APPLICATIONS OF MOLECULAR GENETIC AND REPRODUCTIVE TECHNOLOGIES IN THE CONSERVATION OF DOMESTIC ANIMAL DIVERSITY

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INTRODUCTION

The process of conservation of genetic diversity requires identification of genotypes in danger of erosion or extinction, design of strategies for their preservation, and implementation of a workable and effective programme.

Targeting of populations for conservation efforts can involve several steps, the first being acquisition of evidence to support a need to intervene based on population sizes and threats to their integrity and/or existence. Although it is a debatable issue, it can be argued that in addition, evidence of the potential usefulness of threatened populations should be obtained to justify conservation effort. A broader view is that a defined degree of uniqueness at the level of the genome, that is in its DNA base sequence *per se*, could qualify a population or even an individual for special attention. Whatever the general approach however, molecular genetic characterisation and the estimate of genome rarity which it can provide, is at the very least a useful step. This is especially the case where conservation programmes are resource limited, when it becomes an essential step in the choice of populations to be targeted.

Estimates of genetic uniqueness are usually obtained through measurements of genetic distances. In the first part of this paper we discuss recent advances in molecular technologies for genetic characterisation of livestock populations and consider their technical merits and their potentials to provide the information required to guide population selection. We then review the reproductive technologies which are available and being developed, and which have applicability for the process of preservation, whether through *in situ* or *ex situ* approaches.

MOLECULAR GENETIC CHARACTERISATION

Polymorphisms in gene products such as enzymes (isoforms), blood group systems and leukocyte antigens have now been almost entirely superseded by polymorphisms at the level of DNA, both nuclear and mitochondrial, as a source of information on which to base estimations of genetic distances. DNA polymorphisms may be detected in a variety of ways, the most common being restriction fragment length polymorphisms (RFLPs), variable number tandem repeats (VNTRs) in the form of mini and microsatellites, and randomly amplified polymorphic DNAs (RAPDs). The ultimate form of DNA characterisation is the complete sequencing of the genome. As this is not a practical possibility with livestock species, sequencing of selected regions of both nuclear and mitochondrial genomes is performed and provides a further option for genetic characterisation. These approaches will be described and the factors affecting their application will be discussed.

RFLPs: The first DNA polymorphisms to be widely used for genome characterisation and analysis were RFLPs (Southern, 1975) which detect variations ranging from gross rearrangements to single base changes. The polymorphisms are found by their effects on sites for restriction enzyme-mediated cleavage of preparations of high molecular weight DNA. Following enzymatic digestion, the sizes of particular DNA fragments are

determined by means of gel electrophoresis and identification with appropriate DNA probes. This method has been used extensively to reveal polymorphism in DNA and to characterise populations of a variety of microbial, plant and animal species (Andersen and Fairbanks, 1990; Prince *et al.*, 1992; Karl *et al.*, 1992; Megnegneau *et al.*, 1993). In the case of livestock species, it has not found great favour for nuclear DNA characterisation, probably because it is an expensive and relatively laborious approach for this purpose. In contrast, the small size of the mitochondrial genome lends itself to RFLP analysis (Heindleder *et al.*, 1991). This technique has been effectively applied to reveal polymorphisms in selected mtDNA regions which exhibit relatively high variation, following amplification by polymerase chain reaction (PCR) (Suzuki, Kemp and Teale, 1993). This approach further simplifies mtDNA characterisation because target DNA can be generated from small blood or tissue samples.

VNTRs: For the purpose of this discussion, VNTRs can be considered to comprise two basic types, mini and microsatellites. These differ in the length of repeated sequences at any given locus, possibly in their genome distribution, and in the methods generally utilised to detect them.

Minisatellites: Minisatellites (Jeffreys, 1979) have been used to generate DNA fingerprints typical of individuals within a species (Jeffreys, 1987; Jeffreys and Morton, 1987). A given minisatellite is a sequence up to 60 or so bases in length which can be repeated many hundreds or thousands of times at one unique locus within the genome or, as is often the case, at multiple loci. The attraction of minisatellites is that a single probe based on a chosen repeat sequence could reveal numerous polymorphisms at numerous loci. This methodology involves probing of restriction-digested DNA transferred to a membrane support and, as in the case of RFLP analysis of nuclear DNA, presents a disadvantage in being somewhat tedious and costly by comparison with newer approaches. Further, the complex fingerprints obtained with multilocus probes can be difficult to interpret. Nevertheless minisatellite analysis has proved useful in genetic characterisation of a variety of organisms from lions (Packer *et al.*, 1991) to commercially important plants (Nybom and Hall, 1991).

Microsatellites: Microsatellites (Weber and May, 1989; Stallings *et al.*, 1991; Tautz, 1992) comprise repeats of very simple sequences, the commonest in most genomes being repeats of dinucleotides. They appear to be abundant in the genomes of all complex organisms, including all livestock species which have been examined (Fries *et al.*, 1990; Swarbrick *et al.*, 1991; Ellegren *et al.*, 1992; Wintero *et al.*, 1992; Kemp *et al.*, 1993; Khatib *et al.*, 1993). The simple CA/TG repeat, for example, occurs at many thousands of loci. Polymorphism of microsatellites takes the form of variation in the number of repeats at any given locus and is generally (although not exclusively - see below) revealed as fragment length variation in the products of PCR amplification of genomic DNA using primers flanking the chosen repeat sequence and specific for a given locus (Kemp & Teale, 1991). Microsatellites undoubtedly offer a very promising means of detecting polymorphism in the nuclear genome, because of a number of factors. These include the ease with which they can be identified and sequences of flanking regions determined as a prelude to primer design (Brenig & Brem, 1991; Cornall *et al.*, 1991; Yuille *et al.*, 1991; Baron *et al.*, 1992; Brezinsky *et al.*, 1992; Moore *et al.*, 1992; Koref *et al.*, 1993), and the requirement for only very small amounts of target DNA in the analysis procedure. Moreover, methods are being developed which will simplify detection and analysis of microsatellite polymorphism (Hughes, 1993; Kimpton *et al.*, 1993; Litt *et al.*, 1993; Schlotterer, 1993). A very important attribute is that polymorphisms can be described numerically, and thus the system lends itself to computerised data handling and analysis. Another advantage is that the sequence of relatively short (ca. 20 base) primers, information which is easily shared between collaborators, constitutes the only requirement to examination of a given microsatellite locus. As more studies of microsatellite polymorphisms are undertaken in cattle, for example, it is becoming clear that they offer an excellent means of distinguishing closely related breeds, and it is not proving difficult to identify breed and population specific alleles (Bradley and MacHugh, pers. comm.).

As inferred above, microsatellites can also be used in non-PCR based systems in much the same way as minisatellite probes (Haberfeld *et al.*, 1991; Kashi *et al.*, 1991; Trommelen *et al.*, 1993) and are capable of revealing high levels of polymorphisms. The disadvantage again, however, is that the method requires enzyme-mediated cleavage of target DNA and probing of membranes following Southern transfer of the products of digestion.

RAPDs: PCR amplification on a random basis (Welsh and McClelland, 1990; Williams *et al.*, 1990) has been used extensively for genetic characterisation purposes in a wide variety of organisms ranging from bacteria to mammalian species (Crowhurst *et al.*, 1991; Kleinlankhorst *et al.*, 1991; Welsh *et al.*, 1991; Ballingercrabtree *et al.*, 1992; Levin *et al.*, 1993; Tibayrenc *et al.*, 1993). This technique utilises short (9 or 10 base) primers, usually singly, to amplify nuclear DNA in the PCR. The important feature of this approach is that it does not require knowledge of the sequence of the DNA under study. Primers are designed on a random basis, with the sole constraint being G/C content. The basis of the polymorphisms detected with this methodology is that products are either generated in PCR or not. This is determined by the distribution of sites complementary to the primer sequences in the target DNA. Another principal advantage of the approach is that the levels of detectable polymorphism are generally high, with most primers generating a number of polymorphic products. The principal disadvantage of the methodology is that the PCR results are very sensitive to amplification conditions (Yu and Pauls, 1992) and consequently can be variable between laboratories and even between assays. An important development however, was the demonstration of the effectiveness of RAPD analysis on DNA pools (Michelmore *et al.*, 1991). In these studies, two pools of DNA constructed by mixing the DNAs of individuals of two extreme phenotypes in an F2 plant population were subjected to RAPD analysis, and polymorphisms associated with the phenotypic difference were identified. This so-called 'bulked segregant analysis' approach has been taken further for the purpose of identifying RAPDs characteristic of outbred, but distinct, cattle populations (Kemp and Teale, in press; Gwakisa *et al.*, in press). In these studies, randomly primed DNA segments were identified which proved to be characteristic of the two major sub-species and of breeds within the sub-species. The particular advantage of this refinement to the RAPD method is that by pooling DNAs derived from populations of interest, average sets of products are obtained in the amplification process which mask variability within pools and highlight the more consistent differences between pools.

Once population-characteristic DNA products are identified in pooled PCR they can be cloned and used as probes for PCR products obtained with the relevant primer. In this way many of the disadvantages and virtually all of the variability of the RAPD method can be avoided.

Sequencing: Sequencing of the genome provides the ultimate in terms of genetic characterisation. Hitherto, sequencing even relatively small regions, has been a laborious and expensive exercise. This is gradually changing with the development of automated sequencing hardware and software. To date, however, the direct sequencing approach has not been widely used for genetic characterisation of livestock species, except in the case of variable regions of the mitochondrial genome (Loftus *et al.*, in press). In other species, the mtDNA sequencing approach has been used more extensively and in combination with PCR amplification of target regions such as the D-loop and cytochrome-b gene (Morin *et al.*, 1993; Baker *et al.*, 1993).

Discussion of the various analysis methodologies which can be applied to the data generated by the different molecular genetic characterisation approaches is not within the scope of this paper. However, before leaving this topic, it is important to give some consideration to the interpretation of DNA polymorphism data in terms of the genetic distances which can be derived. It is becoming increasingly clear that different approaches may give different answers, at least in the best studied of the livestock species, cattle. It is for instance apparent that Y chromosomes examined in some populations of *Bos taurus* breeds in Africa are derived from the *B. indicus* sub-species (Bradley *et al.*, 1994; Teale *et al.*, unpublished). Conversely, mtDNA data do not reveal significant *B.*

indicus introgression into African *B. taurus* populations (Suzuki *et al.*, 1993). In comparing African zebu and taurine breeds, microsatellite analysis (predominantly detecting autosomal polymorphisms) provides yet another view of the relationships between these types, which is intermediate between that resulting from the Y chromosome and mtDNA polymorphism studies (Bradley, pers. comm.). This situation may well be the consequence of widespread use of zebu males to introduce zebu genes onto the African continent, rather than the establishment of zebu breeding populations *per se* on a significant scale. Most importantly, this situation demonstrates the need for care in selection of characterisation approaches and in interpretation of the data generated, when assessing genetic distances within species.

REPRODUCTIVE TECHNOLOGIES

Normal reproduction in living stocks for conservation of domestic animal diversity (DAD) is costly and subject to several hazards, including loss due to disease, genetic bottlenecks from fluctuating numbers, accumulative genetic drift, inbreeding depression, contamination from other stocks and changes due to natural selection. High costs and hazards can be avoided by cryopreservation. Importantly, experience with regenerating stocks from frozen semen and embryos has been good.

Reproductive technology has developed rapidly during the past 10 years. Some of the new techniques developed around embryo transfer could be used for conservation of DAD and will increase the efficiency of the storage of frozen gametes or embryos for this purpose. These techniques include *in vitro* production of embryos, embryo cloning, and *in vitro* maintenance of pluripotential stem cells. Here we describe recent developments in these techniques and their potentials in conservation of DAD.

Developments

Cryopreservation of embryos: At present, in all domestic animals except buffalo, offspring have been produced after transfer of frozen-thawed embryos. In cattle, the transfer of frozen-thawed embryos has become a commercial practice and the survival rate of embryos after thawing can be over 80% with a pregnancy rate of around 50%.

Cryopreservation of oocytes: Cryopreservation of mouse ovulated oocytes and live births after fertilisation *in vitro* was achieved in 1977 (Whittingham, 1977). Unfertilised mouse oocytes have been frozen by the vitrification method and birth rates have reached 51% and 25% following *in vivo* and *in vitro* fertilisation, respectively (Kono, 1991). Cryopreserved bovine oocytes have been *in vitro*-matured (IVM) and the *in vitro*-fertilised (IVF) zygotes developed to blastocyst stage (Lim *et al.*, 1991; Xu and Betteridge, 1992). The first human twin pregnancy after cryopreservation was reported in 1986 and many others have followed (Friedler *et al.*, 1988). These results strongly suggest that long-term cryopreservation of mammalian oocytes will be successful.

***In vitro* production of embryos**: A commercial organisation has reported receiving 400 ovaries per day yielding about 1000 usable oocytes (Massey, 1990). Approximately 20% of all oocytes matured and were successfully fertilised *in vitro* to yield viable embryos. Approximately 50% of IVM oocytes can be used as recipient oocytes for nuclear transfer, producing about 20% viable embryos when fused and cultured for 5 days in sheep oviducts. In large-scale IVP (*in vitro* production) of bovine embryos, an IVF rate of 75.6% and a blastula rate of 28.9% have been achieved from IVM oocytes; 12.2 oocytes were collected from each ovary on average, yielding 3.06 blastocysts. This level of production is comparable with that from superovulated cows. Calves have been obtained following transfer of both split and frozen-thawed IVP embryos. Lu and coworkers (Lu *et al.*, 1990) reported pregnancy rates for fresh and frozen-thawed IVP embryos of 58.3% and 50%, respectively.

Embryo cloning: The two principal methods of embryo cloning are embryo bisection (splitting) and nuclear transplantation (NT). In a large proportion of cases, two embryos form if an embryo is bisected at any stage from 2-cells up to, but not beyond, early blastocyst stage (Woolliams and Wilmut, 1989). Hitherto, offspring have been produced from transfer of bisected embryos in sheep, goats, cattle, horses and pigs. This procedure is commonly used in the cattle embryo transfer industry and results in a pregnancy rate nearly equivalent to that with whole embryos, and in nearly double the number of offspring. However, the limit to the number of identical animals produced by splitting is effectively four (First, 1990).

Nuclear transplantation has been shown to be successful in producing viable embryos and offspring in mice, sheep, cattle, rabbits, pigs and goats. Willadsen and coworkers (Willadsen *et al.*, 1991) have achieved one hundred nuclear transplant pregnancies. It has also been reported that several hundred cloned calves, in one case with 11 clones from a single embryo, have been obtained (Bondioli, 1991). Serial nuclear transfer has been accomplished in bovine embryos with the production of sixth generation embryos and third generation calves (Yang, 1991). As many as 190 nuclear transfer embryos have been produced from a single embryo by serial transplantation (Bondioli, 1991). In cattle (Keefer *et al.*, 1993) sheep (Smith and Wilmut, 1989) and rabbits (Collas and Robl, 1991), pregnancy followed by viable births has occurred after NT with inner cell mass (ICM) or morula cells as nuclear donors. Nuclei from frozen bovine embryos have been transplanted into *in vivo* (Bondioli *et al.*, 1990) and *in vitro* (Yang *et al.*, 1993) matured oocytes. Developmental rates to blastocysts were similar to those with fresh embryo nuclei, though pregnancy rate was slightly lower than in the latter case (Bondioli *et al.*, 1990). Calves have been produced by transferring nuclei from *in vivo* embryos to IVM oocytes (Yang *et al.*, 1993; Barnes *et al.*, 1993) and pregnancy has been established through nuclear transplantation from IVP embryos to IVM oocytes (Kono *et al.*, 1993). Transplantation of cultured bovine ICM and ovine ectoderm cell nuclei has led to pregnancy (Sims and First, 1993) and development to blastocyst stage (Rust *et al.*, 1993), respectively.

Embryo stem cells: Embryo stem (ES) cells from the ICM retain the ability to divide in culture, but do not differentiate (Evans and Kaufman, 1981). Stem cells have been isolated from mouse (Evans and Kaufman, 1981) swine (Piedrahita *et al*, 1990) and cattle (Strelchenko *et al.* 1991) embryos, and can be cryopreserved giving 90% viability of the thawed cells. The recent transfer of nuclei from ICM cells of cattle and sheep embryos suggests that it may be possible to use frozen stem cells as donors of nuclei making it possible to multiply embryos rapidly and to create very large clonal families. In addition, transfer of a nucleus from a transgenic ES cell potentially provides an efficient means of obtaining transgenic genotypes (Woolliams and Wilmut, 1989).

Applications and potential

All of the reproductive techniques described above can be used either directly or indirectly in conservation of DAD. Some of them, such as semen and embryo freezing, have already served for this purpose and others will be used at different stages in the future. Semen, mature oocytes, morulae (or blastocysts) and ES cells can all be stored for long-term conservation of DAD. IVP can be used in combination with semen and oocyte freezing to facilitate the re-establishment of breeds. Nuclear transplantation and embryo splitting provide effective means for rapid multiplication *in vitro* of both stored and transgenic or sexed embryos. This significantly reduces the number of embryos to be stored for subsequent re-establishment of a given breed. Stored ES cells can not only be used to produce individuals directly, but can also serve as carriers of transgenes to produce individuals expressing a specific gene. Sexing can also reduce the number of embryos to be stored for re-establishing a breed by reducing the number of male embryos stored.

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