines would be 9:7).
14. White is produced by the dominant allele w, or in the double recessive xxzz. (Expected F_2 ratio is 13:3).
15. Piebald is only produced by the double recessive. (Expected F_2 ratio is 15:1).
16. Piebald is produced by a recessive locus but suppressed by a second recessive locus. (Expected F_2 ratio is 13:3).

Models — Three or four loci each with two alleles (no linkage between loci)

17. An extension of model 13 to three independent loci each with a recessive allele responsible for Piebald.
18. An extension of model 13 to four independent loci each with a recessive allele responsible for Piebald.

Table 3.3 *Schematic description of models 1–16 showing White (W) or Piebald (P) phenotype resulting from each allelic combination. The allele inherited from one parent shown on the horizontal axis, the other parent on the vertical axis. Inheritance model numbers as above.*

Inherited allele	1. w	x		2. w	x		3. w	x	
w	P	P		W	W		W	W	
x	P	W		W	P		W	W/P	
Inherited allele	**4. w**	**x**		**5. w**	**x**		**6. w**	**x**	
w	P	W/P		W	P		W	P	
x	W/P	W		P	W		W	W	
Inherited allele	**7. w**	**x**	**y**	**8. w**	**x**	**y**	**9. w**	**x**	**y**
w	W	W	W	W	W	W	W	W	W
x	W	P	W	W	W	P	W	W	P
y	W	W	P	W	P	W	W	W	W
Inherited allele	**10. w**	**x**	**y**	**11. w**	**x**	**y**	**12. w**	**x**	**y**
w	W	W	W	W	W	W	W	W	W
x	W	P	P	W	P	P	W	P	P
y	W	P	W	W	W	W	W	W	P

Inherited alleles	13. wy	wz	xy	xz	14. wy	wz	xy	xz
wy	W	W	W	W	W	W	W	W
wz	W	P	W	P	W	W	W	W
xy	W	W	P	P	W	W	P	P
xz	W	P	P	P	W	W	P	W
Inherited alleles	**15. wy**	**wz**	**xy**	**xz**	**16. wy**	**wz**	**xy**	**xz**
wy	W	W	W	W	W	W	W	W
wz	W	W	W	W	W	W	W	P
xy	W	W	W	W	W	W	W	W
xz	W	W	W	P	W	P	W	P

Table 3.4 shows each of the proposed inheritance models assessed against the test criteria. The expected incidence rates, and the effect of phenotypic culling were calculated under the assumption that non-piebald animals come from populations in Hardy-Weinberg Equilibrium, excepting the phenotypic culling of piebald animals, with a piebald incidence of 0.005. Further, the F_2 outcome required that the F_1 sire genotype was possible given the F_0 sire genotype. However, in some cases the solution is given as a range because there may be several allele frequency arrangements to give the desired background incidence, and also there may be two or more genotypes possible in the Piebald sire/s.

In Table 3.4, the impact of phenotypic culling is given as the allelic half-life — the generations of phenotypic culling required to halve the incidence. This does not imply that the decline in phenotypic incidence or allele frequency follows a decay curve, but is intended to assist understanding the influence of selection. It might be assumed that to explain satisfactorily the persistence of piebald in a population the half-life should be at least equivalent to that of a simple recessive trait (model 2). Note that the impact of selection could in practice be increased by culling the relatives of affected individuals; also that genetic drift would have a substantial effect on the impact of culling.

Table 3.4 *Assessment of inheritance models against observed features of Piebald inheritance. Expected incidence rates assume that non-piebald animals come from populations in Hardy-Weinberg Equilibrium, with a piebald incidence of 0.005 and phenotypic culling.*

Model	Progeny of		Expected incidence to			Allelic half-life[B]
	White x white	Piebald x piebald	Piebald x piebald	Piebald x non-piebald	F_2 *inter se* [A]	Generation
Field data[C]	W, P	W, P	0.286	0.004–0.295	0.015–0.563	>5?
1	W	W, P	0.75–1.00	0.50–1.00	0.50–0.75	<1
2	W, P	P	1.00	0.07	0.50	6
3	W, P	W, P	0.29	0.04	0.13	12
4	W, P	W, P	0.29	0.05–0.10	0.16–0.54	7
5	W, P	W, P	0.50	0.50	0.50	<1
6	W, P	W, P	0.25	0 00–0.50	0.125	1
7	W, P	W, P	0.50–1.00	0.00–0.09	0.50	7–9
8	W, P	W, P	0.50	0.05–0.08	0.26–0.27	2–5
9	W, P	W, P	0.25	0.02–0.08	0.12–0.16	6–10
10	W, P	W, P	0.75–1.00	0.06–0.16	0.35–0.54	3–7
11	W, P	W, P	0.50–1.00	0.07–0.23	0.25–0.57	5–7
12	W, P	W, P	0.50–1.00	0.01–0.08	0.25–0.53	6–7
13	W, P	W, P	0.50–1.00	0.00–0.09	0.44–0.75	6–9
14	W, P	W, P	0.75–1.00	0.06–0.08	0.38–0.51	6–7
15	W, P	P	1.00	0.07	0.36–0.50	12–16
16	W, P	W, P	0.75–1.00	0.06–0.13	0.34–0.57	7–11
17	W, P	W, P	0.33–1.00	0.00–0.11	0.50	6–10
18	W, P	W, P	0.25–1.00	0.00–0.13	0.50	6–12

[A] Modelled as one piebald F_1 ram x white F_1 ewes.

[B] Generations of phenotypic culling required to reduce piebald incidence from 0.0050 to 0.0025.

[C] From Table 3.2.

3.4 Discussion

Evidence tending to support the hypothesis

The hypothesis of recessive inheritance with incomplete penetrance (model 3) is supported by some of the evidence reported. It is capable of explaining the occurrence of both piebald and non-piebald phenotypes from white x white and piebald x piebald matings. It explains the ineffectiveness of phenotypic culling. The model can also account for the incidence rate to piebald x piebald matings. The hypothesised model of inheritance thus satisfied attributes 1, 2, 3a, and 4.

Evidence tending to undermine the hypothesis

However the proposed model (3) can hardly be said to explain the field data with respect to attributes 3b or 3c — phenotypic incidence from piebald x non-piebald matings. For unselected piebald x non-piebald matings, model 3 predicted a phenotypic incidence of 0.04. In the Sheep CRC Pigmentation Resource Flock the observed frequency was significantly lower. Conversely, in the Pigment Study Flock (Brooker and Dolling 1969) the observed frequency was significantly higher than predicted, either across all sires or for Sire 16 alone. Only when Sire 16 was excluded did the predicted incidence fall within the 95% confidence interval of the observed incidence — an inconclusive finding. The apparent pre-potency of Sire 16 (piebald) mated to unselected white ewes casts doubt on a recessive inheritance because it would imply an unreasonably high proportion of carriers among the ewes. On the other hand, the heterogeneity of piebald incidence was extremely significant and clearly points to a non-random cause. Under a single-locus recessive inheritance model this penetrance factor must be non-genetic.

For F_1 piebald x non-piebald matings the predicted piebald incidence was 0.13. In the Pigmentation Resource Flock, the observed incidence was once again significantly lower. The matings in the Pigment Study Flock (Brooker and Dolling 1969) were slightly different because multiple F_0 sires were used, but under this inheritance model the prediction would be unchanged. The observed incidence exceeded 0.5 and despite a broad confidence interval was significantly greater than the prediction.

Evidence for alternative inheritance models

The inheritance models tested were primarily selected to satisfy attributes 1 and 2. All of the models except for model 15 and the reference models 1 and 2, displayed these attributes. Similarly, most models showed a slow response to phenotypic culling; the exceptions were models 1, 5, 6, and model 8 was marginal in this regard.

By contrast, relatively few of the models satisfied any of the other criteria. Most models predicted a high incidence to piebald x piebald matings, but models 3, 4, 6, 9, 16 and 17

conformed satisfactorily with the field observations. Assessing criteria 3b and 3c was complicated by the heterogeneity observed in the field results. This suggested that there may be two or more genotypes responsible for the piebald phenotype — as in models 1, 4, 7, 10, 11, 12, 13, 14, 16, 17 and 18. None of the models were capable of explaining the low incidence of piebald to an F_2 *inter se* mating in the Sheep CRC Pigmentation Resource Flock, but models 6 and 9 came closest — interestingly, two models including imprinting. However, model 6 was already discounted due to the rapid effect of phenotypic culling, and model 9 could not explain the high incidence from piebald x non-piebald matings achieved by Sire 16 in the Pigment Study Flock.

Some variation in the incidence to piebald x non-piebald matings might be explained by differences in allele frequencies in the background populations. However this cannot explain the most obvious contrast, between Sire 16 and his contemporaries in the Pigment Study Flock. Moreover, we have reliable data on the incidence of piebald in the main source flock for these animals, which is remarkably consistent with present-day incidence across industry flocks. A repetition of Table 3.4 with alternative levels of background piebald incidence may slightly improve the "fit" of some inheritance models, but it seems unreasonable to expect a qualitative improvement in the fit of any particular model/s.

In summary models 3, 4, 9 and 18 appear to show the greatest potential for explaining the field data. As outlined, each of these models has weaknesses but their abilities to explain the data are not sufficiently distinct to support any preferential ranking among them. I would conclude therefore that at this point in time models 3, 4, 9 and 18 should be considered equivalently acceptable, simply because there is no basis to prefer any among them.

Clearly further field observations are required to resolve this issue. I think that additional piebald x piebald matings would be the most informative, particularly repeat matings. Under inheritance models 3 and 9 there is only one genotype resulting in the piebald phenotype, so the outcome of one mating should be independent of the outcome of a repeat of that mating. By contrast models 4 and 18 indicate that the outcome of repeat matings should be correlated to the outcome of the first mating. In these cases the piebald progeny of piebald x piebald crosses would in turn show progressively higher "penetrance" until a pure piebald strain was achieved.

Multi-locus models with linkage were not examined in detail. On the surface, such models could explain the observation of heterogeneity of sire pre-potency. However on closer examination the models consistently fail to explain the observed incidence to piebald x piebald matings.

Of course it would be possible to propose ever more complex models of inheritance until a model might be contrived to explain every field observation. While this course would have the advantage of defining precisely the experimental matings which could disprove each

hypothesis, there is little reason to suppose that the true solution to the puzzle would be brought any closer to our grasp. If the data had been stronger then more advanced statistical techniques such as Maximum Likelihood might have been applied, although this would not have provided a suitable basis for comparing inheritance models except in the limited circumstance of one model being a subset of another, for example comparing putative penetrance rates. As it is, in addition to the small quantity of data, there was no depth of pedigree in the Pigmentation Resource Flock and no genuine pedigree records have survived from the Pigment Study Flock.

Brooker and Dolling (1969) proposed a model with two loci, one a piebald promoter and the other an overriding piebald suppressor — the piebald suppressor allele was presumed fixed in the ancestral Merino but an outcross had introduced an alternative allele. Model 15 depicts this situation where piebald suppression is dominant, and model 16 illustrates for a recessive piebald suppression factor. Both models failed to predict the observed result of piebald x piebald matings, among other failings.

An alternative suggestion is that piebald expression might be masked by white spotting. Certainly there appear to be white spotting factors present in Australian Merino sheep, which are often observed in conjunction with recessive symmetrical (agouti) coat patterns. However a characteristic of *Australian Piebald* is that the pigmented spots are continuous and have rounded edges. It would seem likely that if white spotting of the type described were present in piebald animals then there would be instances of the pigmented spots being a more complex shape, due to the "over-laying" of white. Such cases have not been documented nor, in the author's experience, observed in the field.

Brooker and Dolling (1969) noted a case in which they suspected a black spot was due to a somatic mutation in a lamb heterozygous for recessive black. Interestingly they reported that pigmented fibres on this lamb were particoloured — a common occurrence in recessive black animals but not in piebald. Certainly any confusion between spots caused by somatic mutation and those of *Australian Piebald* would be a huge impediment to inheritance studies. However there is no reason to suspect that misclassification has occurred, much less that it has been common. It will also be obvious that if the F_0 sire in the Pigmentation Resource Flock had been heterozygous for recessive black then dramatic consequences would have been observed in the F_2.

Piebald phenotypes are found in a number of sheep breeds around the world, although the phenotypic definition varies widely, as does the inheritance (for example, Roberts 1926). The wild ancestor of domestic sheep is supposed to have shown a symmetrical pattern of graded colour (Ryder 1987) without random spots. *Australian Piebald* has only been documented in Australian Merino sheep (COGNOSAG 1996) and was not first noted until long after the establishment of the breed on Australian soil (Kelley and Shaw 1942). Either of these facts might be due to a lack of scientific attention to an unwelcome or uninteresting fault, but taken at

face value would point to *Australian Piebald* arising within the Australian Merino. The source might have been a mutation, or an inadvertent introgression from another breed. Sheep of many breeds were introduced to the Australian colonies, from Britain, Europe, Africa, and Asia and some of these were added to Merino flocks and progressively improved by backcrossing; later introductions of Vermont Merino were also made.

For this mutation or introduction to have occurred and then spread widely through Australian Merino flocks, it seems that the genetic agent was most likely a single gene or possibly a group of tightly linked genes, rather than two or more genes spread across the genome — which would make wide distribution of all the elements much less probable. However if the inheritance is to be explained by genetic means then it would appear that the single mutated or introduced gene does indeed interact with one or more loci in the genomic background to give the observed incidence.

Non-Mendelian mechanisms

Several of the non-Mendelian mechanisms listed in Table 3.1 could be excluded — for example *de novo* mutation and sex-limitation. However none of the mechanisms present an explanation any more satisfactory than incomplete penetrance. Another possibility is the presence of phenocopies — that some animals which are phenotypically piebald are genetically normal. For this explanation to be plausible, phenocopies would need to be reasonably common, accounting for as many as half of the piebald phenotypes.

Non-genetic factors

Matings involving piebald parents almost always resulted in a piebald incidence among the progeny that was far higher than occurred in non-piebald matings. This can leave little doubt that the condition is inherited by some means or other, but non-genetic factors may also be at play. Brooker and Dolling (1969) observed that sex did not influence the occurrence of piebald or the location of spots on the pelt. Wright (1926) provides a precedent for an effect of maternal age on pigmentation in the guinea pig. If ewe age (or perhaps parity) was a factor then this could have been an issue for the mating of F_1 ewes in the Pigmentation Resource Flock at 16 months of age. A higher incidence of piebald may have been observed if these ewes had been mated again. At the other extreme, Turner et al. (1959) note that the contemporary practice at the National Field Station was to join ewes from 1.5 years to 10.5 years of age. Thus it is possible that some of the white ewes recruited to the Pigment Study Flock were of considerable age. However it is most unlikely that a non-random allocation of ewes would account for the heterogeneity of piebald incidence between Sire 16 and the other sires.

Implications

The inheritance of *Australian Piebald* may or may not be explained by a single locus model. This has clear implications for current gene discovery efforts, which should avoid designs

exclusively suited to single gene models — that is, methods should be capable of dealing with non-Mendelian inheritance and multi-locus models.

Merino breeders could be justifiably dismayed to find that four decades after the issue of *Australian Piebald* inheritance was first studied, the questions remain unresolved. The monetary implications of pigmented fibre contamination have increased dramatically over that time but research has been funded only in short bursts, with promising programs prematurely terminated. Further modest research investment to resolve definitively piebald inheritance is surely an obvious step to take in the interests of a viable wool industry in this century.

3.5 References

Brooker, M.G. (1968). Pigmentation in the Australian Merino. Master of Science in Agriculture Thesis. University of Sydney.

Brooker, M.G. and Dolling, C.H.S. (1969). Pigmentation of sheep III. Piebald pattern in Merinos. *Australian Journal of Agricultural Research* **20**: 523–532.

COGNOSAG (1996) Mendelian inheritance in sheep. Eds: J.J. Lauvergne, C.H.S.Dolling and C. Renieri. Clamart, France — University of Camerino, Camerino, Italy.

Kelley, R.B. and Shaw, H.E.B. (1942). Note on the occurrence and inheritance of pigmented wool. *Journal of the Council for Scientific and Industrial Research* **15**: 1–3.

Parsons, Y.M, Fleet, M.R. and Cooper, D.W. (1999). The *Agouti* gene: a positional candidate for recessive self-colour pigmentation in Australian Merino sheep. *Australian Journal of Agricultural Research* **50**: 1099–1103.

Roberts, J.A.F. (1926). Colour inheritance in sheep. II The piebald pattern of the Piebald breed. *Journal of Genetics* **17**: 77–83.

Ryder, M.L. (1987). The evolution of the fleece. *Scientific American* **256**: 100–107.

Turner, H.N., Dolling, C.H.S. and Sheaffe, P.H.G. (1959). Vital statistics for an experimental flock of Merino sheep. I. Death rates in adult sheep, in relation to method of selection, age and sex. *Australian Journal of Agricultural Research* **10**: 581–590.

van Heyningen, V. and Yeyati, P.L. (2004). Mechanisms of non-Mendelian inheritance in genetic disease. *Human Molecular Genetics* **13**: R225–R233.

Wright, S. (1926). Effects of age of parents on characteristics of the guinea pig. *The American Naturalist* **60**: 552–559.

Zlotogora, J. (2003). Penetrance and expressivity in the molecular age. *Genetics in Medicine* **5**: 347–352.

4. Homozygosity mapping is a powerful means of gene discovery for Mendelian traits of livestock

4.1 Introduction

The discovery of genes relevant to livestock production, whether affecting discrete or quantitative characters, is of major interest. Elimination of genetic diseases and faults along with enhancement of production will become available to the livestock industries when cheap and accurate gene tests are developed and commercialised. Optimum design of gene discovery experiments will maximise experimental power, conserve scarce research resources and accelerate the application of results to improve industry profitability.

Gene discovery by traditional linkage mapping requires considerable numbers of affected individuals with quite large family sizes. Pedigree information is important and any missing genotypes for parents and grandparents can be an impediment. By contrast linkage disequilibrium mapping can be performed with fewer individuals but requires much greater marker density. Missing parental genotypes can hamper the determination of haplotypes. Thus in different ways these two methods impose practical constraints as well as requiring much genotyping.

Homozygosity mapping (Lander & Botstein 1987) is a method of gene discovery designed for Mendelian recessive conditions which offers the potential to reduce the genotyping effort compared to conventional linkage or linkage disequilibrium mapping methods. The affected and unaffected inbred progeny of an unaffected (presumed heterozygous) ancestor form the experimental material. Affected progeny are expected to have identity-by-descent over a chromosomal region surrounding the causative locus. Therefore marker homozygosity across affected offspring and heterozygosity among unaffected offspring suggest linkage to the locus. The method can locate genes based on small numbers of individuals and a relatively wide marker spacing (Lander and Botstein 1987). Laboratory efficiency can be enhanced further by pooling samples because, ignoring recombination, affected individuals within a family will all be homozygous for the same haplotype carrying the disease allele — the pooled sample should still show a single marker allele at closely linked markers, or an unusual distribution of marker alleles at less-closely linked markers. The HM approach is well suited to humans, with accurate phenotypes and pedigree information available in the general population. Pedigree information is critical to identifying inbred individuals and also for determining statistical significance of linkage. HM has been very widely used for rare recessive conditions in humans, often with little prior biological understanding.

HM would appear to have much to offer the discovery of genes for Mendelian conditions in livestock, in situations where there are not strong candidate genes to narrow the initial search. However there may be impediments to following the exact design of Lander and Botstein (1987). Affected individuals obtained from industry herds may not have pedigree information, and full sibs may either not exist or be impossible to identify. To overcome these problems, a breeding program could be designed to produce inbred affected individuals. Even this course might be impeded by the difficulty in locating an obligate carrier without deliberately breeding it and verifying its carrier status. In addition to these problems, the mode of inheritance may be uncertain.

This study aims to examine the consequences and viability of an altered design for homozygosity mapping, particularly the use of an affected individual as the ancestor in an inbreeding design. The consequences of mating design factors will be examined by stochastic simulation of a gene discovery experiment. The suitability of homozygosity mapping will be tested, and an adaptation will be assessed. This study will clarify the situations where these gene discovery methods are applicable and the conditions under which they are most efficient and most powerful.

This study will test the hypothesis that homozygosity mapping, when applied to a range of Mendelian inheritance models in livestock, provides experimental power for reliable gene detection.

4.2 Methods

This experiment focussed on the design of Mendelian gene discovery based upon animals generated from experimental matings. In this section the complex experimental design will be systematically explained, taking one design variable at a time. First, there are three variants to the breeding design, according to the choice of sire of the second generation. Second, there are three minor variations in the homozygosity mapping method, according to the genotype of the founding sire and the particulars of data analysis. Third, nine different inheritance models for the gene of interest are described. Fourth, three further simple design factors are explained. Fifth, a deterministic approach, used for simpler scenarios, is outlined. Sixth, a description of a simulation approach used to test the full array of scenarios is given. Seventh, the method of sampling animals from the simulated F_2 is given. Finally, Table 4.1 sets out the assumed sire genotypes relevant to the various designs.

Breeding design

In each design an inbred (F_2) cohort was created over two generations. A single founding (F_0) sire was mated to a large group of unaffected F_0 ewes. The F_1 ewe progeny were then subjected to an inbred mating with a single sire under one of three mating options:

a. Backcross to the F_0 sire

b. Half-sib mating to an unaffected F_1 ram

c. Half-sib mating to an affected F_1 ram (where feasible).

Gene discovery — three forms of homozygosity mapping

Homozygosity mapping (HM) is used here to mean a situation where an affected F_0 sire was used, but other aspects are as designed by Lander and Botstein (1987). Among a sample of the F_2, a genome scan identified at each marker individuals homozygous for an allele inherited from an affected ancestor. An excess of homozygosity among affected F_2 was assessed by χ^2 test: a 2 x 2 contingency table was formed according to phenotype and homozygous/not homozygous marker genotype.

Co-inheritance mapping (CM) refers to the use of an affected F_0 sire, and a slightly modified interpretation of the genotype data. Among a sample of the F_2 a genome scan identified at each marker those individuals carrying only allele/s inherited from affected ancestor/s. These alleles need not necessarily be homozygous because homozygosity at the causative allele does not require homozygosity (IBD) at a closely linked marker. An excess of 'co-inheritance' among affected F_2 was assessed by χ^2 test: a 2 x 2 contingency table was formed according to phenotype and co-inherited/not co-inherited marker genotype. Under mating plans *a* and *c* inheritance of one allele from the affected sire was obligatory, so only the co-inheritance of the 'second' allele was of interest, resulting in one entry per individual in the contingency table. For mating plan *b* the inheritance of each allele was of interest so there were two entries per individual in the contingency table. This ensured that the χ^2 test was only dealing with events affected by chance, with the added advantage of maintaining the marginal frequencies of the contingency table at close to 0.5, reducing the disparity between nominal and actual type 1 error rates which can be a problem with χ^2 tests with small numbers of observations (Bradley *et al.* 1979).

Genuine homozygosity mapping (GHM) followed the design of Lander and Botstein (1987) including use of an unaffected carrier F_0 sire. Among a sample of the F_2, a genome scan identified at each marker homozygous individuals, with a χ^2 test as for HM. This option was included only to a limited extent, for comparison.

For all gene discovery methods the χ^2 tests were one-sided, so to achieve a nominal type one error rate of a = 0.05, a *p*-value threshold of 0.10 was used. The gene was considered 'found' and the trial a success if a statistically significant χ^2 result was obtained at any marker/s within 42cM of the disease locus, an arbitrary distance approaching the logical maximum linkage range of 50cM. For all methods it was assumed that the F_1 dams were not genotyped and therefore all alleles detected in the F_2 which could have been transmitted by the sire were indeed attributed to

sire inheritance. Various assumptions were made about the sire genotypes under different scenarios, as shown in Table 4.1.

Inheritance model

A range of modes of inheritance were modelled:

1. Recessive with complete penetrance
2. 75% penetrance
3. 50% penetrance
4. 25% penetrance
5. Dominant with 13% penetrance in heterozygotes
6. 10% penetrance in heterozygotes
7. 7% penetrance in heterozygotes
8. 4% penetrance in heterozygotes
9. Overdominance with maternal/paternal imprinting (3 alleles — see below)

Models 4, 7 and 9 are consistent with observed inheritance of *Australian Piebald* in Merino sheep (Brooker & Dolling 1969, Brash *et al.* 2005). Other models were included so that the sensitivity of gene discovery to the assumed penetrance could be assessed, and in addition so that the results could be confidently extended to characters with various modes of inheritance. In models 5–8, heterozygotes show incomplete penetrance but animals homozygous for the disease allele are fully penetrant. Noting that dominant with zero penetrance in heterozygotes is equal to simple recessive, it may be observed that inheritance models 1–8 form a logical continuum. Under model 9, affected individuals must be heterozygous for the two causative alleles (P, Q), and are only affected if the P allele is inherited paternally and the Q allele inherited maternally. Another possible mode of inheritance was not explicitly simulated — that of several loci each with recessive inheritance and each independently causing indistinguishable piebald phenotypes. The expectations flowing from this model will however be drawn from the simple recessive model.

Design variables and assumptions

i.) Marker spacing. Three marker sets were available spanning the genome at 5cM, 10cM and 20cM spacings. So that the results would reflect the minimum experimental power, the causative locus was always located mid-point between the two closest markers.

ii.) Relatedness. To represent the two extremes of relationship between the F_0 ram and ewes, the F_0 ram was either unrelated to the ewe flock (with 2 unique alleles at each marker) or was a contemporary flock-mate of the ewes, selected only on genotype/phenotype for the condition of interest.

iii.) Ewe carriers. The effect of frequency of the causative allele in the ewe flock was assessed by allowing that the allele was either absent from the F_0 ewes, or present at

two different levels, leading to some F_2 lambs carrying this allele which they had inherited from their maternal granddam through their dam. Within each sample drawn from the F_2 lambs (see below), the number of alleles so inherited was set to either 0, 2 or 8. The distribution of phenotypes of such lambs was determined according to the probability relating to inheritance and penetrance.

Deterministic method

A deterministic approach was applied to a simplified scenario, in which an F_0 sire was mated to unrelated ewes free of the disease allele by each of the three mating plans. The probabilities of disease genotypes and phenotypes among the F_2 were calculated directly for a single marker completely linked to the disease locus, according to Mendelian transmission. χ^2 statistics were tested for indication of linkage. HM, CM and GHM methods were used. Results were cross-validated with the simulation.

Stochastic simulation

To generate the F_0 ram and ewes, a population of sheep was simulated by computer. Flock size was stable at 200 ewes and 5 rams, with 40 ewes and one ram replaced each year. The adults were first bred at two years of age and last bred at six years with no adult deaths, so the generation interval was 4 years for each sex. The actual reproductive rate was irrelevant; there were always sufficient replacements available and mating and selection were entirely random; twins were never co-selected. All affected (diseased) individuals were culled. The flock was closed throughout this phase and continued for either 10 generations (40 years) or 20 generations (80 years) so that the allele frequency distributions, and the effect of selection against the character, would approximate those of field populations. Details of marker allele frequency distributions are presented in the results. The last eight ewe cohorts formed the F_0 group of 320 ewes, which produced approximately 160 F_1 ewes.

The genome was simulated to approximate the lengths of 26 autosomal chromosomes known for sheep, a total length of 3580cM (Maddox and Cockett 2007). The causative locus was placed randomly upon a chromosome, well away from a telomere. In the founder animals, each individual had two unique alleles at each marker locus. Where the disease was to be present in the population, one of the founder rams was a heterozygous carrier for the causative allele/s. At reproduction, chromosomes were passed from each parent to their offspring via Mendelian segregation with the number of recombination events in each interval drawn from a Poisson distribution with the mean equal to the interval length in Morgans. Recombination rates were equal for males and females. There was no mutation at any stage.

Sampling the F_2

For the simulation, the size of the F_2 sample varied from 5 lambs to 120 lambs, because for the successful methods 120 individuals proved to be amply sufficient. The sampled individuals were drawn from a large virtual pool of F_2 offspring. The sample sizes tested increased in graded steps (N = 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 100, 120 lambs) because the computation time increased exponentially with N and because the effect of increasing N tended to diminish rapidly. Within each sample size N, the number of affected individuals p varied through 0, 1, 2, ... , N. The sampling procedure systematically varied the composition of the sample by genotype at the disease locus, filling each genotype quota with randomly-selected individuals, taking 200 sampling replicates of each combination and accumulating statistics weighted by the probability of the phenotype/genotype combination.

Table 4.1 *Assumed sire genotypes. The causative allele is denoted* P *and any second causative allele is denoted* Q.

		HM and CM			GHM		
F_2 Mating Option:		***a***	***b***	***c***	***a***	***b***	***c***
Recessive	F_0 Sire	PP	PP	PP	P-	P-	P-
(Model 1–4)	F_1 Sire	PP	P- (PP[A])[B]	PP[A]	P-	[--, P-][C]	PP
Dominant	F_0 Sire	P- (PP)	P- (PP)	P- (PP)	P-	P-	P-
(Model 5–8)	F_1 Sire	P- (PP)	-- (P-)[D]	P- (PP)	P-	[--, P-]	P-
Overdominant with imprinting	F_0 Sire	PQ	PQ	PQ			
(Model 9)	F_1 Sire	PQ	[P-, Q-][E]	PQ[A]			

[A] Only possible if a causative allele was inherited from a F_0 ewe carrier.
[B] Genotypes in rounded brackets were not considered but were also possible though less likely and more favourable for gene discovery, often genetically equivalent to other mating options.
[C] Genotypes in square brackets were equally likely and phenotypically indistinguishable with differing outcomes; the use of multiple F_1 sires would be required and F_2 families for genotyping could be selected on the basis of incidence rates.
[D] Modelled as [--, P-] for HM under the deterministic method.
[E] Families without affected progeny would be discarded so only P- sire genotypes would remain. (PP was also possible in the simulation with results equivalent to P-.)

4.3 Results

Deterministic: mapping method, mating plan and inheritance

The deterministic approach was used first to shed light upon the influence of mapping method, mating plan and inheritance model, particularly penetrance. A simplified case was examined, with the F_0 ewes free of the causative allele and unrelated to the F_0 ram. Any further assumptions were avoided. Note however that where necessary the rules of inheritance were temporarily suspended to provide an affected F_1 ram for mating option *c*. Sample size was restricted to $5 = N = 40$ because for $N > 40$ the success rate was very high across all options except at extreme values of p, the number of affected individuals. The χ^2 mapping statistics for HM, CM and GHM were calculated for the causative locus only.

For Figure 4.1 the success rate was simply aggregated across N, p combinations, with results for three mapping methods by three mating plans. The nine inheritance models are shown with continuity to illustrate the trends caused by penetrance. For dominance models (5–8) GHM was equivalent to HM, and for overdominance (model 9) GHM and HM were entirely ineffective. The trends for penetrance appear to be close to linear, brought about by the differences in aggregate genotype between affected and unaffected individuals.

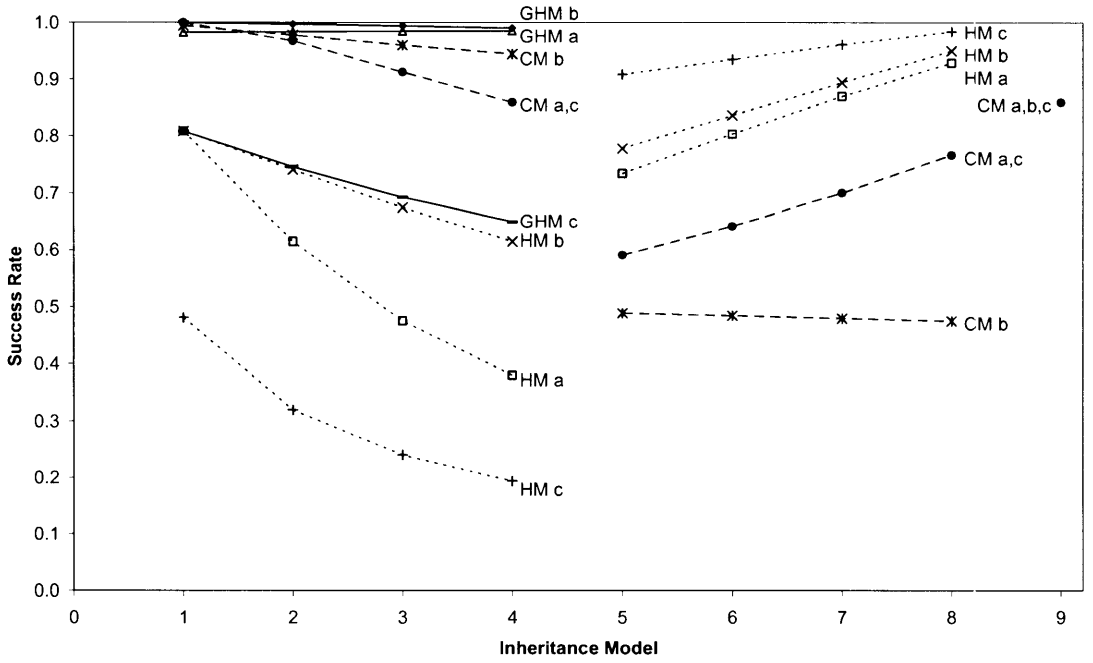


Figure 4.1 *Relative experimental power by analysis method (HM · · · · ·, CM -----, GHM ——) and mating plan (a, b, c) for the nine inheritance models showing continuity across penetrance. Deterministic results averaged across tests for sample size 5 = N = 40.*

In Figure 4.2 the same results are presented with weighting for the probability distribution of p within N; that is, the probable incidence of affected phenotypes within the sample of N F_2 individuals is taken into account. These results are appropriate when the F_2 cohort is smaller than 40 animals. Results are similar for a simple recessive model but the effect of incomplete penetrance is observed to accelerate, due to the impact upon the number of affected individuals born in the F_2.

For recessive models GHM showed the greatest power, and was the least affected by incomplete penetrance, despite the lowest expectations for p. CM is superior to HM. The effect of mating plan was smaller than mapping method and some interaction with mapping method was evident. For dominance models, HM/GHM with mating plan *a* or *c* was superior to CM. Logically, a dominant inheritance model with very low penetrance in heterozygotes approaches simple recessive inheritance. However in most cases the results show a clear discontinuity between inheritance model 8 and inheritance model 1 because different assumptions were made about sire genotypes (see Table 4.1). The result for overdominance (model 9) was unaffected by mating plan and was equal to CM for mating plan *a* or *c* under model 4 (recessive with 25% penetrance).

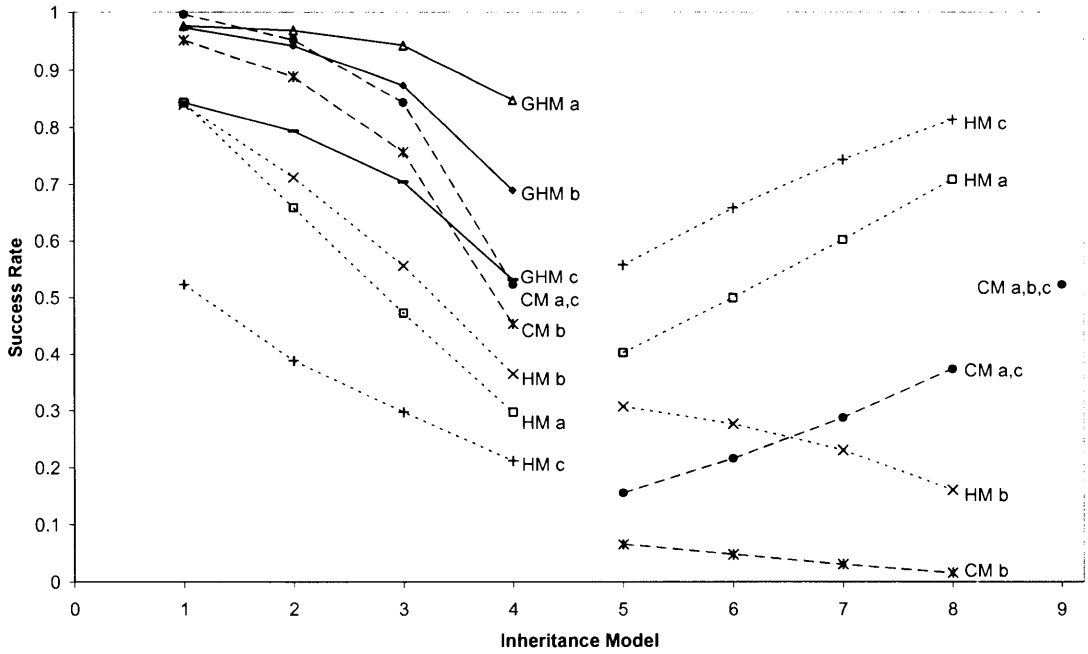


Figure 4.2 *Relative experimental power by analysis method (HM · · · · ·, CM -----, GHM ——) and mating plan (a, b, c) for the nine inheritance models showing continuity across penetrance. Deterministic results averaged across tests for sample size 5 = N = 40 and accounting for the probability distribution of p, the number of affected individuals in the sample.*

Stochastic simulation: sample size

To investigate the effect of sample size and other variables and assumptions, a simulation was used because less-simplified situations could be created more conveniently.

Clearly the number of F_2 animals available for genotyping was a major determinant of experimental power. For a marker spacing of 5cM, Figure 4.3, Figure 4.4 and Figure 4.5 show results for mating plans *a*, *b* and *c*, graphed separately for HM and CM. In each graph the experimental power is charted for each inheritance model, according to *N* the number of F_2 animals available for genotyping. Implicit within each value of *N* are the probability distributions of phenotypes and genotypes within the F_2; as explained, these distributions were sampled systematically rather than randomly.

For recessive inheritance, CM surpassed HM for all but the lowest number of animals, and across all scenarios except when the F_0 sire was related to the F_0 ewes (not shown). As seen in the deterministic results, gene discovery was clearly most efficient when penetrance was high or complete, and for CM the effect of penetrance was magnified with each step. None-the-less, moderate efficiency was achieved with CM for inheritance model 4 (recessive with 25% penetrance) and mating plan *a*, with power exceeding 90% at 40 F_2, 95% at 50 F_2 and 99% at 60 F_2 individuals. Inheritance model 9 tracked the power rate of model 4 under CM but the power of HM was very poor.

For dominant inheritance, the power of HM always exceeded CM for mating plans *a* and *c*. This was significant for intermediate numbers of animals however the power for both HM and

CM generally approached unity with high numbers. In line with deterministic results, lower penetrance slightly increased the efficiency, despite small reductions in the number of affected F_2 individuals, due to increasing distinction between affected and unaffected animals in allele frequency distribution. For mating plan *b* neither HM nor CM could be considered effective for dominance models.

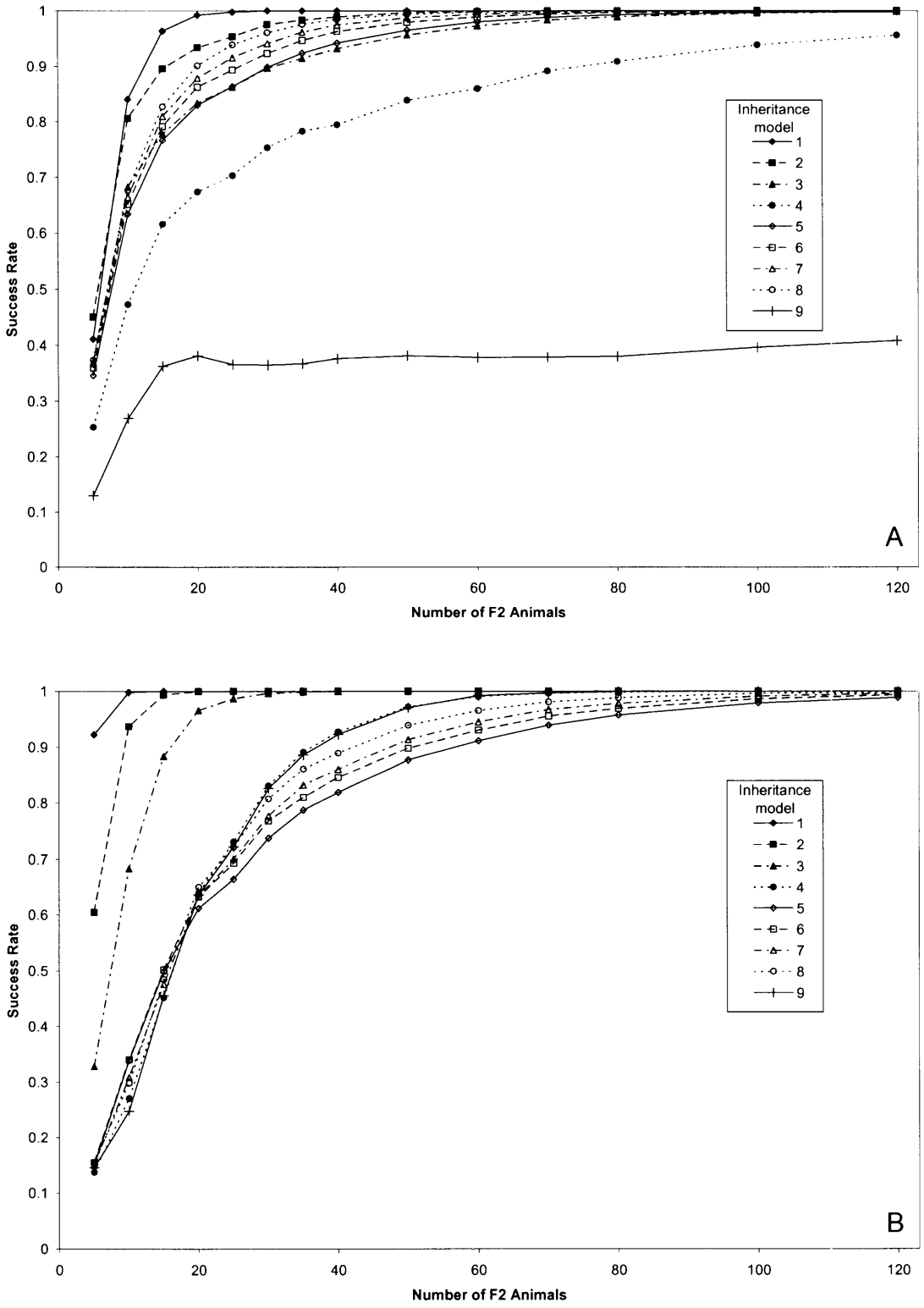


Figure 4.3 *Experimental power for nine modes of inheritance under mating plan a (backcross), against sample size, assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. Marker spacing 5cM. A. HM B. CM.*

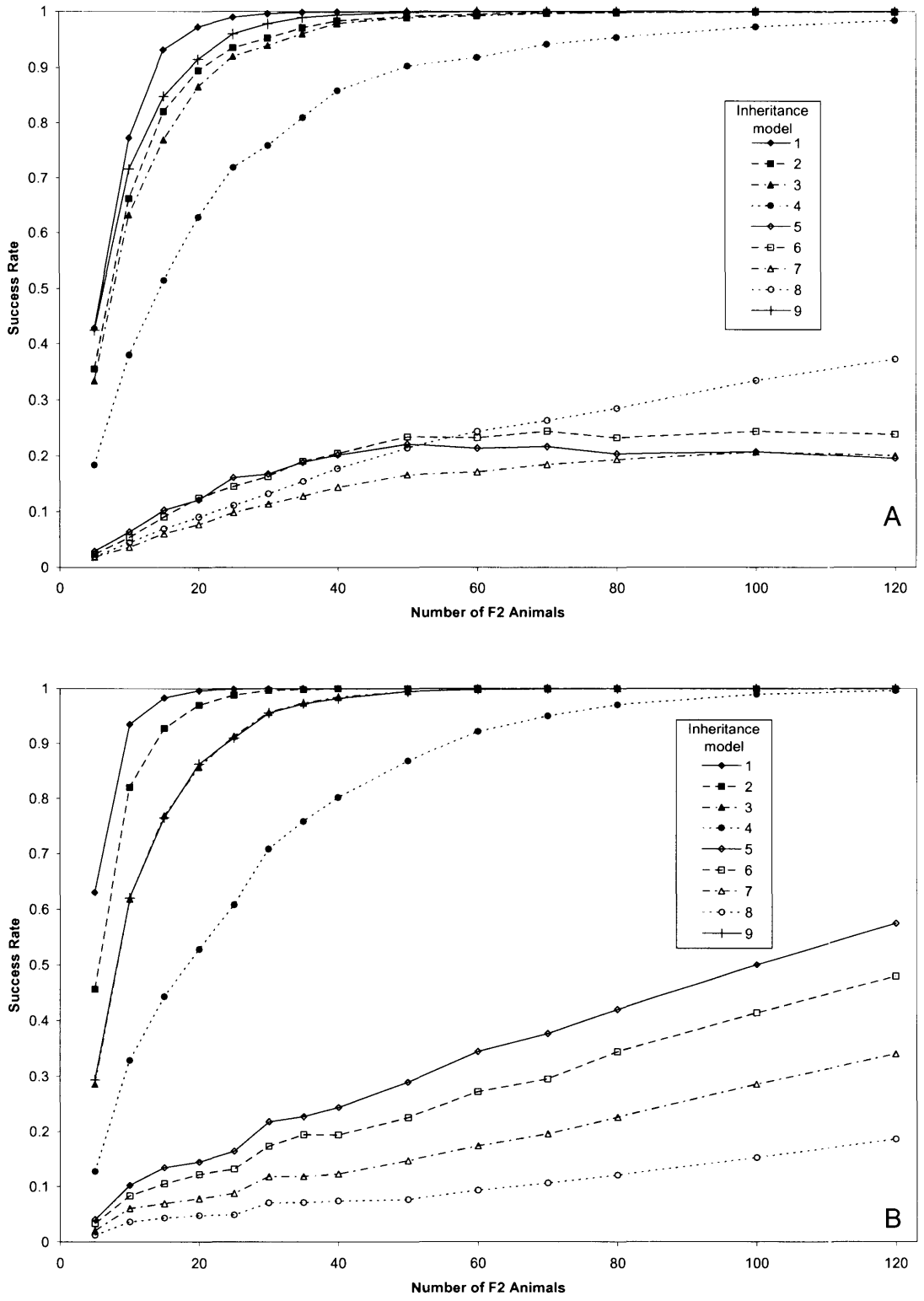


Figure 4.4 *Experimental power for nine modes of inheritance under mating plan b (unaffected F_1 sire), against sample size, assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. Marker spacing 5cM. A. HM B. CM.*

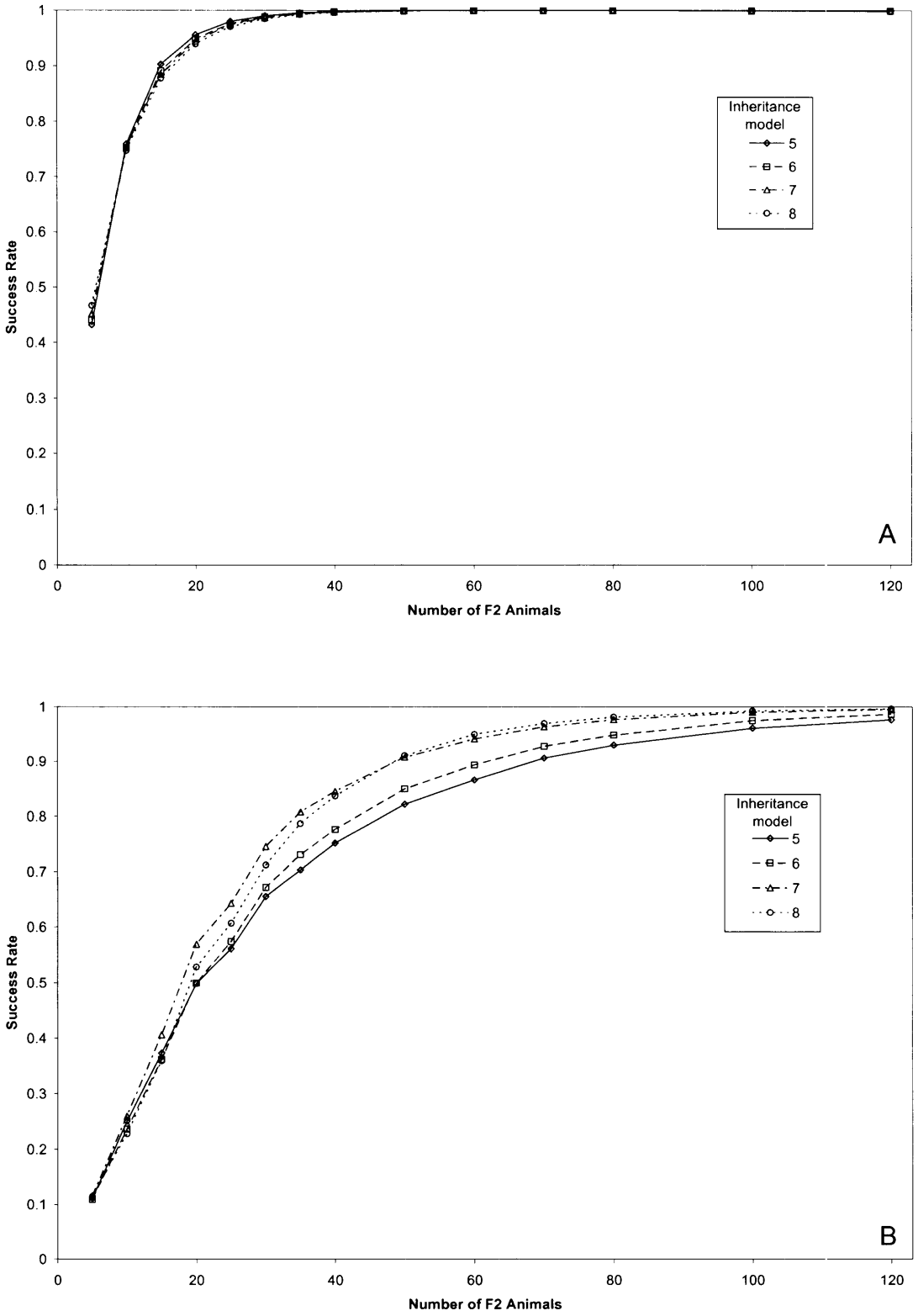


Figure 4.5 *Experimental power for four modes of inheritance under mating plan c (affected F_1 sire), against sample size, assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. Marker spacing 5cM. A. HM B. CM.*

Mating plan

For HM with mating plan *b*, power appeared to be lower for inheritance models 1 and 2 but was improved for models 3 and 4, compared to mating plan *a*. These results are consistent with the

deterministic results (Figure 4.2) when experimental error is considered. For recessive models under CM, the power was lower with mating plan *b* than mating plan *a*. As a result, for mating plan *b*, the power of HM was similar to CM for inheritance models 3 and 4. HM *b* showed higher power in the simulation compared to the deterministic method. In addition, HM for inheritance model 9 and mating plan *b* performed very well despite the deterministic prediction of failure. Both of these discrepancies were explained by recombination in the F_1 sire. For mating plans *a* and *b*, it is expected that homozygosity occurs across 0.125 of each F_2 genome, and that any marker linked to the disease locus would show no more than random homozygosity whether affected or unaffected. However a recombination close to the disease locus during the transmission between the F_0 sire and the F_1 sire, would sharply increase the probability of homozygosity in affected F_2 individuals — homozygosity which would cover a length of DNA extending from the F_1 sire recombination site in the direction away from the causative locus. The probability of homozygosity would potentially approach 1.00 if the distance between the causative locus and recombination site was very small, compared to the background 0.125 probability of homozygosity in unaffected animals. In practice, this phenomenon will create statistically significant homozygosity in all but the rare cases in which there is no recombination in the region of the disease locus. It is important to note that, in this particular circumstance, HM would identify a statistical peak across a chromosomal span delimited by the recombination in the F_1 sire closest to the causative locus, and extending in the opposite direction. This region of interest will not encompass the causative locus and may easily be 20cM or even further removed from it, potentially misleading an investigator proceeding to fine mapping.

For mating plan *b*, CM was slightly less powerful than HM for inheritance model 9. However, CM would be expected to show less variability in results because sire-line recombination has no impact. The CM results tracked those of inheritance model 3 because the expected incidence of affected genotypes and the expected distribution of sire alleles are equal. Note also that for inheritance model 9 with CM, mating plan *b* showed greater efficiency than mating plan *a* — the only inheritance model to do so. This observation was initially surprising because the contingency table cell frequencies were equal. But doubling the total observations in the contingency table gave greater statistical power of the χ^2 test as it applied to mating option *b*, when each animal was represented by two observations.

Mating plan *c* was only applicable to dominance models while the F_0 ewes did not carry any causative allele/s (Figure 4.5). For HM results were superior to mating plan *a* and *b*, and penetrance appeared to have little effect because increasing penetrance gave greater numbers of affected individuals but this was cancelled by the consequent weakening of the expected statistical significance of differences in allele frequency distribution between phenotypic groups. For CM the models showed efficiency comparable to mating plan *a*. The small trend was for power to increase as penetrance decreased, because the balance of the competing influences of penetrance had changed slightly.

It has been demonstrated (and confirmed by many other unpublished results from this study) that for dominance models, HM with mating plans *a* and *c* exceeds the power of CM. Therefore, in the following results only HM *a* and HM *c* are considered for inheritance models 5–8. Similarly, only CM is considered for inheritance models 1–4 and 9 (grouped as recessive models), with the exception that HM is also considered when the F_0 sire is related.

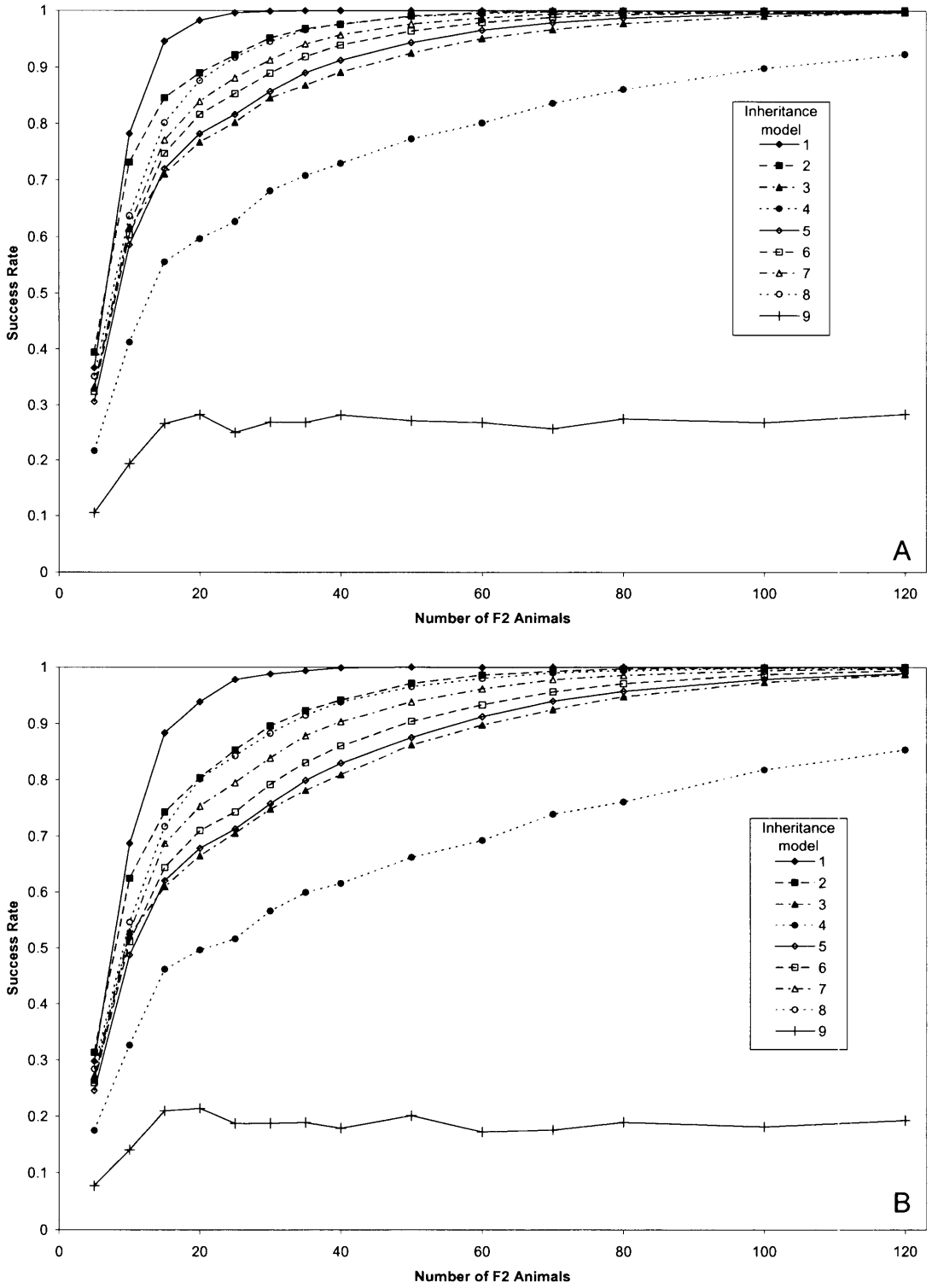


Figure 4.6 *Experimental power for nine modes of inheritance under mating plan a (backcross), against sample size, assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. HM. Marker spacing A. 10cM; B. 20cM.*

Design variables and assumptions

i.) Marker spacing. Figure 4.6 shows for HM and mating plan *a*, the effect of number of animals genotyped on experimental power for two additional marker densities, which may be compared to Figure 4.3A. In similar fashion Figure 4.7 shows results for CM at two marker spacings, comparable to Figure 4.3B.

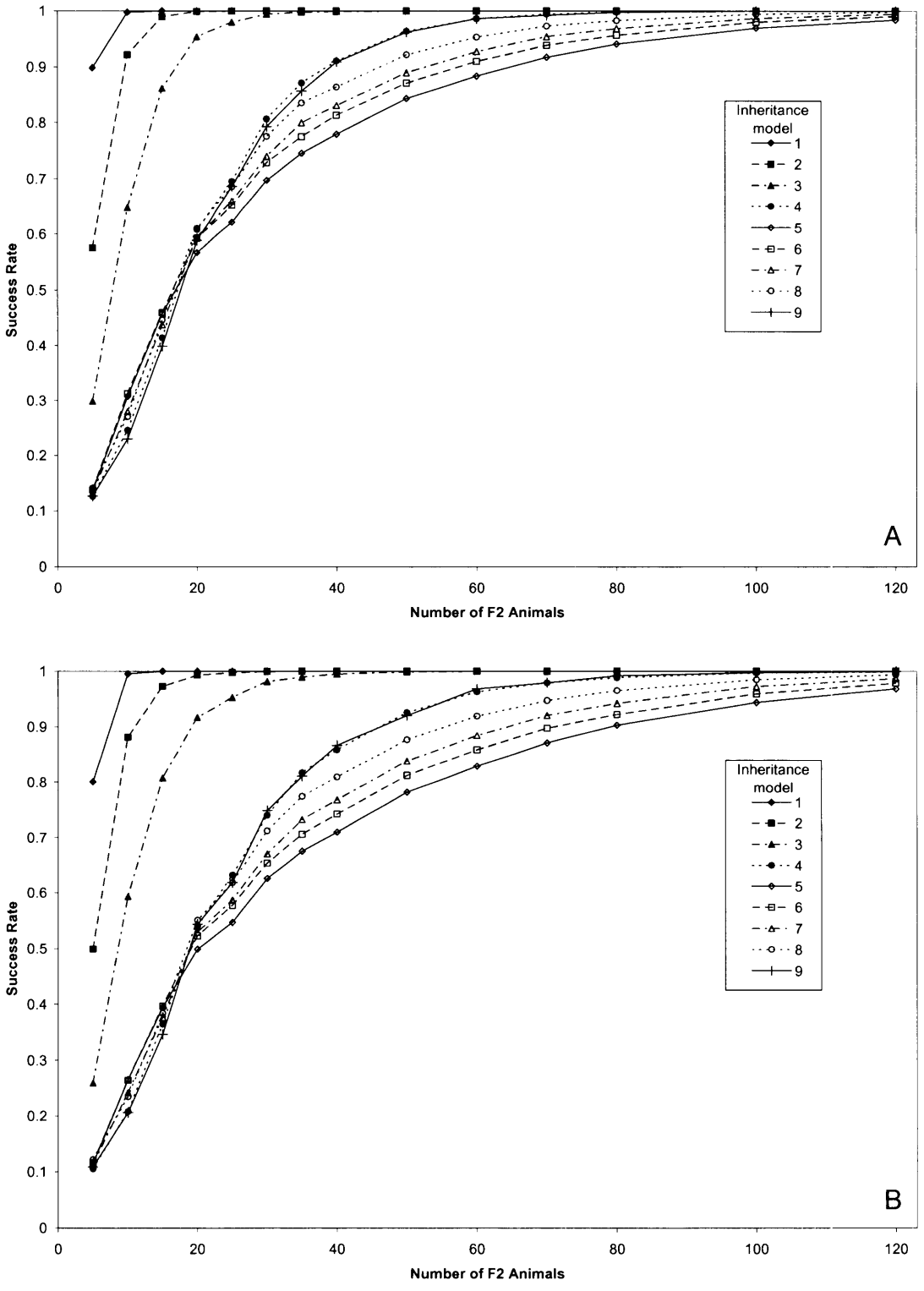


Figure 4.7 *Experimental power for nine modes of inheritance under mating plan a (backcross), against sample size, assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. CM. Marker spacing A. 10cM; B. 20cM.*

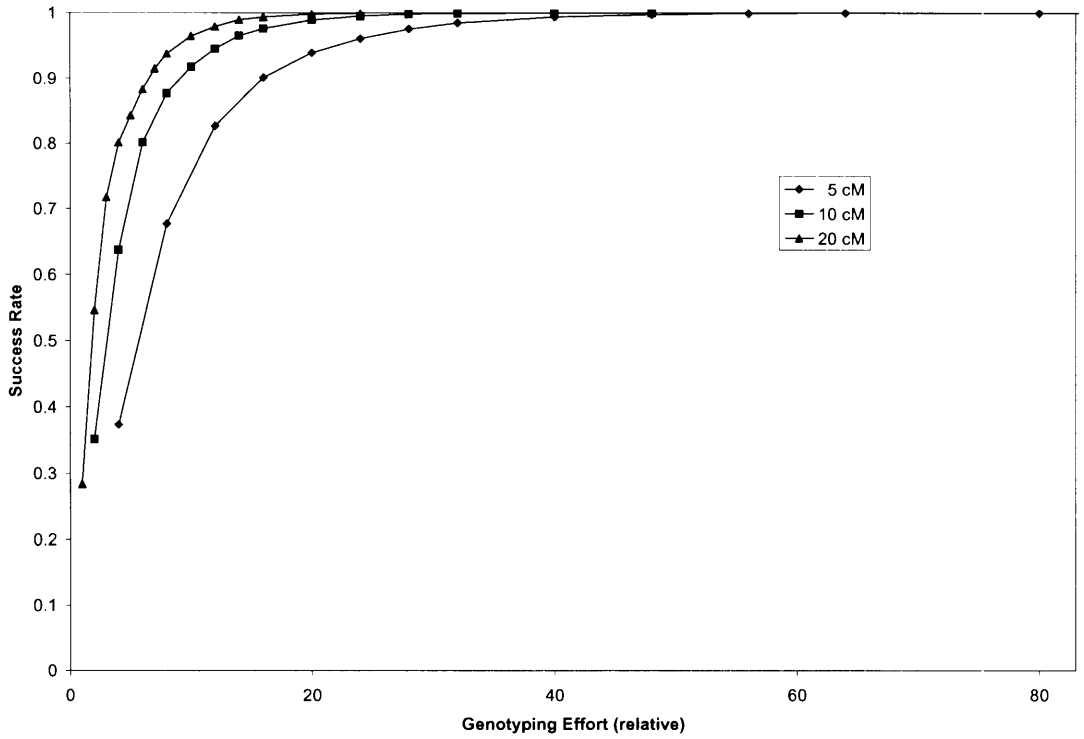


Figure 4.8 *Experimental power for inheritance model 8 under mating plan a (backcross), against relative genotyping "effort" (number of markers* x *number of individuals), assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. HM was used at three marker spacings.*

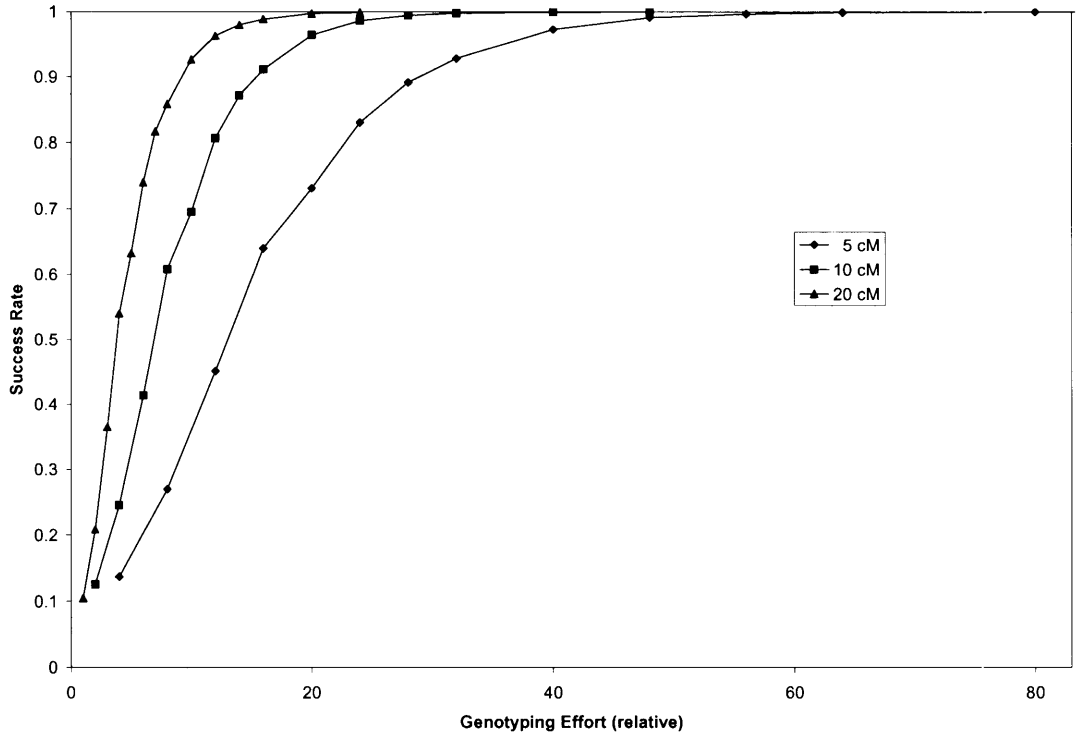


Figure 4.9 *Experimental power for inheritance model 4 under mating plan a (backcross), against relative genotyping "effort" (number of markers* x *number of individuals), assessed by stochastic simulation, accounting for the probability distribution of p, the number of affected individuals in the sample. CM was used at three marker spacings.*

As expected, each reduction in marker density reduced the power. The reductions were greatest in absolute magnitude over the mid range of animal numbers, and diminished at each end of the range. The effect of marker spacing was generally small compared to

the effect of increasing the number of animals, with implications for maximising the efficiency of the genotyping 'effort.' In Figure 4.8 and Figure 4.9 representative examples show the relationship between effort and experimental power over the three marker densities, clearly showing the typical pattern of greater return on effort when genotyping was performed on a greater number of animals at reduced marker densities.

ii.) Relatedness. The effect of sire relatedness was complex. Under recessive models, having an F_0 sire unrelated to the F_0 ewes was, in aggregate, a modest advantage for CM (0.81 vs 0.79). Under dominant models, an unrelated sire was, in aggregate, a disadvantage for HM (0.78 vs 0.82). However, in both cases there were similar interactions at play. As was seen in Figure 4.10, the ewe allele frequency was a factor. Marker spacing was also a player because an unrelated sire was more valuable as the marker spacing increased, due to the lower likelihood of conserving marker associations over greater map distances (Figure 4.11). In addition, for CM and recessive inheritance, the benefit of an unrelated F_0 sire increased as penetrance decreased.

iii.) Ewe carriers. The presence of disease alleles in the historic ewe population had no systematic effect on the power of CM for recessive inheritance models. For HM also, the historic gene presence was neutral for dominant models. These effects were tested independently from the transmission of disease alleles from F_0 ewes to F_1 ewes to F_2 offspring, so would have been due to the effects of selection in the base population causing linkage disequilibrium between the disease locus and markers.

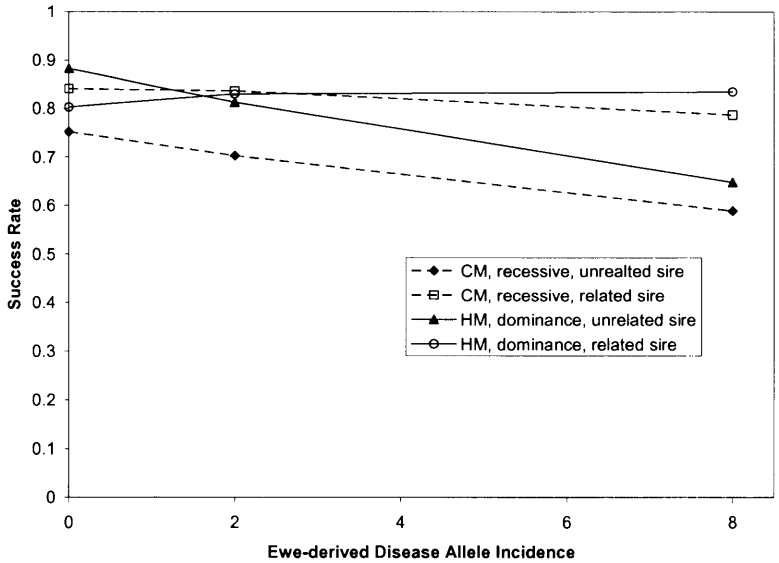


Figure 4.10 *Experimental power: interaction between sire relatedness and ewe gene frequency. Showing HM results for dominant inheritance (models 5–8) and CM results for recessive inheritance (models 1–4), aggregated across all other factors.*

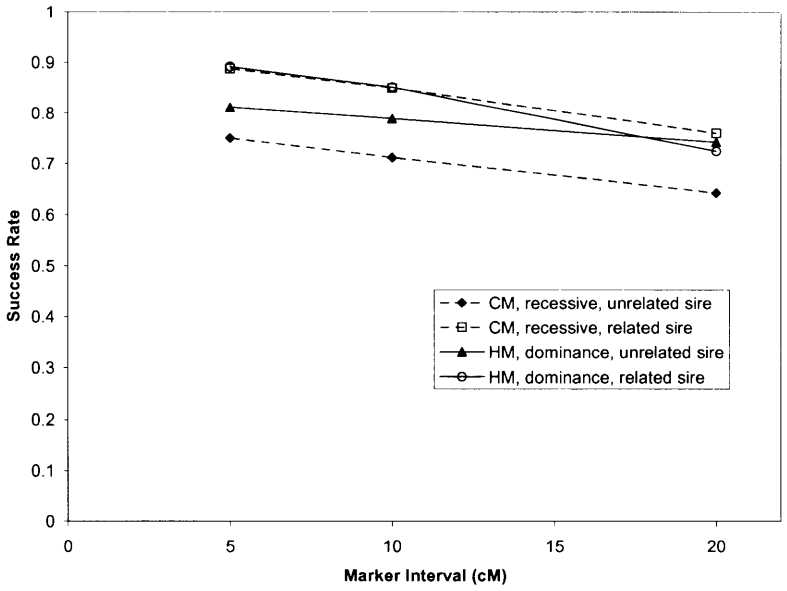


Figure 4.11 *Experimental power: interaction between sire relatedness and marker density. Showing HM results for dominant inheritance (models 5–8) and CM results for recessive inheritance (models 1–4), aggregated across all other factors.*

The presence of ewe-derived disease alleles in the sampled F_2 was systematically deleterious across all inheritance models tested (models 2–8) and both analysis methods, although the negative effect was tempered by the advantage due to the greater number of affected individuals in the F_2 sample, especially when a related F_0 sire was used; ewe-derived disease alleles were likely to be associated with the same marker alleles as those from a related F_0 sire. Figure 4.10 shows that these effects on power were approximately linear for the number of ewe-derived disease alleles in the F_2.

iv.) Population metrics. Across all base populations the increase in inbreeding over time was approximately linear, averaging 0.25 after 10 generations and 0.46 after 20 generations. The proportion of homozygous marker genotypes was similar and averaged 0.253 after 10 generations and 0.454 after 20 generations. After 20 generations the average number of alleles per marker was 8.6, giving a mean marker allele frequency of 0.116. The median marker allele frequency was 0.095. At 0.007 of markers, one allele was fixed, while 0.027 of markers had one allele frequency exceeding 0.98. At the other extreme, 0.186 of all marker alleles had a frequency below 0.02. None of these indicators showed any discernible difference between populations which did and did not carry the disease allele and thus were apparently unaffected by phenotypic culling.

Among populations carrying the causative allele/s (and including those populations where the allele was lost), the allele frequency commenced at less than 0.005 (one homozygote in 205 founder animals). Spreading from the initial carrier ram with no chance of affected offspring in the first generation, allele frequency naturally increased in all populations to average around 0.12 after 2–3 generations. In all cases the average

frequency then dropped, according to the effectiveness of phenotypic culling under each inheritance model, and Table 4.2 shows the average frequency after 10 and 20 generations.

Table 4.2 *Average frequency of disease alleles by inheritance model after 10 generations (40 years) and 20 generations (80 years).*

Inheritance Model	Allele frequency after 10 generations	Allele frequency after 20 generations
1	0.066	0.029
2	0.066	0.035
3	0.073	0.038
4	0.080	0.060
5	0.026	0.004
6	0.031	0.006
7	0.036	0.008
8	0.050	0.016
9 — maternal allele	0.046	0.007
9 — paternal allele	0.034	0.004

As an indicator of inbreeding, the average levels of marker homozygosity amongst affected (culled) individuals were greater than the population averages for inheritance models 1–8, particularly in the early years but still discernible at generation 20. This effect was strongest for simple recessive inheritance (model 1) and was observed to decline as models departed from simple recessive, i.e. through models 2, 3 and 4, and through models 8, 7, 6 and 5. For inheritance model 1, average marker homozygosity of affected individuals after 10 generations was 0.292 and after 20 generations was 0.476. Affected individuals were also much more likely to be homozygous at a single marker 2cM from the disease locus, 0.82 after 10 generations and 0.89 after 20 generations. Inbreeding followed similar trends to general homozygosity but, due to the small number of individuals involved, the means were less reliable. Overall, the number of affected individuals was so small that, as noted, culling had no effect on general population metrics, however the effect on marker homozygosity adjacent to the disease locus was not tested.

The length of the flock establishment phase (10 or 20 generations) had no effect when the F_0 sire was unrelated. For a related sire there was a small benefit across all options for a base population run for 10 generations compared to 20 generations. This was because after a longer period of closed breeding, all of the individuals were more closely related. This effect increased with marker spacing because of the greater importance of the informativeness of each marker.

4.4 Discussion

Homozygosity mapping, having been widely used to map recessive conditions in humans, remains a largely untried resource with respect to livestock species. GHM (Genuine HM, with an unaffected carrier patriarch) was the most successful method for recessive inheritance,

unsurprisingly. However this study demonstrates the broad potential of HM analysis, even when used outside of its conventional parameters for pedigree arrangement and inheritance. An affected patriarch gave very adequate experimental power in many cases, though lower than expected from an unaffected patriarch (Lander and Botstein 1987). Using an affected patriarch is likely to be preferable in many experimental settings, because the only certain way to obtain an unaffected carrier may be by deliberate breeding — requiring an extra generation. However this form of HM has the major drawback that pooling of samples for genotyping is no longer feasible. HM was also found to be effective when the inheritance was dominant with incomplete penetrance — it was the most powerful method for this inheritance.

Co-inheritance mapping was conceived as a derivative of HM. Conceptually, CM is a species within the HM genus and using a distinct nomenclature was merely a matter of convenience for this report. CM maximised the statistical discrimination between affected and unaffected progeny groups of various analyses tested. Making use of an affected patriarch, CM will be applicable for the discovery of many livestock genes. The power of CM exceeded HM for recessive inheritance in most cases, but HM was equal to slightly better only when the F_0 sire and F_0 ewes were very closely related (flock mates). CM was also applicable for co-dominance with imprinting, as was HM with mating plan *b*.

Importantly, the use of pooled samples remains an option with CM to improve dramatically the economic efficiency of genotyping. Rather than looking for a single allele, this test would look for the presence of only two alleles in the pooled sample — those passed down by the F_0 sire. Although this was not tested, any loss of power will be small if a quantitative method is used — silver staining (Jamieson *et al.* 1999) or fluorescent tagging (Winick *et al.* 1999). By contrast, DNA pooling would be ineffective for HM except for markers where the F_0 sire is homozygous — this might however be a viable strategy with dense SNP genotypes.

The choice of sire for the F_2 mating is a key design factor because mating plans must be devised and executed before any useful genotype data can be collected. However there were no clear-cut results for mating option across the various other factors. For dominant modes of inheritance mating with HM, plan *c* (affected F_1 sire) was clearly superior to mating plan *a* (backcross) while mating plan *b* (unaffected F_1 ram) gave virtually no power. For recessive inheritance models with CM, all three mating plans gave effectively equal results. Mating plan *b* was clearly the best option for inheritance model 9, with mating plans *a* and *c* almost equal. On this basis, when the pattern of inheritance is uncertain the safest choice may well be mating plan *c*, accepting that this would give suboptimal results for an overdominance imprinting model. This was confirmed by the deterministic results which suggested that mating plan *c* is likely to give high experimental power across the models tested.

Using an unrelated F_0 sire was often advantageous and allowed for a greater efficiency of gene discovery when the genotyping effort is spread across a greater number of animals at a lower

marker density for the initial genome scan. In these cases the optimum efficiency appeared to be obtained at marker spacings beyond 20cM, while for a related F_0 sire the optimum marker spacing appeared to be in the range of 10 to 20cM. These conclusions are broadly in line with an interpretation of Lander and Botstein (1987), that for close inbreeding (and a design equivalent to GHM) the genotyping effort was minimised at a marker spacing of about 20cM. Clearly the total number of animals available provides a logical limit to improving efficiency by using a wider marker spacing on a greater number of animals.

The ability to type a large panel of SNP markers by SNP chip may make the issue of efficiency and marker density seem less important. However, conservatively equating the information content of one microsatellite marker to five SNP markers, the 736 microsatellite markers (5cM spacing) would equate to a panel of 3680 SNP markers, uniformly spaced. Similarly, 1840 SNP markers correspond to the 10cM microsatellite spacing, and 920 SNP markers to the 20cM spacing. There may well be circumstances where dealing with the smaller number of SNP markers is a cost saving or otherwise more efficient. Importantly, increasing the number of animals sampled would give greater experimental power than increased marker density for a fixed genotyping effort — within the ranges of sample size and marker density considered here.

Ensuring that a sufficient number of F_2 animals will be available is a vital part of the mating design. Clearly the answer depends on a number of factors but the results presented should provide guidance. Achieving an insufficient number of F_2 animals is likely to result in lengthy and expensive delays so a conservative approach would tend to allow an excess or safety margin for lower than expected reproductive success. A greater number of F_2 animals available obviously increases the opportunity to raise the efficiency of genotyping by reducing marker density, as described above.

The degree of penetrance in the inheritance of recessive characters was a major factor in experimental power. Penetrance is therefore a vital consideration in determining the number of F_2 individuals required for gene discovery.

An additional model of inheritance not specifically tested involves the impression of incomplete penetrance being caused by several independent recessive conditions, the affected individuals of each giving indistinguishable phenotypes. If this were the case then the mapping methods would proceed normally as for a simple recessive condition, for the locus at play in the F_0 sire. There might be a small loss of efficiency due to the presence at low frequency of disease alleles from the other loci in the population. The other loci would remain undetected and the discovered gene would only partially account for affected genotypes in the general population.

A special note is warranted for HM under mating plan *b* on recessive inheritance (models 1–4 and 9). As outlined, the experimental power was good but the causative locus may fall outside the zone of the statistical peak, even after saturation marker coverage in the region.

Experimenters should be alert to this possibility and cast a wider net when progressing to candidate genes, for example.

Some of the assumptions underlying the simulation should be borne in mind. First, the unrelated sire was genotypically distinct for both alleles at every marker, and this was clearly unrealistic. However this situation demonstrates the maximum benefit which can be gained from a distantly-related sire; in an intermediate situation the trend displayed between the two extremes of sire relatedness should still hold and could be used to predict informativeness. Under CM, any overlap between marker alleles inherited from the F_0 sire and F_0 ewes is effectively adjusted for, by contrasting the frequency of sire alleles between affected and unaffected F_2 groups. This self-correction occurs to a much more limited extent for HM. Alternatively, where the F_0 sire and F_0 ewes are drawn from a population to which the gene of interest has been recently introgressed (whether deliberately or otherwise) superior results may be obtained for recessive characters.

Second, the regime used for sampling from the F_2 offspring has some limitations. The main weakness of the sampling approach was that while thorough and randomised sampling occurred among the F_2, for each scenario only a single F_0 and single F_1 sire were sampled. The sire genotype at the disease locus was generally controlled (see Table 4.1) but in a number of scenarios the genotype at surrounding markers was critical to the power of gene discovery. An alternative approach involving replication of the entire scenario would be computationally expensive but could be expected to provide more accurate estimates of the treatment effects. Related to sampling, the results were taken for a fixed set of sample sizes and the proportion of affected progeny determined according to probability. In reality these values would be subject to a certain degree of optimisation at the sampling stage.

Third, the causative locus was always positioned mid-point between markers, so in that respect the results represent the minimum experimental power. On the other hand, the locus was not located near the tail of a chromosome, which would cause a decrease in power.

Fourth, the criteria for gene discovery applied here was that a statistically significant χ^2 result was obtained at one or more markers within 42cM of the disease locus. This distance was set arbitrarily and may seem large and the significance threshold may seem low for a whole-genome scan, resulting in an overestimation of experimental power. Conversely, the use of point-wise tests will have lowered the estimates of experimental power compared to multi-point tests. However, the relativity of results would not change, and the primary emphasis is on an initial genome scan ahead of accuracy of location.

It is worth reiterating that this investigation was designed to consider the circumstance of an initial genome scan for gene discovery. Clearly this initial sweep would be followed up by more detailed examination of areas of interest. Therefore, the methods presented are not

intended to be the final word in precise gene location; nor do they provide much protection against false-positive results. However, it is desirable that the causative locus is in the neighbourhood of one of the identified areas of interest. With these factors in mind, several possible enhancements or extensions have not been included, but will rather be left to the later consideration of selected chromosomal regions: for example, maternal genotyping, working with haplotypes, detecting linkage disequilibrium and/or calculating formal LOD scores to assess the statistical significance of linkage. These and other details may be relevant to fine mapping which could extract the maximum information from the F_2 and subsequently utilise unpedigreed affected individuals from the general population; there should be no further requirement for planned matings.

4.5 References

Bradley, D.R., Bradley, T.D., McGrath, S.G. and Cutcomb S.D. (1979). Type I error rate of the Chi-square test of independence in *R* x *C* tables that have small expected frequencies. *Psychological Bulletin* **86**: 1290–1297.

Brash, L.D., van der Werf, J.H.J, Li, Y. and Norris, B.J. (2005). Australian Piebald: incomplete penetrance or incomplete picture? *Proceedings of the Association for the Advancement of Animal Breeding and Genetics* **16**: 188–191.

Brooker, M.G. and Dolling, C.H.S. (1969). Pigmentation of sheep III. Piebald pattern in Merinos. *Australian Journal of Agricultural Research* **20**: 523–532.

Jamieson, C.R., Govaerts, C. and Abramowicz, M.J. (1999). Primary autosomal recessive microcephaly: Homozygosity mapping of MCPH4 to chromosome 15. *American Journal of Human Genetics* **65**: 1465–1469.

Lander, E.S. and Botstein, D. (1987). Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* **236**: 1567–1570.

Maddox, J.F. and Cockett, N.E. (2007). An update on sheep and goat linkage maps and other genomic resources. *Small Ruminant Research* **70**: 4–20.

Winick, J.D., Blundell, M.L., Galke, B.L., Salam, A.A., Leal, S.M. and Karayiorgou, M. (1999). Homozygosity mapping of the achromatopsia locus in the pingelapese. *American Journal of Human Genetics* **64**: 1679–1685.

5. Selection strategies to eliminate pigmentation genes from Merino flocks

5.1 Introduction

Recessively-inherited pigmentation faults impose biological and economic inefficiencies upon Australian Merino flocks. Pigmentation genes persist despite decades of phenotypic culling — a well-established characteristic of recessive traits (Falconer 1985). Implementation of more intensive selection by parental culling has been limited. Sire progeny testing could be effective but is expensive and has practical downsides: the requirement for a large number of white ewes (of unknown genotype), or the maintenance of a special flock of pigmented or carrier ewes; and the unavailability of young rams until they have graduated from the progeny test.

Current research is underway by the CRC for Sheep Industry Innovation and SheepGenomics to locate genes responsible for pigmentation in Australian Merino sheep, particularly for the symmetrical forms of pigmentation. If successful, this work will make diagnostic gene tests available to breeders at reasonable prices. The impact of test availability is difficult to predict and there are only a few precedents relating to undesirable recessive characters of livestock. In the dairy industry, the introduction of DNA tests for the BLAD congenital defect of the Holstein-Friesian breed did not appear to have a dramatic effect and trade in semen from high-index carrier AI bulls continued. However the highly centralised nature of dairy cattle breeding, the expense of progeny-testing and the shortage of direct selection criteria for bulls have in combination led to the elimination of BLAD carriers from young sire cohorts prior to progeny testing.

Merino breeders' individual responses to DNA gene tests for pigmentation may well range from strong aversion to pragmatic tolerance with respect to carrier rams, which will flow through to their demand for the conduct of testing, and the premiums and discounts which may apply to the resulting classes of seedstock (i.e. carrier/non-carrier/untested). Seedstock breeders in turn will face both threats and opportunities: a decision not to test rams may limit marketability but on the other hand an investment in testing may not be recouped; for the first time however, a breeder's attention to removing pigmentation carriers from their flock may be directly rewarded. As is the case with many other animal attributes, the ability to predict and assess the future ram market will remain a key skill of the ram breeder.

Various selection strategies could be applied to reducing or eliminating pigmentation alleles in a ram-breeding flock. For example, using existing technology only or enlisting the power of DNA gene testing; DNA testing rams or both rams and ewes; rapid elimination of pigmentation alleles or a more gradual process to reduce impact on the remainder of the

breeding objective. By assessing the practical and economic consequences of a range of options this chapter aims to provide a scientific basis for guidance to breeders on these points. This chapter will test the hypothesis that through careful choice of strategies, selection for gene elimination can be cost-effective.

5.2 Materials and methods

An Australian Merino ram-breeding flock was modelled by computer simulation. The flock consisted of 1000 breeding ewes mated to 24 rams. Annual reproduction rate to weaning averaged 0.8, 1.0 or 1.2. Adults ewes suffered annual mortality of 0.05 but due to the small numbers, adult ram mortality was ignored. Ewes were in 5 age groups aged 2 to 6 years at lambing. Rams were first bred at 2 years of age and were bred for either 2 years or 4 years.

An inherited form of fleece pigmentation was carried by the flock, with an initial phenotypic frequency in lambs of 0, 0.005, 0.01 or 0.05. Pigmented phenotypes were culled at meat value of $40. Breeding rams were sold for $800, with a 5% premium where tested and free of pigmentation alleles. It was assumed that there was no market for breeding rams tested and found to be carrying pigmentation alleles. Surplus ewes were sold for breeding at $100, again with the addition of a 5% premium if they were known to be free of pigmentation alleles. Tested carrier ewe hoggets were saleable but at a 15% penalty (i.e. for $85). Surplus males were sold as wethers for $70 regardless of carrier status.

Conventional selection strategies were restricted to technology that is presently available, including the constraint that no pedigree records were kept in the flock. Phenotypic culling was exercised universally, and in a subset of simulations, parental culling also operated:

i. DNA fingerprinting used to identify the sire for culling
ii. As in i plus the dam identified by mothering-up and culled
iii. As in i plus the dam identified by mothering-up and confirmed by DNA fingerprinting before culling.

In all applications, mothering-up was assumed to be successful on 0.9 of occasions and accurate on 0.8 of occasions. DNA fingerprinting for parentage identification cost $20 per animal once-off. Where parents of pigmented lambs were identified and were aged younger than the maximum breeding age, they were culled from the breeding flock and sold for $20; a replacement was drawn from the next cohort of hoggets.

Under DNA gene-testing strategies, a single test for all pigmentation alleles cost $30 per animal. Animals were gene tested just prior to selection for breeding, after any other performance measurements had been made — although in practice this sequence might be reversed depending on the relative cost of performance testing and other practicalities. All rams were tested from those born at time zero, and thereafter only non-carriers could be used or sold for breeding.

Ewe DNA gene testing commenced at a (variable) later time, the earliest ewe testing being on the first progeny of DNA gene-tested rams. DNA gene testing for ewes, when applied, was performed on each cohort of selected hoggets prior to breeding. If numbers allowed then all carriers would be excluded, but if not, heterozygous carriers could be selected to maintain flock size. Three levels of constraint were imposed upon any relaxation in selection for the stud's breeding objective to allow for culling of pigmentation carrier ewes: in addition to the normal selection quota, breeding ewes could be drawn from the next 10% of the cohort, the next 20% of the cohort, or the entire cohort. Where pigmented lambs were born to untested ewes (possible under one inheritance model — see below) mothering-up was performed to identify the dam, with confirmation by gene test resulting in culling.

Where ewes of different gene testing status (untested, carrier, non-carrier) were present in the flock simultaneously, it was assumed that the status was permanently recorded and that the status groups were segregated for lambing. Therefore, the ewe progeny of tested non-carrier parents would not themselves require gene testing. Once the undesired alleles were known to have been eliminated from the flock, ewe testing ceased.

The available selection intensity was calculated each year for stud rams, stud ewes and sale rams, relative to what would have applied if there were no pigmentation genes in the flock but equal reproductive success. Actual selection was at random, aside from the treatment of pigmentation genotypes and phenotypes as described above. This approach assumed that there was no correlation between pigmentation genotype and the remainder of the breeding objective. The results for loss of selection intensity could then be put in a practical context with knowledge of the heritability and economic value of any given breeding objective — factors which vary widely in the real world. The heritability of the underlying breeding objective and the selection intensity applied in a flock (a function of reproduction rate and flock age structure) would also affect the selection pressure on undesirable alleles through the actual female generation interval. This effect was modelled deterministically.

Beyond selection intensity, no assumption was made about genetic progress in the flock because industry practice ranges from a high emphasis on measured performance combined in a metric index to pursue a formalised breeding objective, though to almost total reliance upon visual assessment, with subjective combination and a vague objective. This approach meant that it was not feasible to consider a combined index including pigmentation status with the other parts of the breeding objective — which would normally give best results.

Six inheritance models for pigmentation were trialled:

a. Simple recessive
b. Recessive with 0.25 penetrance
c. Dominant with 0.07 penetrance in heterozygotes
d. Overdominance with imprinting (as described below)

e. Four unlinked, independent simple recessive loci

f. Two unlinked loci, one simple recessive and the other recessive with 0.25 penetrance.

The first inheritance model represents the assumed situation for symmetrical (*Agouti*) pigmentation patterns (Parsons *et al.* 1999). Models b, c, d and e represent four models which may apply to the inheritance of *Australian Piebald* (Brooker and Dolling 1969). Note that under model d, there were maternal, paternal and wild-type alleles; a pigmented lamb must have inherited both maternal and paternal alleles from the corresponding parents. Model f represents simultaneous selection against agouti and piebald pigmentation, replicating inheritance models a and b.

While the initial prevalence of undesirable alleles was controlled through the rates of phenotypic incidence, the underlying allele frequency was an important factor, and this was a product of the inheritance model. Table 5.1 shows the initial frequency of undesirable alleles (combined frequency where there were two or more undesirable alleles) for each scenario.

Table 5.1 *Initial pooled frequency of all pigmentation alleles according to the inheritance model and the three levels of initial phenotypic incidence.*

	Initial phenotypic incidence		
Inheritance model	**0.005**	**0.010**	**0.050**
a	0.0717	0.1001	0.2236
b	0.1414	0.2002	0.4475
c	0.0306	0.0540	0.1735
d	0.1425	0.2002	0.4472
e	0.1533	0.2190	0.4353
f		0.2131	

Under one option the flock was selling a large number of breeding rams which meant that culling imposed a cost through the reduction in rams available for sale, but also that the costs of eliminating pigmentation alleles could be offset by the premium available for tested rams. In another option the flock was only breeding its own replacement rams, so there was no positive or negative impact on revenue from ram sales. This scenario would have similar outcomes to any flock not selling rams — whether rams were entirely home-bred, entirely purchased, or some combination of the two — provided always that equivalent selection strategies were implemented in the other flock/s supplying rams.

Sale rams usually comprised the next 50% of the ram hogget cohort, after stud ram selection and excluding known carriers. However additional sale rams could be selected from lower-ranked animals to maintain the number of sale rams at 40% of the total cohort, provided that sale rams could never be selected from the lowest 25% of the cohort. It would be unrealistic to think that this situation adequately reflects year-to-year variation in the volume of ram sales from a stud in the real world, despite the complexity. However it does allow for economic impacts due to pigmented animals and as a result of culling carriers, while avoiding the worst extremes.

In the flock breeding only replacement rams, all gene testing ceased once the pigmentation alleles were known to be eliminated from the flock. However, in the ram-selling flock it was assumed that all sale rams were DNA gene tested, to assure the ram buyers, along with all stud rams as a precaution.

The economics of each set of selection strategies was assessed over 40 years of breeding, relative to a flock with equal reproductive success but lacking pigmentation genes. This benchmark did not represent an alternative scheme but rather was intended to be a fixed point of reference to which the various strategies and scenarios could all be compared. Revenue would vary due to the number and value of rams sold, the number of surplus ewes sold including premium or penalty for pigmentation genotype, wethers sold, and the culling of pigmented phenotypes and adult carriers. The costs of DNA tests for pigmentation genes and DNA fingerprinting were deducted. Financials were accumulated at (undiscounted) cash value and separately as net present value with an annual discount rate of 7% (NSW Treasury 2007). All results presented are the average of 100 replicates, excepting Figure 5.1 which averages 1000 replicates.

5.3 Results

Phenotypic culling

A summary of the results of phenotypic culling is shown in Table 5.2. Where the flock was breeding only its own rams, the only cost was due to phenotypic culling. But for a flock selling rams the cost due to loss of candidate sale rams predominated, consistently four times the magnitude of the culling costs.

In all cases the frequency of pigmentation alleles decreased under the "conventional" culling scenarios. The inheritance model influenced the impact of phenotypic culling with inheritance models a and c providing the best results. The initial incidence rate was also important. From an initial incidence of 0.05, phenotypic culling led to an average 81% reduction in incidence averaged over years 36 to 40. This reduction was 62% from an initial incidence of 0.01, and only 50% from an initial incidence of 0.005. Therefore the impact of phenotypic culling on allele frequency was diminished as allele frequency reduced, but the exact trend differed between inheritance models; for inheritance model e the reduction in incidence was only 21% from an initial incidence of 0.005.

The loss of selection intensity was simply the sum of annual realised selection intensities subtracted from the sum of annual potential selection intensities. Dividing this number by the sex-averaged generation interval would give the loss of genetic progress in standard deviation units. The loss of selection intensity was not entirely negligible, but summed over the 40 years of breeding was at greatest 0.38 of one year's annual average selection intensity of 1.62. The loss of selection intensity was strongly affected by initial incidence, and also by inheritance

Table 5.2 *The practical and financial consequences of a 40 year program of phenotypic culling on inherited pigmentation faults, according to the initial incidence and inheritance model. Relative to a flock having equal reproductive success without pigmentation alleles. Reproduction rate was 1.00 and there were two ram age groups.*

Initial incidence	Inheritance model	Cost due to phenotypic culling [AB] $	Cost due to loss of ram sales [B] $	Net result[A] $	Net result[B] $	Final incidence	Loss of selection intensity
0.005	a	5 775	23 039	-5 775	-28 814	0.0017	0.07
0.005	b	7 194	28 244	-7 194	-35 438	0.0030	0.09
0.005	c	4 601	18 265	-4 601	-22 866	0.0014	0.06
0.005	d	6 967	27 733	-6 967	-34 700	0.0025	0.09
0.005	e	9 196	36 639	-9 196	-45 834	0.0043	0.12
0.010	a	9 174	36 697	-9 174	-45 871	0.0027	0.12
0.010	b	12 670	49 888	-12 670	-62 558	0.0045	0.15
0.010	c	8 414	33 492	-8 414	-41 906	0.0023	0.10
0.010	d	11 366	45 720	-11 366	-57 086	0.0033	0.14
0.010	e	15 695	63 006	-15 695	-78 702	0.0061	0.19
0.010	f	13 005	51 728	-13 005	-64 733	0.0060	0.15
0.050	a	30 611	124 538	-30 611	-155 149	0.0048	0.35
0.050	b	50 151	200 487	-50 151	-250 639	0.0144	0.62
0.050	c	29 840	121 443	-29 840	-151 283	0.0059	0.35
0.050	d	48 296	194 333	-48 296	-242 629	0.0118	0.57
0.050	e	45 023	181 485	-45 023	-226 508	0.0110	0.52

[A] Items affecting a flock breeding only its own rams.
[B] Items affecting a flock breeding sale rams.

models: the models which gave the greatest reduction in phenotypic frequency suffered the smallest loss of selection intensity (correlation 0.95). The additional accumulated loss of selection intensity for sale rams was very small — less than one fifth of the loss within the flock (results not shown).

The results were highly variable between replicates. While on average the allele frequency declines under culling, it can swing wildly in individual cases, affecting the cost associated with culling and the loss of selection intensity. The variability increased with the initial incidence but the coefficient of variation decreased.

Reproductive rate (results not shown) did not influence the impact of phenotypic culling on allele frequency. The effect of reproduction rate upon the costs presented in Table 5.2 was simply due to scale. At a higher reproduction rate culling had a greater absolute impact on selection differentials, from higher base values.

Changing the ram age structure from two age groups to four age groups extended the generation interval and as a result reduced the rate of decrease of pigmentation allele frequency across all scenarios. Costs were also increased, and the loss of selection intensity was slightly greater.

Note that for the phenotypic culling programme represented in Table 5.2, the results for inheritance model f with an initial incidence of 0.010 were in every way almost exactly the sum of its two constituents, that is inheritance models a and b with initial incidence 0.005. In other words, the effect of simultaneously selecting against two inherited forms of pigmentation was entirely linear and neither a benefit nor a penalty resulted.

Phenotypic and paternal culling

The addition of paternal culling (results summarised in Table 5.3) saw an additional category of costs. Culling the sires of pigmented phenotypes involved DNA-fingerprinting plus the expense of replacing any culled sire from the next cohort of ram hoggets. However the cost due to phenotypic culling was reduced, as was the cost due to loss of ram sales, because the allele frequency was reduced more quickly. Combining the costs, the addition of paternal culling represented a substantial saving over the cost of phenotypic culling alone for the ram-selling flock, particularly when the initial incidence was higher. However the flock breeding only its own rams incurred increased total costs when the initial incidence was lower, and only a modest decrease in costs when the initial incidence was higher. Compared to Table 5.3, a discounted financial basis was less advantageous to paternal culling because costs were incurred in advance of savings being reaped.

Table 5.3 *The practical and financial consequences of a 40 year program of phenotypic and paternal culling on inherited pigmentation faults, according to the initial incidence and inheritance model. Paternity was established by DNA fingerprinting. Assessed relative to a flock having equal reproductive success without pigmentation alleles. Reproduction rate was 1.00 and there were two ram age groups.*

Initial incidence	Inheritance model	Cost due to phenotypic culling[AB] $	Cost due to loss of ram sales[B] $	Cost due to paternal culling[AB] $	Net result[A] $	Net result[B] $	Final incidence	Loss of selection intensity
0.005	a	2 332	9 337	4 595	-6 927	-16 263	0.0003	0.25
0.005	b	3 173	12 768	6 788	-9 962	-22 730	0.0005	0.40
0.005	c	2 126	8 614	4 622	-6 747	-15 361	0.0002	0.22
0.005	d	3 037	12 301	5 573	-8 609	-20 910	0.0003	0.28
0.005	e	3 563	14 432	7 471	-11 034	-25 466	0.0005	0.42
0.010	a	3 849	15 651	5 955	-9 804	-25 455	0.0003	0.32
0.010	b	5 153	21 134	9 180	-14 333	-35 466	0.0005	0.55
0.010	c	3 874	15 753	6 781	-10 655	-26 408	0.0003	0.38
0.010	d	4 440	17 936	6 554	-10 994	-28 930	0.0002	0.36
0.010	e	5 844	23 557	9 807	-15 651	-39 209	0.0007	0.60
0.010	f	5 259	21 068	9 150	-14 409	-35 477	0.0006	0.58
0.050	a	15 945	65 014	11 866	-27 811	-92 825	0.0003	0.71
0.050	b	19 471	79 782	17 619	-37 090	-116 872	0.0010	1.29
0.050	c	16 280	66 532	14 080	-30 360	-96 893	0.0005	1.00
0.050	d	19 588	79 920	14 162	-33 750	-113 671	0.0005	0.87
0.050	e	18 572	76 278	16 677	-35 249	-111 527	0.0010	1.19

[A] Items affecting a flock breeding only its own rams.
[B] Items affecting a flock breeding sale rams.

Figure 5.1 illustrates the financial difference between phenotypic culling only, and phenotypic plus paternal culling. Under paternal culling the costs were about 10% higher over years 1–4 but then diminished rapidly as the allele frequency declined.

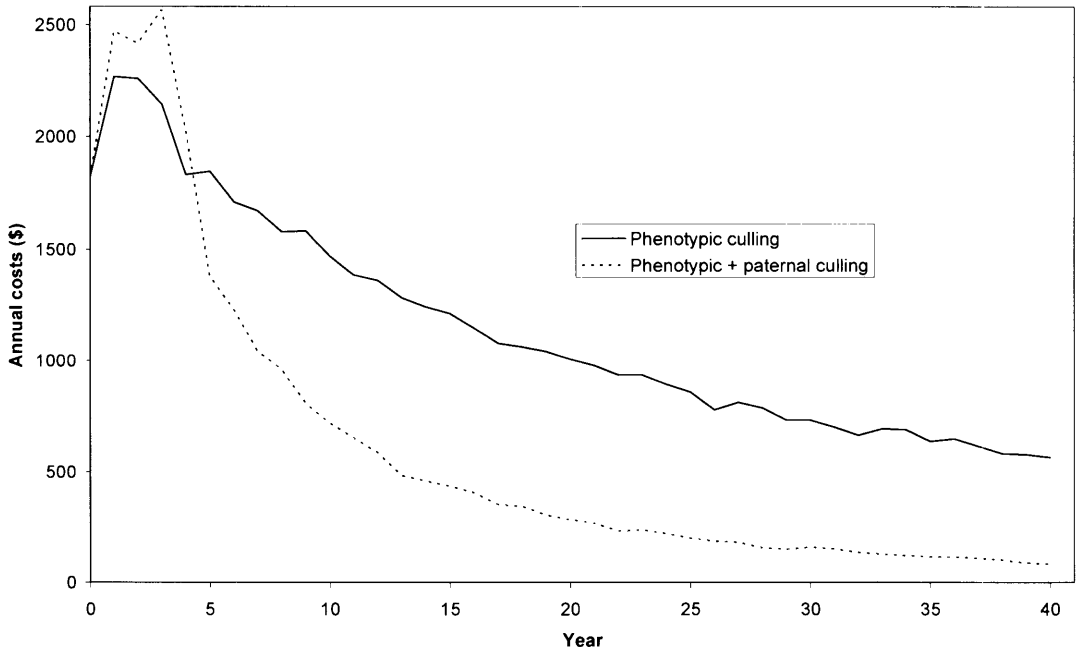


Figure 5.1 *Annual costs associated with two culling strategies over 40 years in a closed ram selling flock. Simple recessive inheritance (model a) with initial incidence 0.01.*

Paternal culling dramatically increased the selection pressure against pigmentation alleles and the final phenotypic incidence was generally less than one tenth of that from phenotypic culling alone. For inheritance model d (imprinting) it was particularly effective in reducing the phenotypic incidence, by placing a higher selection pressure against the paternal allele. Paternal culling greatly increased the loss of selection intensity over phenotypic culling — an average 150% increase. At worst the 40 year total loss represented just 0.02 of the total average selection intensity over 40 years. The additional accumulated loss of selection intensity for sale rams was around half of that within the stud flock. While this loss would not affect genetic progress in the stud flock, it would affect the rate of progress in the flocks of ram-buyers by temporarily increasing the genetic lag.

A higher reproductive rate was an advantage with paternal culling, because higher fecundity increased the chance that a carrier sire was exposed by a pigmented lamb. This led to conflicting effects on selection intensity and no clear trend. For the flock breeding only its own rams, paternal culling was more clearly advantageous at a higher reproduction rate, because while the cost of phenotypic culling increased in proportion to the number of lambs born, the cost of paternal culling increased only slightly with reproduction rate — for any ram the cost of DNA-fingerprinting can only be incurred once, and any ram can only be culled once. Conversely the addition of paternal culling to this flock at a lower reproductive rate was less favourable financially.

Changing the ram age structure from two age groups (L_M = 2.5 years) to four age groups (L_M = 3.5 years) increased the effect of paternal culling across all scenarios, because carrier rams were more likely to be weeded out during their career with the net effect of reducing the proportion of rams in use that were carriers. As the allele frequency and phenotypic incidence fell more quickly, costs decreased in all categories. After 40 years the flock with the longer generation interval had a final phenotypic incidence that was one quarter the magnitude of the shorter. The loss of selection intensity was similar or slightly reduced with four ram age groups.

Compared to phenotypic culling alone, the financial variability between replicates was much smaller. Fluctuations in allele frequency were still possible but on the whole chance was a less significant factor in the costs and in the loss of selection intensity. For selection intensity however, the variability was much increased under this selection strategy. This is because detected carrier rams were necessarily elite and culling them had a larger and more variable effect on selection intensity. Having a higher reproductive rate and retaining males for four years, in addition to their favourable effect on mean results, also substantially reduced the variability between replicates, by minimising the risk that carrier rams were undetected.

Comparing inheritance model f (line 11 in Table 5.3) with the sum of its constituent parts (lines 1 and 2), simultaneous selection against two undesirable genes was more cost effective. The cost of DNA-fingerprinting was reduced by 22% and other costs were reduced by about 5%, giving a summed benefit of 10% for the ram-selling flock and 15% for the flock breeding only its own rams. Benefits were also observed for selection intensity and final allele frequency.

Maternal culling

Maternal culling in addition to paternal and phenotypic culling resulted in a modest average decrease in final phenotypic incidence of 6–7% compared to only paternal and phenotypic culling. There was a reduction in phenotypic culling of about 5% over 40 years and this meant that a greater number of rams were available for sale. However there was considerable cost in replacing breeding females, and a substantial loss of female selection intensity as a result — which was only slightly compensated by better selection intensity for males. DNA-fingerprinting to confirm maternity reduced wrongful culling of dams but almost half of the cost savings were expended on the DNA-fingerprint tests. On balance, the DNA-fingerprinting strategy was more efficient than mothering-up alone, in terms of total costs, selection intensity and final phenotypic incidence. However the net financial position was around 8% worse than without maternal culling in a ram-selling flock (still much more favourable than phenotypic culling alone) and around 27% worse in a flock breeding only its own rams (considerably inferior to phenotypic culling alone).

The power of any culling method to reduce allele frequency was strongly dependant upon the initial frequency. No culling strategy reliably eliminated pigmentation alleles, although the

chance of allele elimination was inversely proportional to the final incidence. Only inheritance model c showed a non-trivial chance of allele elimination, reaching 34% of cases under paternal and maternal culling, rising to 56% when there were four ram age groups. In all scenarios the average time to allele elimination exceeded 30 years.

DNA gene test — selection strategies

A logical selection strategy with a DNA gene test for pigmentation alleles available, would be to commence testing on males. Even under the highest initial allele frequencies, there would be an adequate number of non-carrier rams for breeding and sale. Importantly, the cost of testing breeding rams is far less than the cost of testing ewes, due to the number of animals involved. If no carrier rams are used, then the allele frequency in the ewes is reduced by half each generation. Also, except for inheritance model c, excluding all carrier rams immediately prevents the incidence of any further pigmented phenotypes.

Individually testing ewes is a major cost. If the ewe flock were to be tested at one point in time, the number of animals tested would be the breeding ewe flock plus the next cohort of replacement ewes = 1220 (plus additional replacements for any carriers detected). However, testing older ewes is relatively inefficient because they only have a short reproductive life ahead of them. DNA testing costs can be considerably reduced if some or all of the replacements are tested each year over a number of years. The known non-carriers must be separated from untested ewes at lambing so that their progeny do not require further DNA testing. Under this arrangement the number of females tested would be the annual replacement rate (220) multiplied by the female generation interval (3.9 years) = 858 (again plus additional replacements for any carriers detected). Depending upon the heritability of the flock breeding objective, selection will tend to favour the progeny of younger ewes because these will have greater average merit; advantageously, these animals will also have a lower frequency of pigmentation alleles due to sire testing. This would have the effect of reducing the actual maternal generation interval and therefore the number of ewes requiring testing could be further reduced. The extent of this synergy depends upon both a high heritability of the underlying flock breeding objective and a high reproduction rate. In practice it might be possible, simply by impartially selecting the progeny of younger dams, to reduce L_F to around 3 years, for example with a breeding objective heritability of 0.4 and a reproductive rate of 1.2. Thus, female DNA testing might be reduced to as few as 660 individuals — a little over half the original 1220 animals — with substantial cost savings.

DNA testing of females will be wasteful if the capacity to cull all of the detected carriers does not exist — it will have served a limited purpose, and will require a greater total number of ewes tested before the breeder may be sure the allele has been eliminated. Therefore the extent of ewe testing performed in any year should be restricted by the ability to cull all of the carriers which are expected to be detected by the testing. In practice, a breeder who has already been

gene testing rams should have a good estimate of the proportion of carriers in the flock. If a cohort of ewe hoggets can be segregated by age of dam, then the expectation of a lower allele frequency in each successive generation can be exploited by the breeder — the progeny of the youngest ewes would be tested first because they should have the lowest allele frequency, and the least pressure is placed on the capacity to cull the detected carriers.

DNA gene test — rams only

Table 5.4 shows a summary of results when DNA gene testing was applied to rams only. Phenotypic and adult culling only occurred at time 0, except for inheritance model c. Loss of ram sales reduced income but the 5% price premium for tested (non-carrier) rams more than compensated for this loss in all cases except the highest initial incidence — although the net results varied widely. The cost of DNA gene tests for stud rams varied little between scenarios, slightly increasing with the likelihood of detecting a carrier. The cost of DNA gene tests for sale rams also varied little but was around twenty times the magnitude of stud ram testing costs, due to the numbers of individuals involved. The total cost for a flock breeding its own rams ranged from $15 000 to $20 000 over 40 years; in some cases this was less that the parallel scenario under phenotypic and/or paternal culling. For a ram-selling flock, the net financial impact ranged from a surplus of $23 000 to a loss of $826 000. Considering the financial results in isolation, ram testing compared unfavourably to the phenotypic culling approaches, aside from inheritance model c at the lowest initial incidence.

The flock initially free of pigmentation alleles is useful to illustrate the major avenues of cost and revenue. DNA testing of stud rams cost $360 p.a. and testing of sale rams cost $7 500 p.a. while the premium from sales of tested rams came to $10 000 p.a. In the ram-selling flock testing was profitable from the outset and at year 40 the Benefit:Cost ratio was 1.27. In the flock breeding only its own rams there was no additional revenue to offset the costs.

The first line of Table 5.4 is included because even a flock that is free of pigmentation alleles would incur substantial costs if DNA gene testing were adopted to certify its stud and sale rams. (By contrast, implementing culling strategies in such a flock would carry no costs.) The 5% premium on sale of rams that were free of pigmentation alleles exceeded the total costs of testing for the flock selling breeding rams, but there was no offsetting revenue in the flock breeding only its own rams. By charting the net financial situation against the initial incidence for each inheritance model, it is possible to estimate the "break-even" initial incidence.

Table 5.4 *The practical and financial consequences of a 40 year breeding program incorporating DNA gene testing for pigmentation alleles in rams only, according to the initial incidence and inheritance model. Assessed relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups.*

Initial incid-ence	Inheri -tance model	Cost due to phenotypic & adult culling[AB] $	Cost of lost ram sales[B] $	Premium on tested sale rams[B] $	Cost of DNA testing stud rams[AB] $	Cost of DNA testing sale rams[B] $	Net result[A] $	Net result[B] $	Loss of selection intensity	Allele elimin-ation[C]
0.000	-	0	0	409 563	14 760	307 172	-14 760	87631	0.00	-
0.005	a	460	-149 101	402 108	14 942	306 662	-15 402	-69 057	0.20	84
0.005	b	560	-265 950	396 265	15 356	309 003	-15 916	-194 603	0.41	75
0.005	c	964	-58 227	406 652	15 017	306 807	-15 981	25 637	0.10	95
0.005	d	560	-293 811	394 872	15 386	308 290	-15 946	-223 175	0.42	68
0.005	e	560	-262 634	396 431	15 475	309 952	-16 035	-192 190	0.43	74
0.010	a	820	-209 003	399 113	15 081	306 843	-15 901	-132 634	0.27	79
0.010	b	920	-343 948	392 366	15 684	311 081	-16 604	-279 267	0.54	65
0.010	c	1 879	-109 180	404 104	15 246	306 869	-17 124	-29 069	0.17	93
0.010	d	920	-335 805	392 773	15 619	309 841	-16 539	-269 412	0.51	61
0.010	e	970	-326 330	393 246	15 809	312 235	-16 779	-262 097	0.57	56
0.010	f	970	-342 088	392 459	15 836	312 119	-16 806	-278 555	0.59	63
0.050	a	4 800	-403 591	389 383	16 103	311 424	-20 903	-346 535	0.67	65
0.050	b	4 900	-863 722	366 377	18 750	308 974	-23 650	-829 969	1.43	35
0.050	c	8 517	-287 514	395 187	16 759	309 874	-25 276	-227 476	0.52	80
0.050	d	4 850	-794 594	369 833	17 826	310 060	-22 676	-757 497	1.25	32
0.050	e	4 950	-850 325	367 047	19 257	308 149	-24 207	-815 635	1.45	38

[A] Items affecting a flock breeding only its own rams.
[B] Items affecting a flock breeding sale rams.
[C] Instances of elimination of pigmentation allele/s by year 40 from 100 population replicates.

Almost all of the financial difference between inheritance and incidence scenarios was due to revenue from ram sales. This also meant that the financial differences were small for a flock breeding only its own rams. The variability between replicates for this flock was negligible. However for the ram-selling flock the variability of financial results was slightly greater than for phenotypic and paternal culling. This was because of chance as a factor in the number of carriers detected among potential stud and sale rams, affecting the number of rams sold. By contrast, the variability in loss of selection intensity was much reduced because chance was less of a factor in allele frequency.

The loss of selection intensity was higher than for phenotypic culling alone, but similar to that shown for a combination of phenotypic and paternal culling. However this was in the context of more efficient eradication of undesirable alleles, resulting in complete elimination in many cases. The loss of selection intensity for sale rams was up to three times that under phenotypic plus paternal culling. Clearly the initial phenotypic incidence was a major driver of the financial outcome where ram sales were involved and a high initial incidence was particularly damaging under inheritance models b, d, e and f.

In all cases the allele frequency was very low by the end of the 40 year breeding program, and elimination of the pigmentation allele/s was quite common. The initial incidence and the inheritance model affected the chance of allele elimination. Among those cases where the allele/s were eliminated, the average time to elimination exceeded 30 years, except for inheritance model c where the average was as low as 24 years for the lowest initial incidence.

Varying the reproduction rate had no noteworthy effect. A higher reproduction rate saw a slightly greater loss of selection intensity (from a higher base) and slightly increased the chance of allele elimination. Changing the ram age structure from two age groups to four age groups caused the cost of DNA testing for stud rams to be approximately halved. Otherwise this change had little effect; there was a positive effect on revenue from ram sales when the initial incidence was high but this was an artefact of the rules controlling the number of rams sold, and was of no scientific interest.

DNA gene test — rams and ewes

Adding DNA gene testing of ewes to these scenarios had the practical advantage of speeding allele elimination and allowing the breeder to confirm that pigmentation alleles were no longer present in the flock. Subsequently, although the ram-selling flock continued to test all stud and sale rams, the cost of culling carriers was avoided and all surplus ewes were sold at a 5% premium. In the flock breeding only its own rams, all DNA testing ceased once pigmentation allele/s were eliminated. In order to maximise the benefits, ewe testing should commence as early as practical to avoid wastage in the breeding scheme. Table 5.5 shows a summary of results for schemes including ewe DNA testing, with the initiation of ewe testing optimised..

Table 5.5 *The practical and financial consequences of a 40 year breeding program incorporating DNA gene testing for pigmentation alleles in rams and ewes, according to the initial incidence and inheritance model. Assessed relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups.*

Initial incidence	Inheritance model	Cost due to phenotypic & adult culling[AB] $	Revenue change — ewe sales[AB] $	Cost of lost ram sales[B] $	Premium on tested sale rams[B] $	Cost of DNA test — ewes & stud rams[AB] $	Cost of DNA test — stud & sale rams[B] $	Net result[A] $	Net result[B] $	Loss of selection intensity
0.000	-	0	49 091	0	409 563	28 652	319 052	20 439	110 950	0.00
0.005	a	460	48 293	-105980	404 264	30 036	319 047	17 797	-2 966	0.24
0.005	b	560	47 136	-167505	401 188	32 254	320 877	14 323	-72 872	0.51
0.005	c	627	48 833	-46057	407 260	29 143	318 940	19 064	61 327	0.10
0.005	d	560	46 794	-184846	400 321	32 781	319 963	13 454	-91 034	0.55
0.005	e	560	47 129	-167525	401 187	32 342	321 941	14 227	-74 053	0.53
0.010	a	820	47 864	-140723	402 527	30 995	319 276	16 049	-41 424	0.34
0.010	b	920	46 169	-207435	399 191	33 939	323 306	11 310	-120 240	0.70
0.010	c	1 353	48 444	-79119	405 607	29 873	319 040	17 218	24 666	0.19
0.010	d	920	46 199	-206856	399 220	33 875	322 220	11 404	-118 452	0.67
0.010	e	970	46 380	-202826	399 422	33 933	324 496	11 477	-116 423	0.72
0.010	f	970	46 210	-207201	399 203	34 073	324 207	11 168	-121 038	0.74
0.050	a	4 800	45 760	-256023	396 762	35 121	323 081	5 839	-176 503	0.86
0.050	b	4 900	39 617	-548933	382 116	47 195	321 512	-12 478	-500 807	1.97
0.050	c	6 547	46 913	-195396	399 793	33 693	322 184	6 673	-111 113	0.62
0.050	d	4 850	40 418	-491514	384 987	45 081	322 487	-9 513	-438 527	1.76
0.050	e	4 950	40 970	-545736	382 276	45 581	320 396	-9 561	-493 417	1.90

[A] Items affecting a flock breeding only its own rams.
[B] Items affecting a flock breeding sale rams.

The optimum for a ram-selling flock was always to commence ewe DNA testing at year 2, which meant that the flock would be free of pigmentation alleles by year 8. In the case of simple recessive inheritance (model a) with initial phenotypic incidence of 0.010, each year of delay in implementing ewe testing cost around $7 000 through lost ram sales plus around $700 lost revenue from ewe sales.

The premium available from the sale of confirmed non-carrier ewes made a substantial contribution which declined only slightly as initial allele frequency increased. The cost of DNA tests for ewes and for stud rams (prior to allele elimination) was approximately double the cost of stud ram testing in Table 5.4, with a greater increase for the less tractable scenarios — being inheritance models b, d and e at the highest initial incidence. Across all schemes, the net financial result was much improved for the addition of ewe DNA testing; for the flock breeding only its own rams, most scenarios were revenue-positive on a cash basis. On the basis of net present value, ewe testing remained financially advantageous in the ram-selling flock

For a flock initially free of pigmentation alleles, the costs of DNA testing for rams were unchanged. Ewe DNA testing costs totalled $25 740 over years 2–7 and from then on the premium from sales of certified surplus ewes came to $1 400 p.a. In the ram-selling flock, a break-even point was reached at year 9 on a cash basis and year 10 on a discounted basis, by which time around $112 000 had been spent on DNA testing. The cash flow minimum was passed in year 5 at around -$8 500. At year 40 the Benefit:Cost ratio exceeded 1.31. In the flock breeding only its own rams, cash break-even was reached in year 26 following a cash flow minimum of around -$25 500 in year 7; discounted break-even could not be attained. At a higher reproduction rate, the break-even points were reached earlier — year 8 for the ram-selling flock (cash and discounted) and year 21 (cash) for the flock breeding only its own rams.

By comparison, flocks carrying pigmentation alleles showed slightly higher costs for DNA testing and slightly lower revenue from ewe sales, but the major difference arose from much lower revenue through ram sales.

Ewe DNA testing meant that the loss of selection intensity for the remainder of the breeding objective was increased for ewes but reduced for stud and sale rams. Across rams and ewes, the loss of selection intensity was around 30% greater when ewes were tested. Importantly, all of this selection intensity would be lost in the first eight years, rather than a longer time scale, and this may become a critical consideration for breeders. At the highest initial incidence, and with the less tractable inheritance models, the total loss of selection intensity exceeded that expected from an annual round of selection (1.62) but in the other cases was far less.

The variability in loss of selection intensity between replicates was similar whether or not ewes were included in DNA testing. However extending the testing to ewes did have the effect of

reducing variability of financial outcome, by reducing the extent to which ram sales could be restricted by the incidence of carriers.

For a flock breeding only its own rams the costs of ewe testing exceeded the benefits under discounting because the costs were incurred earlier than the benefits. When combined with the less tractable genetic scenarios (inheritance model b, d or e with initial incidence 0.05) it was more favourable to delay the commencement of ewe DNA testing to year 7. By this time the ram testing had much reduced the number of ewe carriers so that individual testing could be kept to a minimum. This gave a net benefit of around $5 000, compared to ewe testing from year 2, comprising a $6 500 reduction in DNA testing costs for ewes and a $1 500 reduction in revenue from ewe sales.

Increasing the reproduction rate to 1.2 mainly caused scale effects. However there was a considerable financial benefit for the flock breeding only its own rams, through the sale of a greater number of surplus (non-carrier) ewes, while the testing and culling costs increased only a little. Loss of selection differential was unchanged.

When there was a lower reproduction rate and/or a higher forced culling rate among ewe hoggets, the potential to cull carrier ewes was decreased. This meant that the testing of ewes had to be extended over a greater number of years, and the flow of benefits from allele elimination was delayed. For the flock breeding only its own rams, the optimal commencement of ewe DNA testing was later. Otherwise, where these constraints resulted in the required selection of known carrier ewes the overall cost of ewe testing was higher but the penalty in itself was not sufficient to push back the optimal commencement of ewe DNA testing — which was determined to be year 2.

Changing the ram age structure to four age groups once again caused the cost of DNA testing for stud rams to halve. However only about $2 000 of this benefit was realised in the flock breeding only its own rams, before the pigmentation allele/s were eliminated and DNA testing ceased.

Comparing inheritance model f to the sum of its constituent parts in Table 5.5 revealed that eliminating two deleterious conditions simultaneously will save in DNA testing costs, on the assumption that both undesirable alleles are detected by one test at the same price. The revenue from ewe sales was only slightly changed but the revenue from ram sales was around $70 000 higher under joint selection.

Figure 5.2 shows the relationship between initial incidence and the financial impact of the strongest selection strategy, including DNA gene testing of rams and ewes. This relationship is strong and appears to be curvilinear with inheritance model c the most tractable, followed by inheritance model a. The remaining inheritance models show similar results.

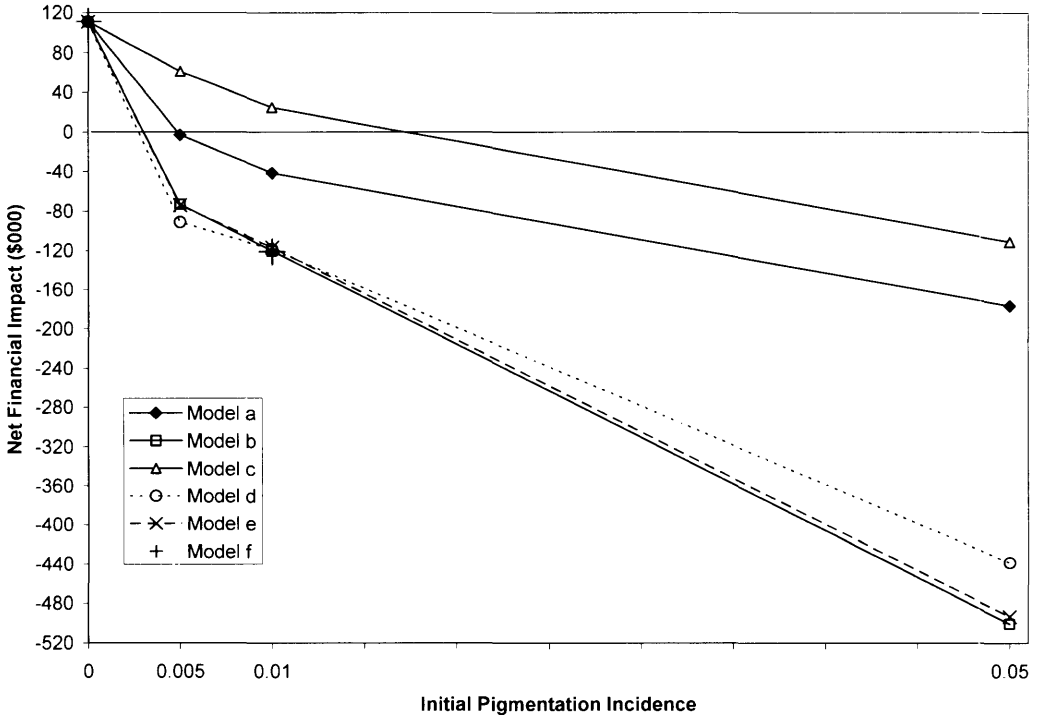


Figure 5.2 *The effect of initial allele frequency (as observed through initial incidence rate) upon the net financial impact of a selection strategy including DNA gene testing of rams and ewes. Assessed over 40 years in a ram-selling flock, for six inheritance models. Assessed relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups, no discounting was applied.*

When the results were charted using initial allele frequency as the independent axis, the results for all inheritance models fell in an approximately linear band and the inheritance model effect was minimal. The linear relationships charted in Figures 5.3 to 5.7 represent correlations of at least 0.75 and in the majority of cases correlations exceeding 0.90.

The relationship between initial allele frequency and the net financial impact of each selection strategy is illustrated in Figure 5.3. The regression was a cost of $12 908 per 0.01 allele frequency under the strategy of DNA testing for rams and ewes. The break-even initial allele frequency was 0.090 when assessed by zero financial impact; however, compared to a similar analysis of phenotypic culling (cost of $4 986 per 0.01 allele frequency) a more realistic break-even initial allele frequency would be the intercept point at 0.140, or compared to phenotypic plus paternal culling (cost of $2 527 per 0.01 allele frequency) a break-even initial frequency 0.107. For DNA testing of rams alone across the regression was $19 734. At high allele frequencies (higher than the break-even points given above) DNA testing was apparently inferior to the culling options, but this assumed that there was a continuing market for untested rams — a doubtful proposition that would be increasingly doubtful over a longer timeframe.

Figure 5.5 shows the same results on a discounted basis. The picture is similar but the intersection between testing options and culling options occurs at a much lower allele frequency — around 0.025.

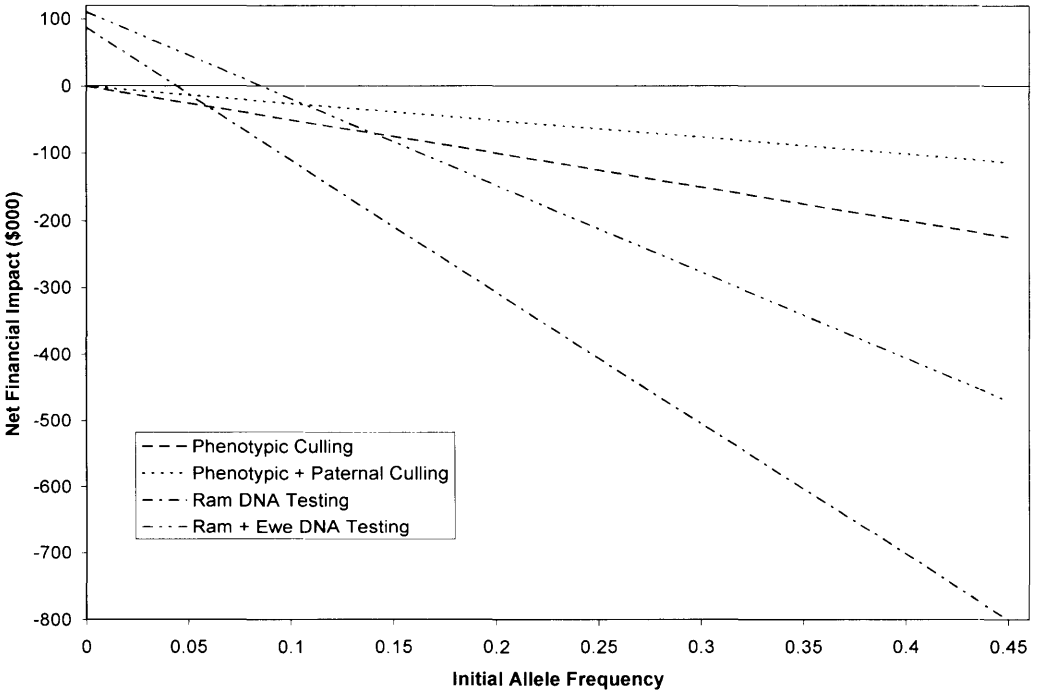


Figure 5.3 *The effect of initial allele frequency upon the net financial impact of four selection strategies to eliminate pigmentation alleles in a ram-selling flock. Assessed across all inheritance models over 40 years relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups, no discounting was applied.*

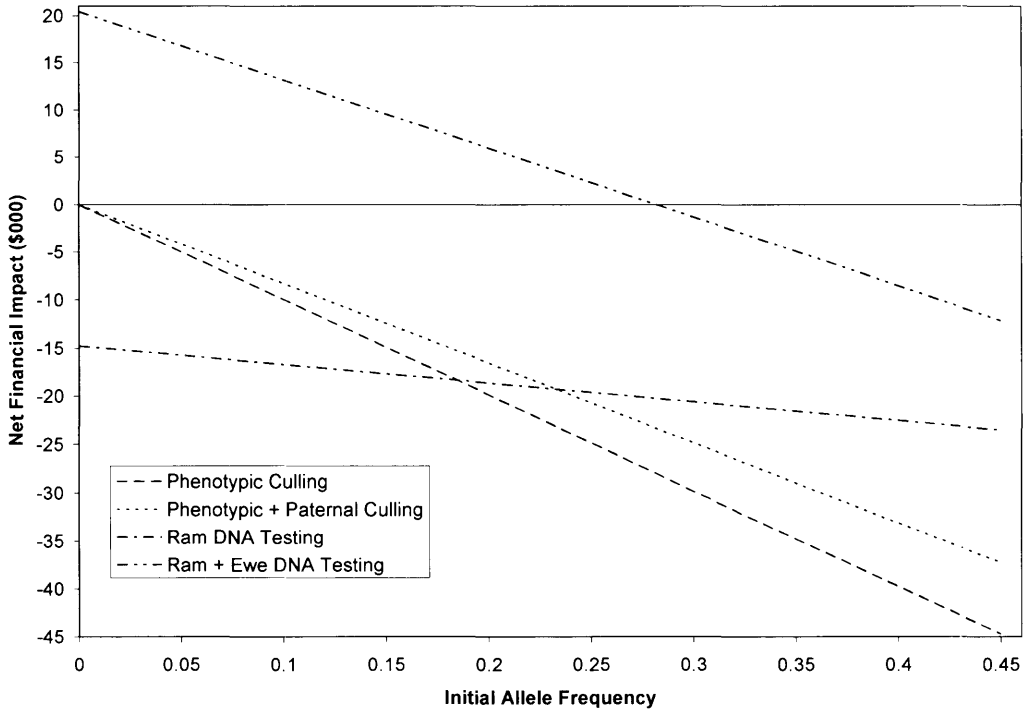


Figure 5.4 *The effect of initial allele frequency upon the net financial impact of four selection strategies to eliminate pigmentation alleles in a flock breeding only its own rams. Assessed across all inheritance models over 40 years relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups, no discounting was applied.*

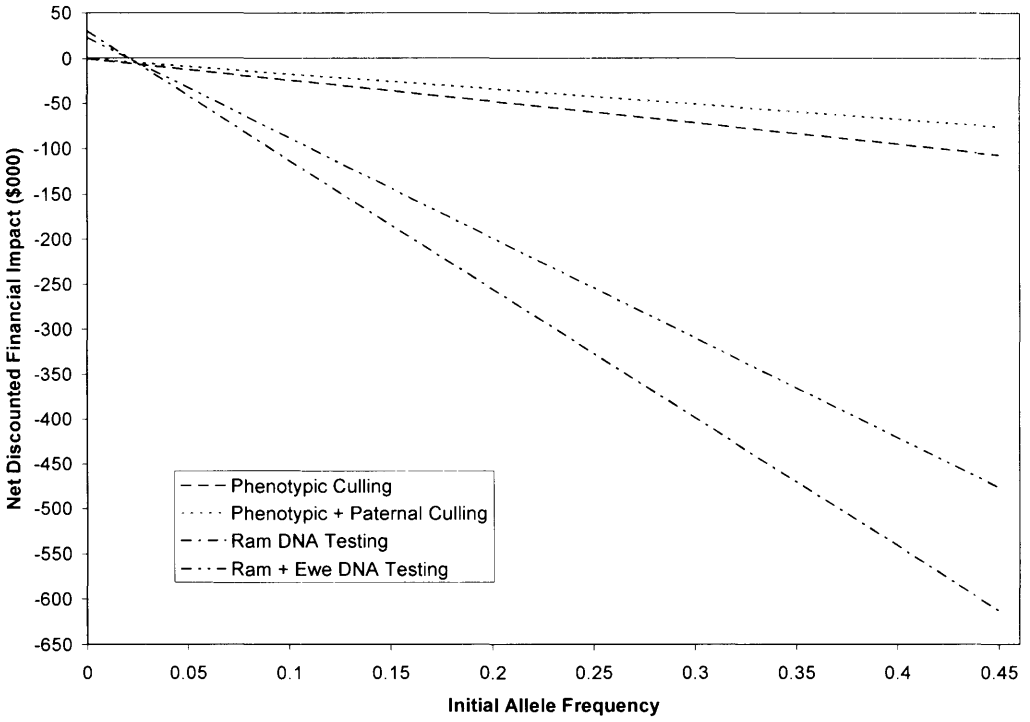


Figure 5.5 *The effect of initial allele frequency upon the net present value of four selection strategies to eliminate pigmentation alleles in a ram-selling flock. Assessed across all inheritance models over 40 years relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups, discount rate was 0.07.*

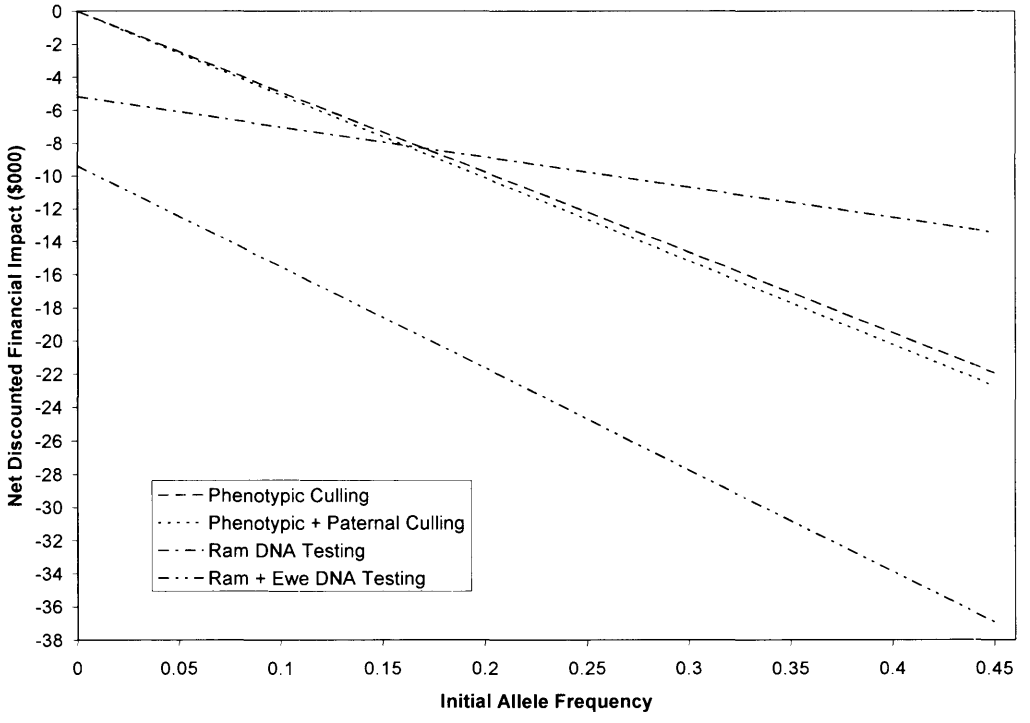


Figure 5.6 *The effect of initial allele frequency upon the net present value of four selection strategies to eliminate pigmentation alleles in a flock breeding only its own rams. Assessed across all inheritance models over 40 years relative to a flock having equal reproductive success without pigmentation alleles and without gene testing. Reproduction rate was 1.00 and there were two ram age groups, discount rate was 0.07.*

Figure 5.4 and Figure 5.6 show the same results for the flock breeding only its own rams. Discounting had a dramatic effect upon the appropriateness of DNA testing for ewes, due to the

high cost involved. The costs for the two culling options were very similar, but paternal culling had the advantage of accelerated decline in allele frequency (Table 5.3). Comparing the culling options to DNA testing for rams, the situation was a reversal of that for the ram-selling flock: DNA testing was only cost-effective if the initial allele frequency was higher than approximately 0.20.

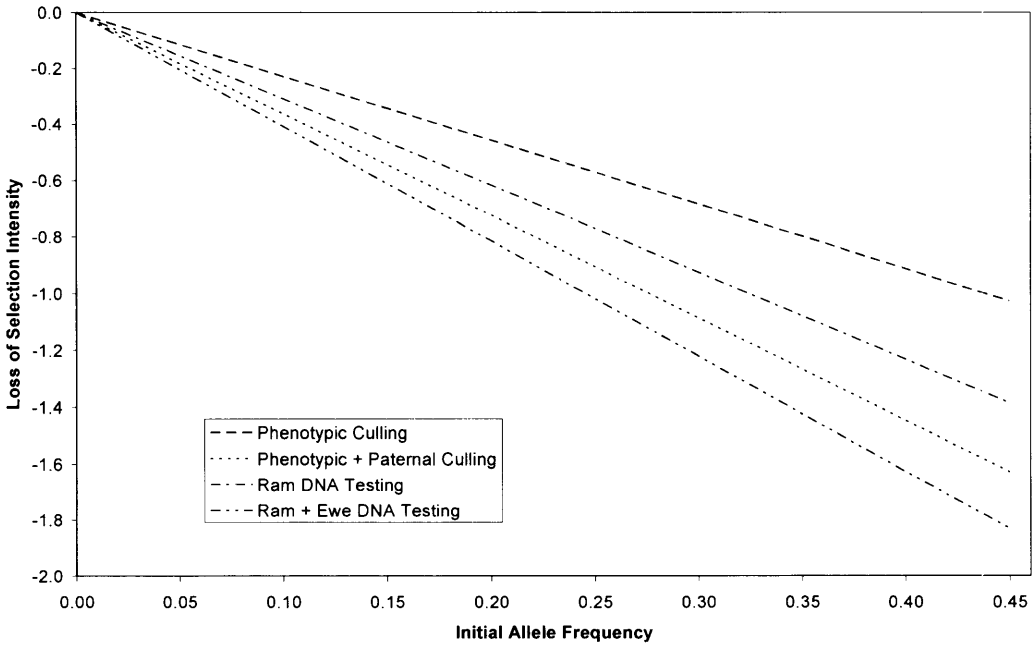


Figure 5.7 *The effect of initial allele frequency upon the cumulative loss of selection intensity under four selection strategies to eliminate pigmentation alleles. Assessed across all inheritance models and in each case selection continued until the undesirable allele/s were eliminated from the flock. Reproduction rate was 1.00 and there were two ram age groups.*

Figure 5.7 shows the cumulative loss of selection intensity for each selection strategy, extended to the extinction of the pigmentation allele/s (inheritance model d was excluded from the culling strategies because only one allele was eliminated). The smallest impact on selection intensity was achieved by the slowest strategy — phenotypic culling. The greatest loss of selection intensity coincided with the most rapid elimination strategy — DNA testing for rams and ewes. DNA testing of rams prior to use was more efficient than paternal culling of older sires because the latter was less than 100% reliable and because a culled sire may have left other carrier progeny in the flock.

Although the exact conclusions depend upon the precise selection strategy, flock age structure and reproduction rate, these results are applicable to other situations where the elimination of undesirable alleles is under consideration.

5.4 Discussion

The steep financial impact of initial allele frequency upon testing for and elimination of pigmentation alleles is incontrovertible. This finding is not necessarily intuitive because the allele frequency would decline exponentially under DNA ram testing, and relatively small

differences in initial frequency should diminish quickly. However the ability to supply non-carrier rams in the early years of the scheme is the major factor determining the financial outcome for a ram-selling flock. The industry as a whole will ultimately bear the costs for eliminating pigmentation alleles, but while the financial impact will be manageable in flocks with a low allele frequency the costs will fall disproportionately upon ram-breeding flocks with a higher initial allele frequency; in some cases this cost burden may be sufficient to threaten the future of a seedstock business (which may however assist in the efficiency of pigmentation allele elimination across the industry). To put this issue in context, an earlier chapter reported on the finding of a survey that the incidence of *Australian Piebald* phenotypes in industry flocks averaged 0.003 and ranged from 0.000 to 0.042, and the incidence of symmetrical (*Agouti*) pigmentation patterns averaged 0.002 and ranged from 0.000 to 0.042.

At least two important secondary implications flow from this result. First, when DNA gene tests for pigmentation traits are released to the market, the frequency of alleles in a ram-breeding flock (relative to other ram-breeding flocks) will largely determine the financial impact of the technology on a ram-breeding business. Second, any delay in adopting the DNA technology will mean that the flock would fall behind other flocks which have adopted. While for any new technology there is often an advantage in early adoption, for this case the disadvantages of late adoption will compound as the allele frequency remains higher and the ability to supply non-carrier rams remains static.

The finer details of the results will depend to some extent upon the validity of assumptions made and the assumption carrying the greatest implications would be the level of premium applying to tested non-carrier animals. Under the 5% premium assumed here, net revenue was clearly far more sensitive to any reduction in the number of rams sold. Note that the effect of a different level of premium can be considered by simply scaling the ram premium totals in Tables 5.4 and 5.5. The premiums available for certified rams and ewes will be largely a product of buyer demand, but are unlikely to be static for two reasons. First, the premium for rams may rise sharply in the initial period when supply is restricted, but could be expected to moderate over time. Second, ram-buyers have varying degrees of loyalty to particular ram-breeding flocks and bloodlines, so that premiums may vary across ram sources at the same time. In addition the supply of sale rams can be adjusted from year to year through adjusting the size of the ram-breeding flock and/or varying the selection intensity — this flexibility was not simulated because the cost of eliminating pigmentation alleles was simply proportional to flock size, and because the supply of additional (but necessarily lower quality) rams would further complicate other assumptions. If the ram-buying market is strong in its demand for rams free of pigmentation alleles, then many ram buyers will have to accept rams of lower average quality in production traits, or pay a competitive premium. If ram buyers in general show less interest in (the lack of) pigmentation alleles then there will still be a market for untested rams and possibly even for carrier rams.

Some Merino ram-breeding flocks routinely collect pedigree records over a majority of their animals. Those flocks would be in a position to utilise segregation analysis to assist in the elimination of pigmentation alleles. Segregation analysis could attribute carrier probabilities to all individuals — leading to either direct culling if the probability is high, or to prioritising animals for DNA gene testing (Kinghorn 1999). These strategies would be particularly useful where several genes are under simultaneous selection (Percy and Kinghorn 2005). However as Fewell (2001) raised the problem that selection against a recessive genetic condition leads to a rapidly diminishing supply of phenotypic data to drive segregation analysis.

The best means for any flock to prepare for the advent of DNA gene tests for pigmentation is to reduce allele frequency by combined phenotypic and paternal culling. This strategy was of equal or greater cost effectiveness to phenotypic culling across all the scenarios tested, while reducing allele frequency at a greater rate and in exchange for a modest loss of selection intensity. In most cases phenotypic culling is unavoidable and paternal culling saves money by reducing the cost of phenotypic culling in future generations. The earliest possible adoption of strict phenotypic culling with paternal culling by DNA finger-printing should be strongly encouraged among ram breeders. Obviously, where sires can be reliably identified without DNA finger-printing, paternal culling will show even greater cost effectiveness.

Phenotypic culling was effective in reducing the frequency of pigmentation alleles. Coupled with paternal culling, simulations showed that allele frequencies could be reduced to very low levels and that elimination was possible, albeit due to a good measure of luck and despite the breeder being unaware of the fortunate outcome. However the time scales involved of 30-40 years were so long as to be barely capable of practical implementation. It should be noted that the main reason phenotypic culling has had little effect on the frequencies of pigmentation alleles across Australian Merino flocks has been the transfers which occur between flocks.

Maternal culling was costly and only marginally effective as a method for reducing pigmentation alleles. However use of DNA-fingerprinting to confirm maternity did improve efficiency when mothering-up was 80% accurate. It might be possible by targeting DNA-fingerprinting at younger ewes, to further improve the efficiency of maternal culling. Given that the value of maternal culling was small or negative, it could reasonably be extrapolated that detecting and culling more distant relatives would have even less favourable outcomes. However under paternal culling the opportunity to cull all male progeny (i.e. half-brothers of the pigmented individual) may have been beneficial to the final elimination of pigmentation alleles. If implemented when the allele frequency had already been reduced to low levels, this option should have a minimal effect on inbreeding. However, in the absence of pedigree records, the cost of DNA parentage testing to identify half sibs could be considerable.

For ram-breeding flocks, DNA testing of ewes should start at an early date and be applied in the most efficient manner possible. Fewer individual tests will be required if successive cohorts are

tested annually and lambing ewes are segregated on test status. However testing within any cohort should cease once the capacity to cull carriers has been reached. By completely eliminating pigmentation alleles sooner, ewe testing leads to increased financial benefits. However for ram-breeding flocks not selling rams, and indeed for commercial flocks, the net effect of ewe testing is small or negative. In economic terms these flocks would be better off simply to ensure that all rams to be used or purchased are tested non-carriers.

Recording the impact of allele elimination on selection intensity separately from the other economic consequences gives flexibility to interpret the results as appropriate to a wide range of breeding objectives in the field. However a potential weakness arises because pursuing a highly heritable breeding objective would tend to favour the progeny of younger parents; and these same animals would carry pigmentation genes at a lower frequency under culling and gene-testing scenarios. Deterministic modelling was used to follow the change in gene frequency by age group in the breeding ewe flock. This was combined with predicted selection fractions by maternal age group to assess the significance of the selection synergy. It was implicitly assumed that each ewe age group had equal reproductive success on average, and that any effect of ewe age on progeny performance was fully accounted-for in selection protocols — minor failure/s on these assumptions would diminish the synergy but on the other hand the synergy would be increased if across-age ewe selection or assortative mating were applied. The heritability of the breeding objective (or in other words the selection accuracy) and the reproductive rate are the determining factors of the extent of selection synergy. Merino breeding objectives typically combine high-heritability traits (e.g. fibre diameter) medium-heritability traits (e.g. fleece weight, body weight) and low-heritability traits (e.g. reproduction and type traits).

Table 5.6 *Effective female generation interval as reduced by selecting progeny of younger dams, due to selection accuracy, at three levels of reproduction rate.*

	Net reproductive rate		
Selection accuracy	**0.8**	**1.0**	**1.2**
1.0	2.94	2.80	2.70
0.9	2.98	2.84	2.74
0.8	3.02	2.88	2.78
0.7	3.06	2.93	2.83
0.6	3.11	2.98	2.89
0.5	3.17	3.05	2.95
0.4	3.24	3.12	3.03
0.3	3.32	3.21	3.13
0.2	3.41	3.32	3.25
0.1	3.55	3.48	3.43
0.0	3.90	3.90	3.90

Table 5.6 shows that, for example, almost equivalent results in effective female generation interval could be achieved with a breeding objective heritability of 0.8 and a reproductive rate of 0.8, or with a breeding objective heritability of 0.6 and reproductive rate of 1.0, or with a breeding objective heritability of 0.4 and reproductive rate of 1.2 — each case would reduce the

effective female generation interval from 3.9 to 3.0 years; which in turn would cause the allele frequency among each selected cohort to be around 0.85 of that predicted by the average allele frequency across all ewes. The benefits of this effect will compound over years. The effect is particularly beneficial from the time that DNA testing of rams commences until the DNA testing of ewes is completed — benefits flow from fewer carriers being culled, which also reduces the number of individuals DNA tested, and additionally the reduced female generation interval means that fewer ewes must be DNA tested. The synergistic effect would be maximised by switching to annual across-year selection in the breeding flock, but few breeders would readily embrace such a significant management change.

There may be opportunities for breeders to employ certain "tricks" on a short-term basis to assist in eliminating pigmentation alleles. First, as described above, allele frequency is reduced and costs are reduced if the female generation interval is tightened. This might be achieved by giving the progeny of younger ewes a slight premium in their breeding values, or simply by increasing the breeding objective emphasis on high heritability traits — either approach would have the net effect of reducing selection intensity applied to the performance breeding objective. Second, a higher reproduction rate helped to detect carriers for paternal culling. Third, it has been noted earlier that paternal culling is more effective and less expensive if sires are retained in the flock for longer. Fourth, by contrast, all other selection strategies benefited from a shorter generation interval so that generational selection gains accrued in a shorter period of years. Fifth, when DNA gene tests are first marketed, breeders may avoid some early financial losses by initially testing stud rams only, and introduce testing for sale rams at a later date. Whether this option is possible in practice will depend heavily upon the attitude of ram buyers; it may be that the market for untested rams diminishes rapidly in volume and value. Breeders should be strongly advised however, against adopting any strategy which would slow the rate of allele elimination in the stud flock.

The exact optimisation of all these considerations would depend upon many factors such as the initial allele frequency and the usual generation interval. It would be possible to use an optimising process such as genetic algorithm to chart the best possible breeding design in terms of both pigmentation alleles and the remainder of the breeding objective.

Design optimisation could also consider the possibility that high-performing carrier rams could be bred in the hope of producing high-performing non-carrier sons. This option was not considered in the simulation because the resulting carrier sons could not have been sold for breeding, costing in the order of tens of thousands of dollars — but in practice a breeder may be able to make accommodation by temporarily expanding the ram-breeding flock.

If the DNA technology is developed to allow for the pooled test of a sample obtained from a number of animals, then this would have the potential to reduce dramatically the cost of gene testing and elimination. Testing of ewes is a large part of the cost of DNA testing, even if the

allele frequency is known to be very low. Allele detection from a combined blood sample from, say, five individuals would reduce the number of tests required by at least 75% provided that less that 1% of animals were carriers. Within the limits imposed by the technology, the number of individuals to be pooled could be optimised according to the expected number of carriers.

As pointed out by Amer *et al.* (2005) the cost of any one application of DNA technology is dramatically reduced if it is used in concert with other applications of DNA technology. For example, a flock that is routinely using DNA-fingerprinting to establish pedigree is likely to be able to add testing for particular undesirable alleles at a small marginal cost. Similarly if a quantitative trait locus (QTL) is to be utilised to rapidly enhance a production trait or disease resistance. As time goes on and the ovine genome slowly reveals its secrets, the opportunities to spread the cost of DNA testing across multiple uses are likely to increase.

The results presented here could be adapted to any Mendelian trait in a situation where undesirable alleles are to be eliminated. One small difference would arise if affected phenotypes were not automatic culls, as for pigmentation traits, but were capable of a normal productive life.

In conclusion, the main findings of this study may be summarised in the form of recommendations to breeders:

1. Start now to reduce the frequency of pigmentation alleles.
2. Paternal culling is cost-effective in the short-term and has long-term benefits in reducing allele frequency.
3. Adopt DNA gene testing for rams at the earliest opportunity to drive the elimination of pigmentation alleles, to maximise ability to supply certified rams, and to take advantage of early premiums for non-carrier rams.
4. For a ram-selling flock, gene testing for ewes should also start early — with the ewe progeny of the first tested rams. In other flocks ewe testing may not be worthwhile.
5. Joint selection for two pigment forms, where possible, will be more efficient.
6. Consider temporary manipulation of the flock structure to maximise the rate of progress in pigmentation and the remainder of the breeding objective — the precise optimisation will depend upon individual circumstances.
7. Devise an efficient testing strategy for ewes.

5.5 References

Amer, P.R., Sherlock, R.G. and Tate, M.L. (2005). Using assortative matings to maximise sales of homozygous favourable sheep sires, a simulation study. *Proceedings of the Association for the Advancement of Animal Breeding and Genetics* **16**: 64–67.

Brooker, M.G. and Dolling, C.H.S. (1969). Pigmentation of sheep III. Piebald pattern in Merinos. *Australian Journal of Agricultural Research* **20**: 523–532.

Falconer, D.S. (1985). Introduction to Quantitative Genetics. Second ed. Longman, England.

Fewell, S. (2001). Strategies to Manage and Eradicate Recessive Genetic Disorders from Animal Populations. BSc Honours Thesis, University of New England.

Kinghorn, B.P. (1999). Use of segregation analysis to reduce genotyping costs. *Journal of Animal Breeding and Genetics* **116**: 175–180.

NSW Treasury (2007). NSW Government Guidelines for Economic Appraisal. http://www.treasury.nsw.gov.au/__data/assets/pdf_file/0016/7414/tpp07-5.pdf, accessed 26 June 2008.

Parsons, Y.M, Fleet, M.R. and Cooper, D.W. (1999). The *Agouti* gene: a positional candidate for recessive self-colour pigmentation in Australian Merino sheep. *Australian Journal of Agricultural Research* **50**: 1099–1103.

Percy, A. and Kinghorn, B.P. (2005). A genotype probability index for multiple alleles and haplotypes. *Journal of Animal Breeding and Genetics* **122**: 387–392.

6. Will the wool industry benefit from elimination of inherited pigmentation?

6.1 Introduction

Research is underway by the CRC for Sheep Industry Innovation to locate genes responsible for pigmentation in Australian Merino sheep. If successful, this work will make diagnostic gene tests available to breeders at reasonable prices. Gene testing could be used to cull carriers of pigmentation alleles and eventually to eliminate those alleles from the flock.

Decisions on whether to adopt gene testing and how to manage the implementation will largely rest in the hands of individual ram breeders, who will each face a different set of circumstances and harbour different attitudes. Chapter 5 has shown that the initial allele frequency in a flock will determine whether adoption of gene testing can be moderately profitable through to extremely costly for individual ram-breeding flocks.

In addition to ram breeders, the attitude of commercial breeders is difficult to predict and will vary widely. This will make it difficult to achieve an industry consensus on where and how gene testing should be used.

While non-economic considerations are also important, the clearest possible picture of the economic impact on the industry as a whole would help to guide the shape of wool industry protocol with respect to gene tests for pigmentation alleles. Each possible avenue of expenditure and return should be considered. In addition, attention should be given to the prospects for revenue streams to be captured on-farm rather than in the processing sector or by consumers.

This chapter will test the hypothesis that thorough economic analysis can clarify the outlook for DNA technology to eliminate inherited forms of fleece pigmentation from the Merino wool industry.

6.2 Internal costs

Many of the costs associated with the occurrence of pigmented sheep in a flock, and also of a program for the elimination of pigmentation alleles, are internal to the wool industry. For example, a sheep carrying fleece pigmentation is unsuitable for wool production or breeding, and exits the industry, sold or consumed as meat. However the vacated position in the flock will be filled by some other candidate, albeit one with a lower level of production. For the individual breeder this substitution represents a loss of value due to pigmentation, because where a surplus animal might have been sold for breeding or wool growing, now that surplus animal is reduced to meat value. But for the industry as a whole the number of animals retained

as breeders, and consequently the number of animals slaughtered for meat, is unlikely to be affected and thus the cost remains internal to the wool industry. The effect on selection intensity will be treated separately.

Similarly for rams or ewes which are identified as carriers of pigmentation alleles — their value as breeders to retain or sell is changed, which affects the returns of the breeder, but within the industry replacements will be recruited and the outcome is revenue-neutral. Conversely if a premium applies to tested non-carrier rams, the extra money changes hands but stays within the industry. Two exceptions arise when demand for surplus breeders exceeds supply and these are considered below.

6.3 Selection intensity — allele elimination with DNA testing

The culling of pigmented phenotypes and known carrier animals affects the future merit of a flock by reducing selection intensity for the remainder of the breeding objective — traits affecting production, quality, reproduction and disease resistance. This effect is extended to the industry as a whole and reduces the profitability of the entire industry.

Allele elimination by DNA gene testing would initially accelerate the loss of selection intensity due to greater selective pressure against the pigmentation alleles. An efficient strategy would complete the elimination in 8–10 years and thereafter all selection intensity will be available for other traits; however the cumulative loss of selection intensity under DNA testing is equivalent to many decades of phenotypic culling. Phenotypic culling can lead to eventual extinction of pigmentation alleles in a closed flock but in practice even rare transfers into the flock are likely to frustrate that objective. In any event the time scales involved are well beyond the scope of business planning. It is to be hoped however, that the wool industry can be encouraged to a long term vision that will inspire short term action to eliminate inherited pigmentation.

Atkins (1993) considered a commercial medium wool enterprise (average fibre diameter 22.5μm) and calculated an optimum rate of genetic progress across the breeding objective of $0.40 per dry sheep equivalent (DSE — a measure of grazing resource) per year (2.7% of gross margin). Atkins (1993) then categorised Merino ram breeding flocks into three levels of genetic gain according to their performance in realising the potential. On average, high gain flocks realised $0.22, medium gain flocks realised $0.15 and low gain flocks realised $0.08 per DSE per year.

Over the years since 1993 the wool industry has regularly been under financial pressure and the Australian flock has reduced in number to historic lows. However the flock reduction has been skewed against the medium and strong wool types, reflecting a higher degree of profitability for fine wool production. Meanwhile, genetic technologies for Merino breeding have been enhanced, mainly in the area of across-flock genetic evaluation. Adjusting the figures of Atkins (1993) for the cumulative average rate of inflation in Australia from March 1993 to June 2007

of 47% (ABS 2007b) gives genetic gains of $0.32, $0.22 and $0.12 per DSE per year for the three levels of stud performance. Clearly these figures should not be regarded as individually accurate but the range will be used for illustration.

A population of 50 000 000 Merino ewes would equate to around 116 million DSE, accounting for the metabolic costs of breeding and growing replacement breeders, but excluding wethers. A typical ram breeding flock with a reproduction rate of 1.00 has selection intensity of around 1.62 applied annually. In losing one unit of selection intensity across the ram-breeding sector for one generation in order to eliminate pigmentation alleles, the wool industry would forego selective improvements in profitability valued at (116 million x 0.32/1.62 =) $23 million, $16 million or $9 million across the range of rates of progress given above. At a reproductive rate of 1.20, the underlying rate of progress would be greater so the losses would be about 9% higher. Conversely for a reproductive rate of 0.80 each would be about 6% lower. This genetic gain would be permanent so the annual loss of profitability must be accumulated over all future years. Using a discount rate of 7%, the Net Present Values were $325 million, $223 million and $122 million per unit of selection intensity.

In Chapter 5 the required loss of selection intensity was charted against the initial allele frequency for different methods of allele elimination. At the highest initial allele frequency (phenotypic incidence 0.05) the total of annual selection intensity expended on eliminating pigmentation alleles was 1.97 units. However at a more typical initial incidence rate of 0.01, only 0.74 units of selection intensity were required even for the least tractable mode of inheritance, ranging down to as little as 0.19 units. For a simple recessive inheritance, as applies to agouti pigmentation patterns, 0.34 units of selection intensity were required. In each case the total loss of selection intensity must be divided by the generation interval (taken to be 3.2 years in ram-breeding flocks) to allow for the number of selection events per generation.

For the time being there remains uncertainty as to the inheritance of *Australian Piebald*. While allele frequencies are not well estimated, they are also expected to vary substantially from flock to flock. The rate of genetic progress will also vary greatly between ram breeding flocks. However taking the moderate rate of genetic progress as representative of the industry as a whole and assuming an initial phenotypic incidence of 0.01, the loss of selection intensity would be valued at around (223 million x 0.34/3.2 =) $24 million for the simple recessive case up to (223 million x 0.74/3.2 =) $52 million in the worst case.

6.4 Selection intensity — phenotypic culling

Simulations predict that phenotypic culling can eventually lead to elimination of pigmentation alleles in a closed flock, but over an implausible time scale (Chapter 5). However most Merino flocks are not entirely closed in the long term which raises two related impediments to eradication. First, to the extent which interlinked flocks represent a single population, the need

for good fortune in the eradication of every last carrier increases with population size. Second, because allele frequency can fluctuate over time and is known to vary considerably from flock to flock at any point in time (Chapter 3) even low rates of transfer between flocks will tend to equalise allele frequencies, thus having greatest negative impact on flocks which have — through management or good fortune — had greatest success in reducing pigmentation alleles. These problems would tend to mean that the long-term impact of phenotypic culling predicted in the closed flocks simulated in Chapter 5 may be optimistic.

In some respects the elimination of undesirable alleles may be likened to the eradication of weed infestations. Panetta and Timmins (2004) state a key factor in the feasibility of weed eradication is the ability to detect individuals even at low densities. Recessively inherited traits demonstrably fail this test — at the extreme if there is only one breeding adult in the flock which is a carrier, then there is zero chance of detection by the production of a pigmented lamb.

The loss of selection intensity due to phenotypic culling was small (Chapter 5), but the effect on genetic progress would be repeated annually and the effect on the genetic mean would accumulate. Following the same assumptions as the previous section, this loss of selection intensity would have a net present value to the wool industry of $15 million under the high gain scenario, $10 million under medium gain and $6 million under a low gain assumption.

Because breeders have no choice but to cull pigmented phenotypes, the cost of phenotypic culling should be deducted from the cost of alternate strategies. Assuming the industry averages a medium rate of genetic gain and with other assumptions in the moderately good range, the value of net loss of selection intensity for allele elimination by DNA testing could be as little as ($24 million - $10 million =) $14 million. Or, if a higher initial allele frequency was assumed this might be ($52 million - $13 million =) $39 million. Thus a net cost for DNA testing is estimated in the range of $14–39 million.

6.5 Fleece contamination risk

Merino sheep found to have fleece pigmentation will generally be culled, although the thoroughness of surveillance and the determination to cull vary among commercial flocks; small patches of pigmentation may not be detected until shearing, or not at all. Pigmented fleece patches can be removed on the shearing board but dark fibres shed from such patches are likely to be dispersed through the lamb fleece. Hansford (2003) reports that Merino lamb fleece typically carries pigmented fibres at a relatively high rate, such that it should not be directed to sensitive end uses (i.e. light coloured fabrics). It is therefore unlikely that problems would be caused by additional small quantities of pigmented fibres in lamb fleece.

Following the introduction of Awassi, Damara, Dorper and Karakul breeds to Australia, the potential for cross-contamination has been a major concern. White Merino ewes can pick up contamination from exotic breed rams during mating, and also from pigmented cross-bred

lambs (Hansford 2003). The opportunity certainly exists for dark fibre transfer between pigmented Merino lambs and their mothers' fleece wool. Indeed Hatcher *et al.* (1999) found in a two-week pen trial that transfer of pigmented fibres between black Merino ewes and white Merino ewes was several times greater than between Awassi ewes and Merinos. Fleece contamination from black Merinos averaged 21.9 pigmented wool fibres per 10 grams compared to the control of 0.3 fibres/10g. Furthermore, they found that while worsted processing tended to remove the coarse Awassi fibres, the black Merino contamination had similar fibre properties to the host white wool and fibres were retained through processing thus having a greater relative impact in finished fabric.

Accurate tests exist for detecting dark fibres in raw wool and tops (Burbidge and McInnes 1994). Benchmarks have been established for the frequency of dark fibres in top such that 100 dark fibres per kilogram is considered the commercial limit for white and pastel end uses (Hansford 2003). Sampling of top for this testing is effective due to the mixing that occurs in early stage processing. However raw wool is much more difficult to sample adequately because some sources of contamination (e.g. physical transfer from pigmented animals) result in dark fibres distributed throughout a sale lot, while others (e.g. fleece pigmentation on the animal itself; urine stain). result in discrete clumps of affected wool (Hansford 2003). There is also a disconnection between results for raw wool and those for top because fibres may break during early processing, increasing the number of fibres per unit of weight. Therefore only loose benchmarks are possible for raw wool, whereby 10 or fewer dark or medullated fibres per 10 grams is classified as a low count, 11-20 fibres/10 grams a medium count and more than 20 fibres/10 grams a high count.

Dark fibre test results on random sale lots of Merino adult fleece wool (not identified by the classer as a dark fibre risk) bear out these considerations (AWTA 2007, unpublished data). The average of 9747 tests was 1.39 dark fibres/10g with 45% of samples returning a zero result. The distribution was similar to the Poisson distribution, or more realistically a combination of Poisson distributions, predominantly with low means (i.e. 0–2 dark fibres/10g). However a small number of results were much higher than predicted by this distribution: 0.6% of samples fell into each of the medium and high count categories and the highest result was 430 dark fibres/10g. The worst 1% of samples (greater than 12 dark fibres/10g) accounted for 44% of all dark fibres found. The distribution of low count results could be modelled by as few as four discrete Poisson distributions but the medium and high count results could not be adequately described in this way. Thus the count distribution gave no clue to distinguish between uniform contamination and clumps of contamination; the only assessment can be the expectation that the probability of clumps of contamination in the sample was higher when the dark fibre count was higher.

The 1.2% of test results exceeding the low count threshold would be expected to represent a range of contamination sources. The Federation of Australian Wool Organisations' Merino Dark and Medullated Fibre Risk Scheme Decision Tree (appended in Section 6.11 — Hansford 2003) distinguishes contamination risk based on sex between adult ewes and wethers due to the risk of urine stain where shearing occurs more than three months after crutching. This is based upon the limited investigations of Burbidge and McInnes (1994); it appears that the issue of Merino lambs as a potential source of ewe fleece contamination has not received further consideration — surprisingly, given that lamb fleece is classified as the much higher risk level 4 (Hansford 2003).

Unpublished data was made available to this study from a survey of Merino breeders conducted by the Australian Sheep Industry Co-operative Research Centre. Of 101 flocks surveyed, 19 flocks had an incidence of pigmented lambs exceeding 0.01 at their latest lambing. Extending this proportion to the industry as a whole, it is clear that this rate of incidence does not typically lead to dark fibre test results in the medium or high risk range. Rather, as might be expected, dark fibre transfer to ewe fleece wool from a few pigmented Merino lambs was much less than that caused by an entire crop of lambs from an exotic breed cross.

Ultimately there is insufficient data available to assess reliably the economic impact of the elimination of inherited pigmentation in Australian Merinos via fleece contamination. While it would be hoped that there will be a positive influence, the available evidence suggests the effect will be small in terms of detectable contamination in raw wool or top.

However it should be borne in mind that a single dark fibre contamination "episode" typically carries a very high cost. The wool processor suffers the cost of hand-picking fabric or dramatic devaluation of the processing batch and failure to meet orders (likely to involve payment of compensation), plus the cost of decontaminating machinery and loss of reputation. The wool in a processing batch is worth millions of dollars even at farm gate prices and much more as it moves through the processing chain. Moreover, a series of contamination episodes could threaten the future of a wool processing enterprise, simply due to bad luck. The whole industry ultimately shares any economic loss and any effort which eliminates a single contamination episode is positive, additionally contributing to economic stability within the industry.

6.6 DNA testing costs

Based on a test cost of $30, DNA testing for the eradication of pigmentation alleles from a Merino ram-breeding flock of 1000 ewes has been estimated to cost around $35 000 for the least-tractable inheritance models (although this figure was relatively insensitive to the inheritance model or initial gene frequency) plus an annual cost of around $8 000 to certify sale rams, making a 40 year total cost of around $360 000 (Chapter 5). Such a flock could produce rams capable of covering approximately 45 000 commercial ewes; thus DNA testing would

have cost the industry $8 per commercial ewe. This cost could be reduced if a more favourable inheritance model was at play, if it were possible to test pooled samples, or if ram buyers adopted a more trusting attitude and were prepared to forego individual testing after a stud flock is known to be free of genetic pigmentation. In any year, a proportion of commercial Merino ewes are mated to other breed sires, meaning that the cost of certified rams may be spread over a larger number of commercial Merino ewes. Some ram-breeding flocks are aligned with "daughter" or multiplier flocks selling rams to commercial flocks; this would extend the reach of the "parent" stud but any savings in DNA testing costs in the multiplier flock would be small. To take a more negative point-of-view, it is also possible that industry inefficiencies in breeding structures and the design of elimination strategies would push up the cost of a DNA testing solution.

Elimination of pigmentation alleles across the Merino seedstock sector would require in the order of 13 million DNA tests. This would seem to provide substantial scope for economies of scale, which may see the test cost reduced to $20 or even $10. The ideal might be a form of the test which could be used in the field with minimal expertise giving instant results -- perhaps a card that changes colour in the presence of blood from a carrier. This would be expensive to develop but would offer many practical advantages along with a low unit cost. It may be less difficult to design a form of the DNA test which could be used on a pooled sample from several animals. This would greatly reduce the cost of testing when the gene frequency is low — pooling strategies could be designed to minimise testing costs. The ability to pool up to five animal samples could reduce total DNA testing costs by more than half.

6.7 Crossbreeding

Many commercial Merino breeders opportunistically mate non-Merino rams to a portion of their Merino ewes when it is more profitable to do so. Effectively this means that rather than having surplus Merino ewes and wethers for sale they have crossbred progeny for sale either as meat animals or as breeding ewes for lamb production. When wool prices are low relative to sheep meat prices, this can be an important income boost. Sheep meat prices have been consistently good over recent years due to increasing demand.

Eliminating pigmentation alleles from Merinos would mean that fewer Merino x Merino matings are necessary to produce the required number of replacement Merino ewes. Thus a greater portion of the flock can be available to exploit crossbreeding opportunities. An assumed national flock of 50 million ewes requires the annual replacement of 10 million ewes. With an incidence of pigmented phenotypes of 0.005, it would be expected that at least 50 250 pigmented ewes must be culled to leave 10 million breeding ewes — even before allowance for any other selection. With a reproduction rate of 0.8 and balanced sex ratio, these pigmented ewes account for an extra 125 600 parental matings. In the absence of pigmentation genes these matings could have been switched to meat-breed sires and with a crossbred reproduction rate of

0.9 this would lead to around 113 000 lambs. If Merino lambs grown for slaughter were worth $70 and crossbred lambs were worth $100 then the combination of extra lamb numbers and more valuable crossbred carcases would gain the industry $4.3 million annually, equal to a net present value of $61 million (discount rate 0.07).

While particular circumstances would vary widely, an extension of these calculations can predict the value of a non-carrier ram compared to an untested ram on average. Each non-carrier ram in a commercial flock would return an extra $20.50 over its lifetime from the increased crossbreeding opportunity alone. This gives a partial basis for estimating a sensible premium on tested sale rams.

6.8 Limits on flock growth

Culling of pigmented phenotypes and known carriers reduces the supply of breeding rams and ewes to industry. This is not a limiting factor in the ordinary course of events because there are alternative sources of breeding stock within the industry. However in times when the Merino flock is rapidly expanding on a national or regional basis, demand for breeding ewes in particular may exceed supply. This might occur when the industry is experiencing a boom, or is recovering from a major drought.

A national Merino flock free from pigmentation alleles would have a higher net reproduction rate with respect to breeding females — a small effect but of non-trivial magnitude across the industry. This would allow the industry to respond more quickly to favourable economic signals, and to recover more quickly following drought. These benefits would occur irregularly and are difficult to quantify. Market forces would push up the price of a replacement Merino ewe such that a Merino x Merino enterprise was more profitable than meat breed x Merino — therefore if players are acting logically their revenue can only increase. Thus the increased flexibility provided by increased net reproduction could be expected to raise the long-term profitability of wool-growing by a small margin, while reducing the negative financial impacts of drought.

6.9 Discussion

Elimination of pigmentation alleles from the Merino population will cost the industry in terms of DNA tests and lost selection intensity, and provide returns through a reduction in contamination risks, and through increased flexibility in breeding to expand flock size or turn-off crossbred lambs when desired. Table 6.1 lists the best estimates of each category of costs and returns. The cost of individual DNA tests can be predicted reasonably well because equivalent technology is already on the commercial market, and dramatic cost reductions are unlikely. However the predicted cost of $30 per test may be reduced to $20 or even $10 as a lab-based test, and possibly reduced further as a field test. The way the technology is applied could also have a large effect on the total costs incurred. It is reasonable to expect that in future

DNA testing will serve an increasing range of purposes and an increasing proportion of elite animals will be tested for some purpose. As Amer *et al.* (2005) points out, if DNA testing is being performed simultaneously for dual or multiple purposes then the effective cost is reduced. To summarise possible cost reductions for DNA testing and a rough estimate of the extent of such savings:

- Economies of scale (33–66%)
- Pooled testing (50%)
- Efficient ewe testing strategy (40%)
- Combination with other DNA tests (40%)
- Market assurance scheme in place of continued sale ram testing (80%)
- Segregation analysis to better prioritise animals for culling and/or DNA testing (15%).

Both these categories of costs — DNA testing and loss of selection intensity — will be entirely borne by the on-farm sector of the industry. However the loss of selection intensity is not a cash expense and is only incurred as a loss of revenue to the industry.

At $30 per test, DNA testing to allele elimination is estimated to cost around $8 per commercial ewe, and the net loss of selection intensity is estimated to add less than $1 per head. In 2007 the Eastern Market Indicator of the price of wool is around 800 cents, so $8 equates to the value of a typical kilogram of clean wool. At $8 per commercial ewe, a population of 50 000 000 Merino ewes would cost the industry $400 million in DNA testing. The total cost is around 24% of the annual gross value of Australian wool production (ABS 2007a). This cost would be spread over at least 8–10 years and produce long-term benefits; it would appear to be an investment within the reach of the industry.

Table 6.1 *Costs and returns associated with elimination of pigmentation alleles from the Australian apparel wool industry. Discount rate 0.07.*

		Net present value	
		Total ($ million)	**Per commercial ewe ($)**
Costs	DNA testing	400	8
	Selection intensity	14–39	0.28–0.78
	Culling	internal	internal
Returns	Contamination	70 ?	1.40 ?
	Cross breeding	61	1.22
	Flock expansion	< 5	< 0.10

Returns to the wool industry will flow from greater net reproduction leading to flexibility in the capacity to expand the Merino flock or boost the sheep meat supply as economies dictate. Increased scope for crossbreeding is worth at least $1.22 per commercial ewe in net present value, while the value of faster expansion of the Merino flock is likely to be much less. The change would have a very small impact on the national supply of sheep meat, so the on-farm sector could expect to reap most of the benefits.

Returns from reduced dark fibre contamination risk across the industry are very difficult to measure. To reach a "break-even" point these returns must reach in the order of $350 million net present value which would equate to an annual value of $24.5 million. This magnitude of return could accrue through preventing 4–8 top contamination incidents per year; these numbers would seem to be at the uppermost range of what may be attributed to this pigmentation source. Similarly, an annual return to growers of $24.5 million would require an average market premium across the entire Australian clip of around 1.5%, which seems optimistic — for Table 6.1 this return was set to one fifth of that value.

The costs and returns presented in Table 6.1 relate to the entire Australian Merino flock. Naturally, the technology may be adopted by certain segments of the industry and not others. However, to the extent which the segmentation applies evenly across the ram-breeding and commercial sectors, the economic values are essentially scalable so that the overall economic picture would not change.

On this basis the economic case for elimination of pigmentation alleles is certainly not strong, with an overall net present value likely to be zero or less — with the assumed individual DNA testing at a cost of $30; there are several possible means for reducing the cost of DNA testing which could reshape the picture. So far, no account has been taken of the cost of research to locate the genes and devise diagnostic tests. It should be noted however that research costs are likely to be much less than the total cost of DNA testing — as a point of comparison, the major Australian ovine genomics collaboration, SheepGenomics, has a 5-year budget of $30 million.

Unpublished data made available from a survey of Merino breeders conducted by the Sheep CRC indicated strong support for DNA testing at a projected test cost of $30. From a total of 101 responses, 87% of stud breeders said they would expect the test to have been performed in relation to any of their ram or semen purchases. Of commercial breeders, 79% said they would require testing on their ram purchases while 62% said they would DNA test rams after purchase.

In a free market context, it may well be that some ram breeders and commercial wool producers will enthusiastically embrace DNA testing while others do not. The cost calculations here can be scaled to any fraction of the national flock. If the use of tested rams is incorporated into existing quality assurance protocols, then those who adopt the technology will have the opportunity to achieve higher wool prices from risk-averse processors.

Non-tangible benefits may arise through enhancing the reputation of Australian wool for quality. The nature of the wool/fashion industry means that consumers are not particularly sensitive to the price of the end product provided that their perceptions of quality are satisfied. By working to eliminate pigmentation genes as a means to reduce dark fibre contamination, Australian wool growers would be seen to be taking a strong stand in favour of wool quality in future decades. Broad support from the wool industry for elimination of pigmentation alleles

when the DNA testing technology becomes available would comprise a strong vote of confidence in the long term future of the apparel wool industry. This is in addition to the financial and practical advantages which could accrue through a co-operative approach and efficient allele elimination strategy across the Australian Merino flock.

By contrast, failure to tackle inherited pigmentation even when the means is at hand, could send an unfortunate message to wool processors and the fashion industry: that growers are indifferent to an issue which regularly causes serious problems throughout the supply chain and can even threaten the livelihood of its participants.

6.10 References

Amer, P.R., Sherlock, R.G. and Tate, M.L. (2005). Using assortative matings to maximise sales of homozygous favourable sheep sires, a simulation study. *Proceedings of the Association for the Advancement of Animal Breeding and Genetics* **16**: 64–67.

Atkins, K.D. (1993). Benefits of genetic improvement to the Merino wool industry. *Wool Technology and Sheep Breeding* **41**: 257–268.

Australian Bureau of Statistics (2007a). Australian farming in brief. ABS Catalogue No. 7106.0.

Australian Bureau of Statistics (2007b). Consumer Price Index, Australia, Jun 2007. ABS Catalogue No. 6401.0.

Burbidge, A. and McInnes, C.B. (1994). Dark fibre risk and prediction. Proceedings Woolspec 94 Seminar: Specification of Australian Wool and its Implications for Marketing and Processing. CSIRO Division of Wool Technology and International Wool Secretariat, Sydney.

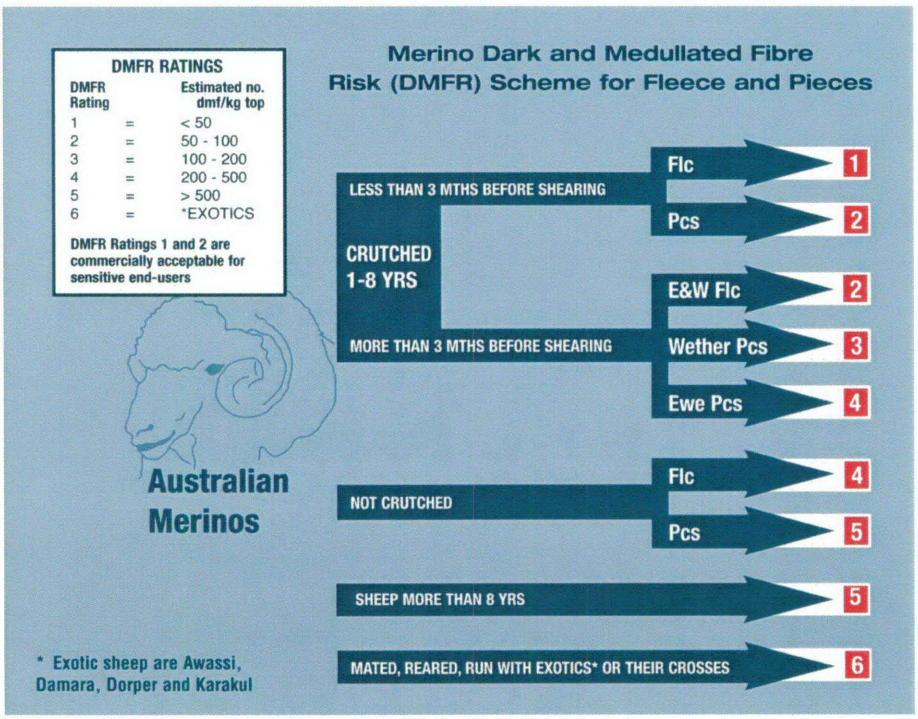
Hansford, K.A. and Australian Wool Industry Working Group (2003). The development of a quantified risk factor for the presence of dark and/or medullated fibres in Australian Merino wool. International Wool Textile Organisation, Technology and Standards Committee, Istanbul, Report RWG7.

Hatcher, S., Foulds, R.A., Lightfoot, R.J. and Purvis, I.W. (1999). The relative wool contamination potential of Awassi and black Merino sheep when penned together with white Merinos. *Australian Journal of Experimental Agriculture* **39**: 519–528.

Panetta, F.D. and Timmins, S.M. (2004). Evaluating the feasibility of eradication for terrestrial weed incursions. *Plant Protection Quarterly* **19**: 5–11.

6.11 Appendix: Federation of Australian Wool Organisations' Merino dark and medullated fibre risk scheme decision tree (after Hansford 2003.)

This decision tree allows the risk of dark and medullated fibres to be determined for wool from adult ewes (E) and wethers (W) in fleece (Flc) and pieces (Pcs) lines, according to crutching status, age and contact with pigmented breeds.



7. General discussion

7.1 Inherited problems

It is disappointing that 40 years after the CSIRO's first investigations of the inheritance of *Australian Piebald* (Brooker and Dolling 1969), very little progress can be reported. While work is continuing for the symmetrical forms of pigmentation, it can only be hoped that some benevolent agency can in future allocate the necessary resources to run a few dozen sheep over a period of 3–4 generations and thus provide the means to finally resolve piebald inheritance.

The low incidence of piebald in the Sheep CRC Pigmentation Resource Flock meant that insufficient animals were generated to have any realistic chance of mapping the causative gene. Financial and practical issues prevented the retention of F_1 ewes for further matings, and so that resource is now lost, save for a collection of frozen blood samples and semen from the F_0 sire.

The outside observer may consider that it was unwise for the Sheep CRC to have committed resources to breeding for a character of unknown inheritance, and that view has some validity. However risk is always an element of research, and surely a scientist should not be criticised for allowing their optimism occasionally to override eternal caution. In any event, a breeding trial to establish the mode of inheritance would have caused unreasonable delays; there remains no indication that this question will be taken up elsewhere.

Given the inadequacy of simpler models to explain piebald inheritance, it seems likely that molecular genetics will have an eventual role to play in unravelling the mystery. However the current state of knowledge requires that a conventional breeding experiment is the logical next step. Recording the outcome of further piebald x piebald matings and their repeatability would seem to be the most informative option.

7.2 Gene discovery

Homozygosity mapping has great potential compared to other mapping methods based on the relative flexibility of requirements as to pedigree structure and the modest demands as to number of individuals. The theoretical particulars form a sound basis, which has been built upon by the experience of widespread use in humans and extensive success in gene discovery.

Homozygosity mapping promises similar advantages in livestock and other species, although special constraints may apply in any new situation — for example a lack of pedigree information, typical pedigree structure and the ability to conduct planned matings specifically aimed at gene discovery. These considerations will determine whether experimental material can be drawn from existing populations, or alternatively that it can be generated from planned matings. If planned matings are necessary then the requirement for a heterozygous carrier as

patriarch of the pedigree could impose an additional burden and delay on the experiment. There may therefore be benefits if an affected patriarch can be substituted without substantially affecting the experimental power.

Simulation studies presented here have demonstrated this potential and have also shown that other departures from the homozygosity mapping design of Lander and Botstein (1987) can be tolerated. Importantly, various inheritance models beyond simple recessive were amenable to homozygosity mapping. The background frequency of disease alleles would ideally be low or zero, but departures were quite manageable. Use of widely-spaced markers across a greater number of individuals was found to be most efficient. The simulation will also inform other elements of the breeding design and genotyping strategy.

The use of DNA pooling for homozygosity mapping in humans seems to have been unreasonably restricted, and a less cautious approach would be encouraged across all species. With the potential to slash the genotyping effort and cost, DNA pooling makes gene discovery a realistic possibility in many new situations. Theoretically, DNA pooling could even be used with SNP genotyping but this has not been reported in the literature. The current trend appears to be towards individual genotyping by SNP chip because this is an efficient means of generating dense genotype data. However the use of widely spaced, highly informative microsatellite markers with homozygosity mapping may be a more efficient means of gene mapping.

While a number of pitfalls have been documented with homozygosity mapping, and they should always be guarded against, their occurrence has been quite rare — it is not necessary for practitioners to be excessively cautious or for rigid constraints to be imposed on experimental design.

7.3 Adoption of DNA testing to eliminate pigmentation genes

The most important factor affecting adoption of gene testing in a ram-breeding flock was the expected level of demand for certified rams (tested free of pigmentation alleles). The level of demand would affect the price premium available for certified rams, and would impact revenue from ram sales through the ability to meet the demand for certified rams. Within that parameter, the economic consequences of gene testing were directly affected by the initial allele frequency in each flock. A lower allele frequency was advantageous and the net financial outcome declined in an approximately linear relationship with increasing allele frequency. Implementing an efficient testing strategy was beneficial through reducing the number of individual gene tests required.

At an industry level, the test cost was the most important variable in the assessment of the costs and returns of widespread adoption of DNA gene testing. At $30 per test, the total cost of testing was estimated at $400 million if adopted by all ram-breeding flocks, or $8 per

commercial ewe; returns were considered unlikely to exceed the costs. However these values can be simply scaled to show the consequences of altering the test price. A price of $10–15 per test would obviously be much more competitive. In addition, if testing of pooled samples was technically possible for the same price, then at least half of the testing costs could be avoided. The cost of lost selection intensity was modest compared to the cost of DNA testing.

The economic effects of eliminating inherited pigmentation as a source of dark fibre contamination are extremely difficult to estimate. However contamination episodes are very costly to the processing sector, and ultimately to wool growers. Therefore there are potential indirect gains from maintaining and improving the quality perceptions of Australian wool through taking a proactive stance on pigmentation. This could apply at the industry level if a consensus can be forged to eliminate pigmentation, or it could apply to individual growers marketing their wool with quality assurance documentation.

7.4 DNA technology to eliminate inherited pigmentation

Homozygosity mapping could certainly be applied as a primary search tool in locating the *Australian Piebald* locus or loci. Such a search could utilise relatively modest numbers of animals generated from planned matings and efficient genotyping with DNA pools and widely-spaced markers.

Supposing that this search resulted in a single critical region of around 5cM, significant work would be required through a combination of fine mapping, candidate gene search, and sequencing for mutation detection. This aspect has not been investigated here, but should not be underestimated. However the continually expanding knowledge of the sheep genome (Maddox and Cockett 2007) tends to make the task less arduous. In addition, the on-going accumulation of a library of DNA samples from piebald sheep will be a valuable resource.

This phase of work is approaching conclusion for the *Agouti* locus which causes symmetrical pigmentation patterns in Merinos. It is hoped that in the near future the Sheep CRC will able to release a conclusive gene test for the white *Agouti* allele.

Reliable information for Merino breeders will be important to assist decision-makers at the flock level to take appropriate action when gene test/s are available, and indeed in anticipation of gene testing. Of course, individual ram breeders must judge the future level of demand from their clientele for tested rams. In general ram breeders would be advised to pre-empt gene testing by increasing selection against pigmentation alleles through paternal culling, and to adopt at least within-flock gene testing at the earliest opportunity.

Adoption of gene testing in the wool industry will effectively increase reproductive efficiency and improve the productivity of breeding flocks to produce both Merino replacements and crossbred animals for meat production. This would lead to increased revenue, but would also tend to improve the flexibility of the industry and enhance responsiveness to economic signals.

7.5 References

Brooker, M.G. and Dolling, C.H.S. (1969). Pigmentation of sheep III. Piebald pattern in Merinos. *Australian Journal of Agricultural Research* **20**: 523–532.

Lander, E.S. and Botstein, D. (1987). Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* **236**: 1567–1570.

Maddox, J.F. and Cockett, N.E. (2007). An update on sheep and goat linkage maps and other genomic resources. *Small Ruminant Research* **70**: 4–20.