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Genomic characterization of animal genetic resources

Practical guide

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Foreword

The diversity of animal genetic resources for food and agriculture (AnGR) is an international public good that must be managed properly to help ensure the sustainable contribution of livestock to world food security and nutrition. For that reason, FAO Members developed the *Global Plan of Action for Animal Genetic Resources*,¹ (Global Plan of Action) which was adopted in 2007 and reaffirmed in 2017.

The Global Plan of Action contains 23 Strategic Priorities assigned to four Strategic Priority Areas. The first Strategic Priority Area is the characterization, inventory and monitoring of trends and associated risks of AnGR; which recognizes that management of AnGR will be successful only if decisions are made on the basis of sufficient and accurate information about the AnGR subject to management. Among this information are details about the genetic constitution of the breeds, which can be measured on the molecular level by characterization of a representative sample of animals. To assist countries and their scientists in this task, in 2011 FAO published guidelines on *Molecular genetic characterization of animal genetic resources*² and distributed them widely.

Molecular biotechnologies have developed rapidly and substantially since the release of these previous guidelines. The livestock sector has been an active participant in the so-called “genomic revolution.” Advancements in the sequencing of genomes and related genotyping methods have created opportunities for gathering much more information on the molecular level than ever possible, at a faster rate, and for exponentially decreased costs.

Scientists and other livestock stakeholders in countries with highly developed economies have been among the main beneficiaries of this revolution. However, the greatly decreased costs of genomic applications have also created opportunities in countries with developing and transitional economies. In many instances, the lack of awareness of these opportunities and in the technical capacity for the full application of genomic tools may represent a greater obstacle to their utilization than the expenses involved.

The development of this document and the subsequent application of the information contained herein may help to bridge this gap, bringing new opportunities to light and transferring knowledge that can be used to increase the genetic characterization of AnGR and subsequently improve the sustainable use and conservation of livestock genetic diversity.

The document first presents the rationale for characterization of AnGR, including a look at the history of molecular characterization and prospects for the future. The document then presents the basics of genomic characterization studies, reviewing the steps from initial planning, to sample collection, to laboratory and data analysis, to dissemination and utilization of the information in AnGR management. Next is a review of the main genomic tools, including DNA sequencing and genotyping of single nucleotide polymorphisms

¹ **FAO.** 2007. *Global Plan of Action for Animal Genetic Resources and the Interlaken Declaration*. Rome. www.fao.org/3/a1404e/a1404e.pdf

² **FAO.** 2011. *Molecular Genetic Characterization of Animal Genetic Resources*. FAO Animal Production and Health Guidelines. No. 9. Rome. www.fao.org/3/i2413e/i2413e00.htm

(SNP), and of the statistical and computational methods to prepare the resulting data for analysis and application. These applications are then described in the subsequent section. Assessment of genetic variation within populations is first introduced and then expanded to making comparisons across populations. The approaches to use data to draw inferences about population histories and development are described. This section continues with a discussion of how genomic data can be integrated with information about phenotypes to obtain more knowledge about the AnGR being studied and then provides examples on how this knowledge can be and has been applied. The document wraps up with conclusions and recommendations based on the key points from the various sections. Several appendices provide a greater level of detail on specific issues.

The preparation of this document represents a contribution to Strategic Priority 2 of the Global Plan of Action, which addresses the need to develop international technical standards and protocols for characterization of AnGR. The document was reviewed by an independent group of experts and by Members of the Commission on Genetic Resources for Food and Agriculture, as well as its Intergovernmental Technical Working Group for Animal Genetic Resources.

The document aims to present the state of the art in genomic characterization of AnGR, but the technology will continue to advance. It will thus be refined and updated in accordance with new technological developments and as experience with in the field is accumulated. The assistance of the National Coordinators for the Management of Animal Genetic Resources and their country networks will be particularly important to this process of revision.

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The International Society of Animal Genetics (ISAG) has been an important and valued partner with FAO in the application of molecular genetics for management of AnGR. The ISAG-FAO Advisory Group on Animal Genetic Diversity has been a Standing Committee of ISAG for more than 25 years. Members of ISAG have previously served as authors of previous guidelines on *Measurement of Domestic Animal Diversity*³ and *Molecular genetic characterization of animal genetic diversity*.⁴

Members of ISAG were thus regarded as logical partners for the preparation of this document. The editors were past and current members of the ISAG-FAO Advisory Group. The editors, in cooperation with FAO, selected the authors of the different sections, most of who were also ISAG members. Finally, current members of the ISAG-FAO Advisory Group that were not among the editors or authors reviewed the document for technical quality and accuracy.

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³ Simianer, H. & Meyer, J.H. 2003. Past and future activities to harmonize farm animal biodiversity studies on a global scale. *Archivos de zootecnia*, 52: 193–199. <https://dialnet.unirioja.es/descarga/articulo/1420891.pdf>

⁴ FAO. 1993. *Secondary guidelines: measurement of domestic animal diversity (MoDAD)*. Rome.

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In addition to the reviewers listed above, document was assessed by members of the Commission on Genetic Resources for Food and Agriculture. In particular, detailed reviews were provided by representatives from Brazil, Canada, Germany, Spain and United States of America.

Considering the editors, authors and reviewers, process involved the inputs of more than 30 people from 20 countries.

The document was prepared under the supervision of Paul Boettcher, with the full support of the Head of FAO's Animal Production and Genetics Unit, Badi Besbes, and of officers of the Animal Production and Genetics Unit: Roswitha Baumung and Grégoire Leroy. Administrative and secretarial support was provided by Antonella Falcone and Kafia Fassi-Fihri, Enrico Masci took care of the layout.

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Goal and structure

This document has been developed to update and largely supercede the content of the previous FAO Guidelines on *Molecular characterization of animal genetic resources*.⁵ In particular, the content of the previous guidelines was used as a template for the preparation of Sections 1 and 2, as well as for several of the appendices. As with the previous guidelines, the broad objective of this document is to provide guidance on performing molecular characterization studies on AnGR by employing the most advanced technologies that are feasible considering the given context.

The document generally targets the scientists who are considering to undertake a molecular genetic characterization study of their local AnGR. However, all or parts may be of interest to other stakeholders. For instance, the discussion of rationale for characterization studies may be important for policymakers or potential donors of support of such studies.

The document consists of five main sections plus appendices.

Section 1 begins by outlining the rationale for characterization of AnGR in general, by pointing out that information about an animal population is necessary for its proper management and noting that complete information is still lacking for many AnGR. Molecular characterization provides more precise information about the genetic diversity within and among breeds than is available by other forms of characterization. The section also reviews the history of molecular genetic characterization and previews the future prospects offered by the use of genomics.

Section 2 outlines the complete process of genomic characterization. It starts by highlighting the importance of preparation, describing all of the planning that should be done to ensure a successful study. This is followed by a summary of activities to be taken in the field, in the laboratory, and then once the data are obtained. Utilization of the results to improve the management of the characterized population is emphasized.

Section 3 describes the main methods for transforming biological samples into genomic data. These methods include genotyping of SNPs and whole genome sequencing. The concept of data imputation is introduced as a method to increase the amount of genomic information per sample. The section also lays out the step-by-step process of editing raw data to obtain a working dataset to which statistical analyses can be applied.

Section 4 explains the most common applications for the use of genomic data for characterization of populations. These range from assessment of the genetic variation within a single population to analysis of the genetic similarity and differences among members of a group of breeds. The section explains how inferences about the history of a breed can be drawn exclusively from the genotypes of currently living animals and refers readers to the most appropriate software for each application. It also includes subsections on selection signatures, genome wide association studies and landscape genomics that explain how complementing genomic data with phenotypes and/or geographical information can create

⁵ FAO. 2011. *Molecular Genetic Characterization of Animal Genetic Resources*. FAO Animal Production and Health Guidelines. No. 9. Rome. www.fao.org/3/i2413e/i2413e00.htm

new knowledge about the genetic basis for traits, including adaptation. The section concludes with a brief introduction of how genomics can contribute to the sustainable use and/or conservation of AnGR and provides some case studies.

Section 5 contains the main conclusions and recommendations. It is a summary of the main take-home messages from the preceding sections. Finally, nine appendices are included that provide in-depth information to complement the main sections.

Abbreviations and Acronyms

AB	ascertainment bias
ABC	approximate Bayesian computation
aDNA	ancient DNA
AFS	allele frequency spectrum
AIM	ancestry informative marker
AMOVA	analysis of molecular variance
AnGR	animal genetic resources (for food and agriculture)
Bp	base pair
CBD	Convention on Biological Diversity
CNV	copy-number variation/variant
DAD-IS	Domestic Animal Diversity Information System
DNA	deoxyribonucleic acid
D_r	Reynolds genetic distance
EAAP	European Federation of Animal Science
FA	factor analysis
Gb	Gigabase
GBS	genotyping-by-sequencing
GWAS	genome-wide association study
HDP	high-density panel
H_e	expected heterozygosity
H_o	observed heterozygosity
HWE	Hardy–Weinberg equilibrium
IBD	identity by descent
IBS	identity by state
ILRI	International Livestock Research Institute
indels	insertions and deletions (of nucleotides)
ISAG	International Society for Animal Genetics
K	number of genetic groups in model-based clustering analyses
kb	kilobase
LD	linkage disequilibrium
LDP	low-density panel
LRS	long-read sequencing
MAF	minor allele frequency
MAT	mutually agreed terms
Mb	Megabase
MCMC	Monte Carlo Markov Chain
MDS	multidimensional scaling
MSMC	multiple sequentially Markov coalescent method
MSY	male-specific part of the Y chromosome
MTA	material transfer agreement

mtDNA	mitochondrial DNA
N	sample size
N_b	number of breeding animals
N_e	effective population size
N_j	neighbour-joining
PAR	pseudoautosomal region
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
π	nucleotide diversity
PIC	prior informed consent
PSMC	pairwise sequentially Markovian coalescent method
QTL	quantitative trait locus
ROH	runs of homozygosity
SD	standard deviation
SNP	single nucleotide polymorphism
SPA	Strategic Priority Area (of the Global Plan of Action)
SPAG	spatial areas of genotype probability
SRS	short-read sequencing
SV	structural variation
θ	population mutation rate
WGS	whole-genome sequencing

SECTION 1

Introduction

Johannes A Lenstra, Utrecht University, the Netherlands

RATIONALE FOR CHARACTERIZATION OF ANIMAL GENETIC RESOURCES

Domestic animal diversity is an important component of global biodiversity. About 40 species of domestic animals and poultry contribute to meeting the needs of humankind, providing meat, fibre, milk, eggs, draught animal power, sport and recreation, skins and manure, and are an essential component of many mixed farming systems. Within these species, around 8 800 breeds and strains constitute the animal genetic resources (AnGR) that are of crucial significance for food and agriculture (FAO, 2021).

The present pattern of diversity of AnGR is the result of a long and complicated history that started with animal domestication. Depending upon the species, domestication occurred 10 000 to 1 000 years ago. Since then, domestic livestock have spread with human migration and trading to all inhabited continents. Local adaptation, artificial selection, mutation and genetic drift turned the genetic diversity captured with domestication into a vast array of differences in appearance, physiology and agricultural traits. During recent centuries, this differentiation has been accentuated by the emergence and development of breeds – more or less isolated populations that were subject to systematic selection. This development has been most pronounced in the temperate zones where the demands of food supply led to a rationalization of agriculture. The last 50 years have seen the global spread of a few highly developed breeds, most of which originated in Europe. A well-known example is the high-yielding Holstein-Friesian breed of dairy cattle, which has become by far the most widely dispersed cattle breed in the world.

The global diffusion of these specialized breeds is endangering or even risking the extinction of many well-adapted local breeds/populations. This trend is occurring in both moderately to highly intensive production environments and in marginal areas (Godber and Wall, 2014; Sponenberg *et al.*, 2018), where local husbandry practices are being abandoned (Köhler-Rollefson, Rathore and Mathias, 2009). Local breeds are usually much less productive than the highly developed international transboundary breeds when raised in optimal conditions, but are adapted to the local climate (Mirkena *et al.*, 2010) and do perform in a natural environment without intensive management. Indiscriminate cross-breeding and introduction or increased use of the specialized exotic breeds have been reported as the two most important causes of genetic erosion at global level (FAO, 2015).

This erosion of diversity of AnGR has become a major concern (Hodges, 2006; FAO, 2007a; Bruford *et al.*, 2015). The negative consequences of genetic erosion and inbreeding depression have been amply documented and may be manifested by loss of viability, fertility and disease resistance, and the frequent occurrence of recessive genetic diseases (FAO, 2007b; Taberlet *et al.*, 2008; Howard *et al.*, 2017). According to the report on the *Status and trends of animal genetic resources – 2020* (FAO, 2021), approximately 7 percent of reported livestock breeds have become extinct, and more than 25 percent are considered

to be at risk of extinction. Moreover, the situation is presently unknown for more than 50 percent of breeds, most of which are reared in developing countries.

Several recent developments have strengthened the interest in local breeds. First, we now realize that their local adaptation will become even more essential in view of the ongoing and escalating climate change (Hoffmann *et al.*, 2010). Second, there is a growing interest in the restoration of ecosystems (UN, 2020), including “rewilding” of abandoned agricultural areas, in which domestic ungulates are kept under feral or semi-feral conditions and occupy the niche of mega-herbivores (Carey, 2016). For this purpose, only animals that are adapted to extensive management are suitable. Third, the global COVID-19 pandemic has increased the awareness about zoonotic diseases, and the potential benefits (Liverani *et al.*, 2013) of a high biodiversity of wildlife, livestock and production systems (Simianer and Reimer, 2021).

FAO has a history of working with countries and other stakeholders to improve the productivity of livestock and the livelihoods of their citizens while maintaining AnGR diversity (FAO, 1990a; 1990b). Specific priorities for AnGR management are set out in the *Global Plan of Action for Animal Genetic Resources* (Global Plan of Action) (FAO, 2007a), the internationally agreed policy framework for management of AnGR.

One of the Strategic Priority Areas of the Global Plan of Action is the characterization, inventory and monitoring of trends in AnGR diversity. In brief, this Strategic Priority Area (SPA) addresses the gathering of information to increase the knowledge about AnGR. Knowledge is necessary to properly assess the value of breeds and to guide decision making in livestock development and breeding programmes. Assessments of the capacity to manage AnGR have revealed that a lack of knowledge is a major constraint (for example, FAO, 2007b; 2015)

In this document, the words “breed” and “population” will be used almost interchangeably. Technically, “population” is a more general term and includes both well defined “breeds” and groups of animals that have not yet been defined as a breed. The breed is the most common operational unit in the conservation of genetic resources. However, the biological breed concept to describe groups of animals having particular genetic characteristics is not always applicable. Many breeds originating from industrialized countries are well-defined and phenotypically distinct, and have been largely isolated genetically throughout the course of their development. In contrast, a significant proportion of other breeds, especially those in Asia and Africa, correspond to local populations without structured breeding programmes, and therefore differ only gradually according to geographical separation. In addition, breeds with different names may have a recent common origin (Felius *et al.*, 2011), and crossbreeding has been common since the invention of breeds in the eighteenth century.

It is often argued that breeds, by being associated with a group of people, is a social construct rather than a biological concept (“A breed is a breed if enough people say it is,” K. Hammond as cited by Oldenbroek and Van der Waaij, 2015). On the other hand, recent developments in biotechnologies have greatly increased the power of molecular analyses to discern the genetic makeup of organisms and identify differences and similarities among populations. Molecular genetic analysis can now complement phenotypic characterization and indigenous knowledge to help identify the breeds or groups of breeds that have

retained their uniqueness, and to gather information to help guide their future management. Genomic characterization is a useful tool regardless of the precision with which a “breed” is defined.

MOLECULAR CHARACTERIZATION – HISTORY AND PROSPECTS

Scientists began use molecular data for livestock in the early 1990s, and since then, these data have become continually more relevant for the characterization of genetic diversity (Groeneveld *et al.*, 2010; Bruford *et al.*, 2015). In 1993, an FAO working group proposed a global programme for characterization of AnGR, including molecular genetic characterization, and formulated the *Secondary guidelines: measurement of domestic animal diversity (MoDAD)* (FAO, 1993) with recommendations for the molecular analysis of domestic animal diversity on a global scale.

The FAO MoDAD report succeeded in creating awareness of the need to monitor AnGR diversity. In addition, the proposal of the program helped motivate many nationally funded research projects and larger regional and international projects supported by organizations such as the European Commission, the Nordic Council of Ministers, the International Atomic Energy Agency (IAEA), the International Livestock Research Institute (ILRI) and the World Bank.

Scientists in many countries have undertaken studies to characterize locally available breeds, while large-scale international efforts on breed characterization have built comprehensive molecular datasets for most livestock species. The study of genetic diversity of livestock at the molecular level has developed into an active area of research that frequently contributes to the capacity building of young scientists, and receives considerable attention in scientific press and at the conferences of organizations such as the International Society for Animal Genetics (ISAG) and the European Federation of Animal Science (EAAP).

The first MoDAD guidelines were followed-up in 2011 by the FAO report *Molecular genetic characterization of animal genetic resources* (FAO, 2011). At that time, the state-of-the-art technology was still largely based on the use of microsatellites as a genetic marker, which, after 1990, had revolutionized the science of molecular genetics. However, this category of markers has two major disadvantages for studying genetic diversity. First, allele calling is difficult to reproduce across laboratories. Second, in spite of the efforts of FAO and ISAG towards standardization, many laboratories continued to use private panels of genetic markers, which precluded the joining of datasets and seriously decreased the impact of such studies.

Starting about 10 to 15 years ago, these markers have, for almost all research applications, become outdated by the availability for most livestock species of whole-genome sequences. The genomic sequences allowed the identification of millions of single nucleotide polymorphisms (SNPs) and other types of genetic variants such as insertions and deletions (indels), structural variation (SV), copy-number variation (CNV). The invention of “bead arrays” allowed the simultaneous genotyping of 10 000 to 1 000 000 SNPs (Nicolazzi *et al.*, 2015). Subsequently, whole-genome sequencing (WGS) (Eusebi, Martinez and Cortes, 2020) became more and more affordable. Section 3 will describe these approaches in more detail.

These developments have generated an ongoing “tsunami” of genotype and sequence datasets, and have been accompanied by the development of a multitude of new statistical

analyses and programs for data management and analysis (Biscarini, Cozzi and Orozco-Wengel, 2018). Another consequential development has been the substantial progress in the analysis of ancient DNA (aDNA) (McHugo, Dover and MacHugh, 2019). All these developments have contributed to the realization of most of the original MoDAD objectives, albeit with different markers than envisaged originally:

- The wild ancestral species of most livestock species were already identified based on mitochondrial DNA (mtDNA). More detailed studies, often using aDNA, have yielded more details on the interaction of several livestock species and their wild ancestors during and after domestication (MacHugh, Larson and Orlando, 2017).
- The differentiation of breeds as well as their homogeneity can now be assessed unambiguously. This is, in itself, relevant, but also guides downstream analyses that assume breeds to be homogeneous populations.
- The genetic constitution of breeds can now be more accurately assessed via quantitative measures of diversity, admixture or subdivision, inbreeding, introgression and assortative mating.
- The evolutionary history of species and populations can be reconstructed on the basis of the phylogenetic relationships of breeds. The resulting data are commonly visualized by a “tree-like” (hierarchical) topology, but may also indicate gene flow between breeds with different histories.

For the most important domestic species, we have obtained a fairly comprehensive global view of diversity – that is, also including Asian, African and South American breeds as well as those from high-income countries – by integrating national or regional datasets of SNP genotypes or whole genome sequences, even if these datasets originated from different laboratories (in contrast to studies based on microsatellites).

Other positive developments are the realization of more effective cryoconservation programs (Paiva, McManus and Blackburn, 2016; De Oliveira Silva *et al.*, 2019) and the globalization of the diversity studies with active participation of institutes from all inhabited continents. The genomic characterization of breeds also led to the insight that livestock breeds have never been static phenomena, but rather are the result of a continuously dynamic history that has involved selection pressures that change over time, and has often included crossbreeding (Feliuss, Theunissen and Lenstra, 2015). As a consequence, attempts to establish priorities for conservation of breeds on the basis of neutral genetic markers (Lenstra *et al.*, 2012) have been de-emphasized.

Instead, attention has shifted toward the adaptive variation, SNPs, indels and CNVs that possibly contribute to environmental adaptation or other relevant traits. At least for monogenic traits, most of the adaptive variation resides in the structural genes (Nicholas and Hobbs, 2014), but there are interesting examples of intergenic mutations (Zappala and Montgomery, 2016; Aldersey *et al.*, 2020). In addition, several methods have been developed for detection of a local perturbation of the diversity across the genome that may suggest the effect of selection, the so-called selection signatures (Maynard-Smith and Haigh, 1974; Randhawa *et al.*, 2016; Friedrich and Wiener, 2019). In all these studies, the limiting factor is the validation of the signals found by genome-wide analysis or by studies of gene expression in terms of direct causative effects. Such genome-wide association studies (GWAS) have also been facilitated by the availability of new genomic tools.

Genomics are continually creating new opportunities for increased response to selection and improved management of genetic diversity (Bruford *et al.*, 2015; Oldenbroek and Van der Waaij, 2015).

From a fundamental scientific view, it will also be most interesting to link phenotypes to non-coding RNA molecules (Weikard, Demasius and Kuehn, 2016) and to epigenetic phenomena (Giuffra and Tuggle, 2019). Because of the multitude of genes, traits and breeds, this promises to remain a large and rewarding area of research.

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SECTION 2

The basics of genomic diversity studies

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HOW TO CARRY OUT GENOMIC DIVERSITY STUDIES

This section provides a general overview of the steps to be undertaken when conducting genomic studies of animal genetic resources (AnGR). The process of genomic characterization can usually be broken into a series of activities that are distinct in terms of time and space, but coordination among the steps is critical. Although the genotyping technologies have changed substantially since the previous guidelines (FAO, 2011b), the basic steps for any genetic diversity study have remained largely the same.

PREREQUISITES

Consider the national context for the management of animal genetic resources

Genetic characterization is often undertaken as an academic research activity, which has value on its own, but the potential impact of the study will be greatly increased if undertaken in coordination with national framework for the management of AnGR (FAO, 2011a). Nearly all countries have identified an organization to serve as its National Focal Point for Management of AnGR, and have nominated an individual to serve as a National Coordinator (FAO, 2021a). Contacting the National Coordinator prior to the study and keeping him or her informed as the study progresses is recommended for several reasons. Characterization is a key action in national implementation of the *Global Plan of Action for Genetic Resources* (FAO, 2007) and countries are regularly requested to report their activities to the FAO (e.g. FAO, 2021b). Many countries also have national strategies and action plans for management of AnGR (FAO, 2009), and characterization studies should be consistent with such plans. The National Coordinator should also be aware of the stakeholders that will be interested in the results and their application in management of the characterized populations. To maximize the efficiency of your data collection efforts, phenotypic characterization (FAO, 2012) of the populations and their production system should be undertaken in concert with sampling of biological material for genomic characterization.

Know your breeds

Characterization is itself an information-gathering activity, but efforts should be made to obtain as much background knowledge as possible about the target populations while planning your study. Sources of information will include the comprehensive breed encyclopaedia of Porter *et al.* (2016), scientific literature, national technical reports, popular press articles, breed databases such as the Domestic Animal Diversity Information System (DAD-IS) (FAO, 2021a), the Breeds of Livestock (Oklahoma State University, 2015) and websites of breed

organizations. This information will help to formulate objectives, plan sampling strategies and develop hypotheses to be tested.

Written information should be complemented with input from local experts, livestock keepers and representatives from breed associations that are familiar with the herd books and breed history. These stakeholders may be able to share anecdotal information about breeds and provide contacts for sampling of biological material and measurements of phenotypic traits.

Define clear objectives

Genomic analyses can be used to address a wide range of objectives and can be applied in many ways (see Section 4). These objectives are important for deciding on the number and type of animals to be sampled, the optimal genetic markers to be used, and complementary information to be collected during sampling. A common objective is to elucidate the relationships of a group of local breeds and their relationships to other breeds in the same geographical area, especially if these breeds are ancestral to one or more of the local breeds. For example, Kim *et al.* (2020) analysed the ancestry of indigenous African cattle and compared these cattle with European and Asian breeds. They found that the seemingly distinct “breeds” shared a common history of gene flow. Many livestock breeds have already been subject to genomic characterization, and the data from those studies is often in the public domain. Therefore, comparison of targeted breeds with other breeds may not require sampling and genotyping of the complementary breeds. However, such analyses require a preliminary assessment to ensure that genotyping approaches were similar and shared common markers.

Design the sampling

As noted earlier, genomic characterization will ideally be done in concert with phenotypic characterization and evaluation of the production environment. This will especially be the case when the study represents the first time that a population is characterized, and the objective is to capture the range of variability. To accomplish this, the sampling plan should consider the structure of the production environment, geographic locations and familial relationships (as much as possible). The following recommendations will help ensure a genetically diverse sample:

- Preferably, sample in the areas that are close to the site of the development of the breed(s).
- Depending on the objectives of the study, relevant populations in other local regions or countries (transboundary breeds) may be sampled as well.
- If applicable, sample animals that represent all different subtypes or subpopulations from different agroclimatic zones (which should be recorded in the complementary data, see below).
- Typically, to improve representativeness, avoid sampling of animals that are more closely related than the population in general, e.g. no more than 10 percent of any one herd or village population should be sampled and at most five animals should be sampled from any single herd; and
- Avoid sampling animals with common grandparents.

When planning for collection of samples from animals in different agroclimatic zones or any large area, a systemic approach is recommended. Two possible options are grid-sampling and sampling along linear transects. With grid-sampling, a “grid” of squares is formed by drawing sets of equally spaced North-South and East-West running lines over a map of the sampling area. Equal numbers of animals are then sampled from each square. With linear transects, straight lines are drawn across the sampling area and animals are sampled (as much as possible) from the farms or herds that fall along (or close to) the lines.

Choose the genetic marker technology

The type of genetic marker technology used will influence the types of inferences that can be drawn from a genetic characterization study, and thus must be chosen judiciously. As a basic rule, the most advanced technology that is available for the species to be studied should be chosen, because this method will generally be the most informative. However, practical factors must be taken into consideration to address various trade-offs. The three technologies that are most frequently used for genetic characterization (in order of increasing sophistication) are: (i) microsatellites; (ii) single nucleotide polymorphism (SNP); and (iii) whole genome sequencing (WGS). These technologies were briefly introduced in Section 1.

Because SNP and WGS are more suited to assaying the entire genomics of organisms, and are thus much more appropriate for “genomic characterization,” these technologies are emphasized in this document. Nevertheless, microsatellites have a long history for genetic characterization and are still being utilized in situations where practical considerations take centre stage (e.g. Yadav, Arona and Jain, 2017; Madilindi *et al.*, 2020), and thus merit some discussion (see Box 1).

BOX 1

Microsatellites: glory and decline

Microsatellites are a type of genetic marker discovered in the 1980s. They consist of repetitive units of 2 to 6 base pairs and are thus also known as “STRs” for short tandem repeat or “SSR” for short sequence repeat. Because of their abundance and fast PCR-based scoring, they revolutionized the genetic localization of heritable traits. In the 1990s, hundreds of microsatellites were discovered and published for the most common livestock species. Since 2000, they have been used for many genetic diversity studies.

They can be credited with a considerable scientific progress and have also been

relevant for the management of livestock genetic resources:

- rapid tests of paternity and identity (still being done);
- genomic localization of loci corresponding to monogenic traits;
- quantitative estimates of the relative diversity of a breed via the expected heterozygosity (H_e);
- reasonably accurate indication of non-random mating via the F_{IS} heterozygote-deficit parameter (see Section 4); and
- estimates of genetic distances and differentiation between breeds, revealing common descent, crossbreeding and geographic clines.

(Cont.)

These analyses have been performed for global, continental or national populations of most livestock populations.¹

On the other hand, microsatellites have several limitations, which affect the analysis of molecular diversity:

- With typically no more than one marker per chromosome in most characterization studies, microsatellites do not attain genomic coverage.
- Scoring alleles cannot be automated and is error prone.
- Estimates of genetic diversity depend on the panel of microsatellite markers, so comparisons are valid only within a dataset. Panels recommended by FAO² have not been universally adopted.
- Absolute allele sizes are not consistent across laboratories, even if the same equipment is used. Consequently, results of different labs with the same marker panel can only be combined by sharing reference samples or by using allelic ladders as a size standard.
- Because of the low number of markers used in most studies, typically 15 to 30, genetic distances between individuals cannot be estimated reliably. Also due to the limited number of markers, localization of genetic traits is very imprecise.
- They are not suitable at all for the recently developed and statistically powerful modes of analysis.

Since the publication of the previous FAO Guidelines on *Molecular characterization of animal genetic resources*,² microsatellites have been largely replaced by genome-wide bead arrays, whole-genome sequences and genotyping by

sequencing (see Section 3). These more informative approaches generally yield superior results.

On the other hand, indirect benefits of microsatellites should not be overlooked. They raised high expectations for genetic characterization and thus catalysed the formation of a network of international collaborations and consortia, which still are in place. This paved the way for a rapid and most productive implementation of genome-wide and whole genome sequencing.

Do microsatellites still have a place in genetic characterization? They remain appropriate for standardized routine paternity testing. They may also be the only practical option if local options for SNP genotyping or WGS are not available or unreasonably expensive. There are also special cases for their use when merging new information with data from genotypes of historic samples that are no longer available (although this requires retyping at least some of the historical samples as a reference). However, for the reasons outlined above, microsatellites cannot anymore be generally recommended for genome-wide characterization of genetic variation. In situations where microsatellites are the preferred or only option, utilization of markers from the standard FAO panel² is strongly recommended.

¹ Groeneveld, L.F., Lenstra, J.A., Eding, H., Toro, M.A., Scherf, B., Pilling, D., Negrini, R., et al. 2010. Genetic diversity in farm animals – a review. *Animal Genetics*, 41(s1): 6–31. <https://doi.org/10.1111/j.1365-2052.2010.02038.x>

² FAO. 2011. *Molecular genetic characterization of animal genetic resources*. FAO Animal Production and Health Guidelines. No. 9. Rome. www.fao.org/3/i2413e/i2413e00.htm

Regarding the choice between SNP and WGS, the latter yields much more information and thus provides potentially more precision in subsequent genomic analyses. WGS also provides information about other forms of polymorphism, such as insertions and deletions

(indels) and structural variation (SV). However, WGS is much more expensive, and the greater amount of information produced requires substantially greater human and technical capacity for data storage, computing and analysis, which may be out of reach in many circumstances, especially in developing countries. Nearly all of the analyses described can be undertaken with data from SNP arrays, so this will be the most appropriate technology for most of the users.

Numbers that count

For reliable estimation of allele frequencies of biallelic markers such as SNP, at least 20 animals per breed should be typed (Hein, Schierup and Wiuf, 2004). Smaller sample size decreases the precision of estimates of allelic frequencies, a parameter that underlies many genetic characterization analyses. This imprecision may inflate estimates of genetic distances among other breeds, for example. For multiallelic markers, at least 40 animals are required. A larger sample size is also recommended for subdivided breeds.

For specific objectives, smaller (larger) samples sizes may be sufficient (required). Based on a comprehensive simulation to evaluate the properties of different statistical methods, a reasonable power to identify selection signatures (see Section 4) can be achieved with a smaller sample size (around 15 animals per population) when using a high-density SNP genotypes, e.g. > 1 SNP per kb (Ma *et al.*, 2015). For genome-wide association studies (GWAS), more samples will likely be needed (Ball, 2013) (see Section 3 and Section 4). Collection of more samples than these minimum values will help to increase precision of inferences and provide a backup in case of low-quality samples.

Know the rules

Genomic characterization involves the collection and analysis of genetic resources, and may generate data of relevance for intellectual property claims. Therefore, it is recommended that a formal agreement for acquisition and exchange of the material is made with the provider of the material prior to the start of the study. The standard approach is for all involved parties to sign a material transfer agreement (MTA), which stipulates the terms of the material exchange, describes how the material will be used and handled after characterization and analysis. The provider may already have a standard MTA and provide access to the genetic resources only upon acceptance of the terms of the MTA. The MTA should also create legal certainty as to what can be done with the material (such as storage or distribution after the study), as well as with any data resulting from the study. Both parties must be informed about any limits to the rights that the provider can cede to the receiver of the material, in accordance with national regulations. In principle, the providers of the material are to maintain ownership throughout the duration of the study. The providers of the genetic material should be regularly informed about the progress of the study and results obtained. An example MTA is provided in Appendix 2.

Furthermore, international exchange of research material involves a specific set of rules. First, AnGR are considered sovereign to their country of origin. Both the providers of material and their collaborators must therefore adhere to the terms of each country's regulations implementing the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (Nagoya Protocol) of the Convention on

Biological Diversity (CBD), such as the signing of an access and benefit-sharing (ABS) contract negotiated on mutually agreed terms (MAT). More information about the Nagoya Protocol can be found in Box 2 and in other specialized documentation prepared by FAO (2019). Second, because biological material is being exchanged, collaborating scientists must be aware of any sanitary regulations involving international shipments and obtain the necessary permits.

BOX 2

Nagoya Protocol: In brief

Hartmut Meyer

The Nagoya Protocol was developed to ensure the fair and equitable sharing of the benefits arising out of the utilization of genetic resources across countries, one of the three objectives of the Convention of Biological Diversity.¹ This requires appropriate access to genetic resources and appropriate transfer of relevant technologies, considering all rights over those resources, technologies and appropriate funding. The Nagoya Protocol was adopted by the CBD in 2010 and has since been ratified by more than 125 member governments.

As confirmed by the Nagoya Protocol, countries, in the exercise of their sovereign rights over natural resources, may provide that access to genetic resources for their utilization, which shall be subject to their “prior informed consent” (PIC). Many countries have decided to not restrict access to AnGR. However, where domestic legislation or regulatory requirements require this, users need to obtain permission prior to accessing and using genetic resources for research and development, and share the benefits arising from this utilization of genetic resources on mutually agreed terms (MAT). The Nagoya Protocol, as well as many national access and ABS laws, do not go into any detail as to what constitutes “utilization” of a genetic resource, and therefore requires PIC and MAT. Certain activities, such as taxonomic research which may or may not be further developed commercially, could require PIC and MAT in one country and not in another. It is therefore important to specify in detail the intended activities being undertaken and to clarify with the competent authority of the

country that provides the genetic resources if and what kind of permit is required.

In some countries, access to traditional knowledge associated with genetic resources that is held by indigenous peoples and local communities is also subject to their PIC or approval and involvement, and MAT have to be established.

Countries that ratify the Nagoya Protocol are expected to ensure that genetic resources utilized within their jurisdiction have been accessed in accordance with PIC and that MAT have been established, as required by the ABS measures of the country from which the resources were obtained. It is therefore crucial to comply with the applicable ABS measures irrespective of where the genetic resources are actually used for research and development. This is because parties to the Nagoya Protocol are under an obligation to provide that any research and development activity on genetic resources within their jurisdiction complies with the ABS measures under which the genetic resources have been accessed.

Through so-called “checkpoints” parties to the Nagoya Protocol ensure that research and development on genetic resources within their territory are based on the PIC of the party that provided these genetic resources, as applicable. Once PIC has been granted and the permit or its equivalent has been made available to the ABS Clearing House, they constitute an Internationally Recognized Certificate of Compliance.

¹ CBD. 2011. *Nagoya Protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization to the Convention on Biological Diversity*. Montreal. Cited 14 September 2020. www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf

BOX 3**Incorporation of compliance to national ABS frameworks into project planning and implementation***Karen Marshall*

Compliance with ABS measures adds a level of administrative complexity to the execution of research and development projects involving AnGR. The ABS measures require specific planning, but may help avoid eventual disputes regarding the sharing of benefits arising from such projects that could otherwise occur in the course of the project.

The following points regarding ABS compliance should be considered in preparation for genomic characterization studies involving international exchange of AnGR:

- (i) Account in the project timeline for the time required to negotiate the ABS contract and obtain PIC, the national ABS permit and other applicable permits (typically 2 months to 2 years, depending on the country).
- (ii) Be aware that it may not even be possible to negotiate the required contract.
- (iii) Include in the project budget sufficient funds to cover whatever benefits have

been negotiated with the providing country. Expected benefits are variable by country, and including provider countries as project collaborators may be advantageous, inasmuch as some of the benefits will be in-kind, rather than monetary.

- (iv) Consider carefully both present and potential future uses of the AnGR samples and prepare the wording of the contract accordingly. If you want to use the resources for a purpose other than that stated in the original agreement, a new agreement will be required.
- (v) Keep all relevant documentation associated with the negotiation of the contract and granting of PIC and the ABS permit. These documents will be crucial for demonstrating compliance with the ABS laws of the providing country if the country in which you are using AnGR has adopted ABS compliance laws (e.g. European Union and the United Kingdom of Great Britain and Northern Ireland).
- (vi) Keep a record of all benefits (both monetary and non-monetary) obtained by both the provider and user of the genetic resource.

The ABS contract and MAT define the limits of utilization and the sharing of benefits. In general, obtaining these contracts involves a phase of negotiation that must be considered before one starts a genomic characterizations study. Box 3 provides key points to consider regarding this negotiation process.

IN THE FIELD**Collecting samples**

For this most crucial step, the following considerations are relevant:

Almost all cells or tissues may be used for DNA analysis: blood, semen, hide, bone, tissue (e.g. ear tissue), plucked hair (only the root cells contain nuclei, but cut hairs can be used for analysis of mitochondrial DNA) and feathers.

High-quality DNA is most easily obtained from samples of peripheral blood, organs or other tissues. Most convenient are blood samples collected in an anti-coagulant (EDTA or natrium citrate). A protocol for blood collection is provided in Appendix 3.

Collect enough material for present and future studies (considering ABS agreements for future studies). This process may either include the collection of multiple samples per animal, or of a sufficiently large sample that can be aliquoted into separate portions in the laboratory. For most PCR-based applications, including high-density SNP arrays and whole genome sequencing, 5 ml of blood is adequate. Note that poultry species have enucleated erythrocytes and, therefore, much less blood (~1 ml) is required.

Blood samples can be transported at ambient temperatures, but in tropical regions, samples should be processed within 36 hours.

For longer storage, samples can be placed in a room-temperature preservative such as Queen's buffer (0.01 M Tris/HCl, 0.01 M NaCl, 0.01 M EDTA and 1 percent n-lauroylsarcosine, pH 8.0) (Seutin *et al.*, 1991).

Tissue samples of 1 cm squared should be minced to 1 mm squared pieces and placed in Queen's buffer or 70 percent ethanol. Air-drying of ethanol-treated samples allows long-term storage and easy transport of samples. Alternatively, pieces of tissue may be dehydrated directly by placing them in vials on crystals of silica gel.

Hair samples should be desiccated as soon as possible and stored dry.

FTA® cards can be used for collection of genetic material with DNA to be amplified by PCR, but special protocols are required for some species to obtain double-stranded DNA. Moreover, the single-stranded DNA obtained with standard isolation protocols is not suitable for all other applications.

Samples that are to be used for cloning and Southern blotting require double-stranded DNA of high molecular weight.

Labelling of samples should be unambiguous and permanent. Whenever possible, the official identification number of the animal should be recorded.

Bank it: store all samples and gather and organize all relevant information unambiguously in such a way that it can be retrieved and understood, even by persons not involved in the sampling.

Collecting data

Recording the following information for each sample is essential:

- animal identification, preferably a herd book registration number, if available;
- date;
- location, preferably based on global positioning system (GPS) coordinates;
- name of collector;
- breed;
- sex of animal; and
- type of sample (blood, hair, etc.).

Collection of the following information is strongly recommended and will be necessary for some types of analyses (e.g. phenotypes will be necessary for studies to determine genomic regions having influence on traits):

- age, or date of birth;

- any relevant phenotype(s);
- basic pedigree information (i.e. parents, if known);
- digital photograph(s) of animal, showing any interesting morphological features, and including a measuring stick to evaluate body measurements;
- size of herd; and
- notes about any recent change in geographic location of the animal.

An example of a sampling form is provided in Appendix 4.

In addition to the information recorded for each sample, for each breed, a form like the example in Appendix 5 should be compiled on the basis of the information available. This form addresses breed origins, farming practices, basic production information, and features of the breed such as productivity, disease resistance or adaptation to local conditions. For further advice on collecting data on breeds' phenotypes and production environments, consult the *FAO Guidelines on Phenotypic Characterization of Animal Genetic Resources* (FAO, 2012).

IN THE LABORATORY

Technological advancement in genomics has been accompanied by the emergence of private companies providing custom genotyping or sequencing services. For many genomic applications, all laboratory procedures subsequent to sample collection can be outsourced, such that a traditional "wet lab" is not strictly needed. Outsourcing saves the investment in expensive and specialized equipment, which tends to become quickly obsolete. Nevertheless, several issues need to be considered even if the experiments are outsourced.

Extracting DNA

Extraction of DNA is a key step for in-house genotyping and for analyses outsourced to companies that require DNA instead of raw samples. Extracted DNA is also subject to less stringent sanitary regulations than are biological samples when shipped across borders.

Several reliable protocols for DNA extraction are available. Older protocols are based on Proteinase K/SDS lysis of cells, organic extraction and alcohol precipitation. Salt precipitation avoids organic solvents, but the long-term stability of the DNA samples is problematic. Convenient commercial kits based on the specific binding of DNA to resins are available for several kinds of tissues and usually perform well. Whenever a new DNA extraction procedure is used, a practice run on test samples should be performed to gain experience and ensure that it works properly before being applied to the field samples.

DNA assay

Section 3 discusses protocols for genotyping and sequencing in detail. In general, these protocols are highly automated and straightforward, but the indicated procedures need to be followed precisely. The following are general suggestions that will be applicable in many situations:

- Genotyping methods that are sensitive to variation between laboratories, such as microsatellite typing, should include at least one reference sample shared by the laboratories involved.
- The inclusion of a duplicate sample should be considered to permit evaluation of accuracy.

- The quality of the resulting data must be checked critically, even for outsourced analyses.

DATA ANALYSIS

Sections 3 and 4 address specific issues of data analysis according to the type of data (i.e. individual loci or sequence data) and study objective. For the many scientific objectives, software is freely available and greatly simplifies the tasks associated with data analysis (see Appendix 6). The easy access to software does not, however, relieve the researcher from the responsibility for understanding the genetic and statistical principles underlying the analyses. Each scientist must understand whether and why a specific approach is or is not suitable for his or her data and objective. Instead of copy-pasting computer output into manuscripts, the researcher should evaluate the results critically. For many analyses, multiple options for software are available and repeating an analysis using other options or another program can be informative. When software requires the input of parameters, the effect of parameter changes should be evaluated, especially if true values of the parameters are unknown. Analysis of simulated data is another option for validation of an algorithms. Results of the data analyses should be interpreted in the context of existing biological, genetic and historical knowledge.

PUBLISH IT

Let the world know

Publish your findings in a scientific journal. Explain how the results are relevant in a greater context for the management of AnGR. Open-access journals are recommended because of their wide diffusion and free accessibility.

Share the credit

Properly acknowledge contributors of samples and/or data. In general, include all colleagues who supplied samples and/or data in the preparation of any scientific publications so that they are fully aware of the key results and can be recognized as co-authors.

Share the data

After publication, deposit your data in a public database and/or comply with requests to make datasets available as already requested by many journals.

For each of the three tasks listed above, it's important to ensure that all activities are addressed in the MTA.

TRANSLATE THE RESULTS

Genomic characterization studies are excellent capacity building activities and contribute to academic knowledge, but they should also be used to improve the management of the AnGR involved. The country's National Coordinator for the Management of Animal Genetic Resources should be informed about the study, and should be provided with data that can be uploaded to the Domestic Animal Diversity Information System (DAD-IS) (FAO, 2021). Researchers should communicate their results and conclusions to the main stakeholders of the breeds evaluated, and, if requested, provide recommendations.

The following phenomena can be detected through genomic analyses and may be relevant for breed management:

- **Original diversity.** Populations located in or having originated near their species' centre of domestication are presumed to have the greatest genetic diversity. The level of diversity can be measured by the amount of heterozygosity, the number of unique alleles and haplotypes, or the nucleotide diversity.
- **Unique origin or history.** In many instances, populations from the same region have relatively high genetic similarity, even if they have distinct phenotypic characteristics, suggesting that they share a common origin or history. Breeds with a distinct origin are assumed to carry unique alleles and thus be of greater value for conservation.
- **Pedigree errors.** Genetic tools now allow for errors in recording of parentage to be identified and remedied; this is especially useful in the case of unknown paternity.
- **Crossbreeding or hybridization.** Breeds that have been subject to recent crossbreeding are often considered to have decreased conservation value relative to "pure" breeds because of lower distinctiveness, even if they still maintain a substantial portion of unique genetic diversity. This is especially the case when the crossbreeding or hybridization involved one or more international transboundary breeds. On the other hand, systematic selection may have created a stable and valuable combination of traits from two breeds, typically a high productivity from one breed and strong adaptation to a tropical climate from another breed. Well-known examples are the Girolando dairy cow and the Dorper sheep.
- **Consanguinity.** Conservation activities should avoid inclusion of related animals as much as possible. For example, gene banks should avoid selecting related donors and *in vivo* programmes should select sires that are as unrelated as possible. Genomics can be used to determine the genetic relatedness of potential donor animals.
- **Inbreeding depression.** High homozygosity within a breed often leads to decreased fitness and survival. Genomics can be used to diagnose excessive homozygosity and to plan matings that decrease the incidence of inbreeding depression. In the worst-case scenario, strategic crossing with another breed may be required to introduce new genetic variation and genomics may be used to identify the most suitable breed.
- **Distinctive phenotypes.** Breeds are often defined by one or more distinctive genetic traits, which may justify their conservation.
- **Genetic defects.** Many genetic defects are determined by mutations of one or a few genes. Identification of the genomic locations of these genes can facilitate elimination of the defects, and determination of the precise mutation may allow for the development of control therapies. Deficit or absence of a homozygous genotype for a given locus may indicate lethal recessives. With the novel CRISPR/Cas technology (Jinek *et al.*, 2012) gene defects may in the future become correctable, and the same technology can also introduce mutations that confer desired traits (Menchaca *et al.*, 2020), although genetic modification is not yet permitted in many countries.

Genomic information will rarely be the only tool available for the management of a breed, but can often complement factors such as productivity, breed demographics and cultural significance in making decisions on AnGR management.

INTERNATIONAL COORDINATION

Results from genomic characterization studies have potentially high value not only for national management of the breed from which the samples originated, but also for other breeds and countries. Thus, sharing of the resources associated with such studies is strongly recommended, whenever possible, under the terms of use agreements and contracts and the Nagoya Protocol. As already mentioned, the data from such studies should be made available, preferably in a public repository such as DRYAD (2021) or Figshare (2021). DNA samples should be stored in a biobank for use in follow-up studies, including international collaborations. Once again, the rules of the source country and conditions imposed by the original owners of the animals must always be respected.

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SECTION 3

Genomic tools and methods

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Since the appearance of the previous guidelines on *Molecular genetic characterization of animal genetic resources* (FAO, 2011), there has been a spectacular growth in the use of genomic technologies, which are still becoming more and more powerful and accessible. As an initial step in application of these technologies, whole genome sequencing (WGS) was performed on selected animals from each major livestock species. This sequencing established initial reference genomes, which then allowed the development of multi-locus bead arrays of single nucleotide polymorphism (SNP) for most domestic species. These arrays fulfilled the objectives originally envisaged for microsatellite genotyping and have remained extremely useful. The next development was a much wider adoption of WGS technologies. More animals were fully sequenced and reference genomes were considerably improved. Approaches such as resequencing and genotyping-by-sequencing (GBS) were developed and applied, and now allow the collection of WGS datasets for tens or hundreds of individuals, yielding vast multi-locus genotype datasets. All these processes are self-reinforcing, since the availability of many high-density genotypes allows the elevation of low to high density datasets by imputation. This section explains these various developments and approaches in more detail.

SNP GENOTYPING

Single nucleotide polymorphism (SNP)

A SNP is a deoxyribonucleic acid (DNA) sequence variation that occurs by substitution of a nucleotide at a specific position in the genome. The SNP is the most common type of polymorphism and is estimated to occur with a frequency of one SNP per 1 kilobase (kb) in most mammalian genomes. SNPs have increasingly become the marker of choice and have largely replaced microsatellite markers to assess genetic diversity, structure and relationships among populations; and particularly to identify genomic regions associated to economic traits. As noted in Section 1, SNPs have several advantages over microsatellites,

including: (i) stable inheritance; (ii) distribution throughout the genome at a much greater density; (iii) location in coding regions that can possibly alter protein function and phenotypic expression; (iv) location nearby or within quantitative trait loci (QTL) of interest; and (v) suitability for high throughput genotyping.

Candidate SNP genotyping methodologies

Discovery of a SNP at the candidate gene level is done by targeted resequencing of specific regions of the genome in a subset of unrelated individuals representing the species or the populations of interest. Several methodologies are available to genotype one or a few SNPs identified in specific segments of candidate genes, including exons, introns, promoters and untranslated regions. Most of these methods are based on conventional or real-time polymerase chain reaction (PCR) procedures. A few examples include: (i) traditional gel-based approaches, such as amplification refractory mutation system (ARMS; Little, 2001), restricted fragment length polymorphism (RFLP; Jarcho, 1994), single strand conformation polymorphism (SSCP; Dong and Zhou, 2005), denaturing gradient gel electrophoresis (DGGE; Strathdee and Free, 2013); and (ii) medium throughput approaches, such as competitive allele specific polymerase chain reaction (KASP assays; He, Holme and Anthony, 2014), exonuclease detection (Taqman assay; Holland *et al.*, 1991), mass spectrometry-based primer extension detection (Sequenom) and high resolution melting (Bradić, Costa and Chelo, 2011).

SNP microarrays

Advances in next generation sequencing technologies and rapidly decreasing costs of WGS have made SNP discovery at the genome level possible in most livestock species. The process includes WGS of individuals from a subset of populations of interest and selection of polymorphic loci for additional genotyping in a larger pool of animals, often using high throughput SNP microarray technology. The SNP microarrays consist of allele-specific oligonucleotide probes fixed on a solid support, such as glass slides or silicon quartz wafers. The target DNA (whole genome) samples are fragmented and labelled with a fluorescent dye, and the fragments are then amplified and hybridized to these oligonucleotide probes for single nucleotide extension or ligation. The labelled SNPs are visualized by an immunohistochemistry assay to increase the signal intensity and infer the genotypes accurately. Box 4 provides some technical details about the SNP genotyping platforms offered by two main commercial suppliers.

BOX 4

Commercial options for SNP genotyping

Two major commercial SNP array platforms are currently available for genome-wide SNP typing of livestock species: (i) Illumina's Infinium iSelect Microarray or BeadChip, based on single nucleotide extension or allele-specific primer extension,¹ and (ii) Affymetrix's GeneChip or AxiomArray, based on molecular inversion probe hybridization.² The two platforms differ in their array fabrication and protocols. The BeadChip uses 50-mer (i.e. 50 nucleotides) allele-specific probes, while the GeneChip uses 25-mer and/or 30-mer probes for hybridization with target DNA samples.

The BeadChip is produced by etching out "wells" on a silicon wafer, each of holds a spherical bead. Each of the beads is coated with a specific oligonucleotides (short strand of DNA) containing 29mer address tags and 50-mer SNP specific primers. Every oligonucleotide is complementary to a different strand of DNA adjacent to a given target SNP. The DNA sample to be tested is fragmented and combined with labelled single molecules for each of the four nucleotides in DNA and then passed over the chip. The fragments in the sample that are complementary to oligonucleotides on the beads attach to them by hybridization and are then extended by a single labelled nucleotide (i.e., matching the sequence at the site of

the SNP). The genotype at the SNP is then determined by measuring the intensity of fluorescence after exposure to a laser.

The GeneChip is produced by *in situ* synthesis of oligonucleotide probes on a chemically protected array surface. The method relies on a photolithographic process, meaning that each nucleotide in each oligonucleotide is printed on the chip through exposure to or masking from ultraviolet light. The sequential application of specific lithographic masks determines the order of the probe sequence synthesized on the array. As with the BeadChip, the sample DNA is first fragmented, and then passed over the chip. The subsequent automated genotyping process then consists of four steps: (i) hybridization of fragmented DNA to the oligonucleotide probes on the chip; (ii) staining to differentiate among nucleotides; (iii) rinsing away of the residual DNA and stain; and (iv) imaging to determine genotypes.

¹ Illumina Inc. 2016. *Infinium® iSelect® Custom Genotyping Assays. Guidelines for Using the DesignStudio™ Microarray Assay Designer Software to Create and Order Custom Arrays*. Technical Note. Cited 02 February 2021. www.illumina.com/documents/products/technotes/technote_iselect_design.pdf

² Affymetrix Inc. 2020. *Mitigating Sequencing Errors, Monomorphs, and Poor Performing Markers during de novo SNP Selection for Genotyping Applications*. Technical Note. Cited 28 December 2020. https://assets.thermofisher.com/TFS-Assets/LSG/brochures/mitigating_genotyping_appnote.pdf

SNP array design

The process of SNP array design starts from SNP discovery, and includes validation of *de novo* variants, selection of marker loci, validation of draft SNPs and synthesis of arrays. To fully understand the process of SNP array design, one must have a grasp of some basic concepts for WGS. These concepts and associated terminologies are explained in Box 5. A more detailed discussion of WGS is found later in this section.

BOX 5

Basic concepts of whole-genome sequencing

The process of WGS involves three basic steps:

Fragmenting the genomic DNA strands into segments. The WGS technology is rapidly improving and the length of an individual DNA segment that can be sequenced varies. Depending on the technology used, the DNA in a given sample is either kept as entire as possible or is broken into fragments. A DNA sample contains many copies of an animal's genome, which are fragmented by physical means (e.g. sonication) and thus break in random locations. This results in many fragments, each of which is fully or partly overlapping with other fragments.

Sequencing individual segments. The sequence of an individual segment is called a "read." Because a given sample includes many copies of DNA, sequencing all fragments is neither possible nor necessary. However, sequencing more fragments helps to ensure that as much of the genome is sequenced as possible and results in a greater accuracy of the final sequence. Two terms that characterize the quality and quantity of sequencing data are "coverage" and "depth". These terms are related and are often used interchangeably, but also have distinct meanings. When expressed as a percentage, coverage refers to the proportion of genome that has been "read" (i.e. sequenced). Depth (or coverage

depth) is essentially the average number of times that a random nucleotide in the genome has been included in one of the sequenced reads (by chance, some regions will have been sequenced more often, some less often). Depth is usually expressed as value "nx" and can be calculated by dividing the amount of data produced in the sequencing assay by the data size of a single genome sequence. For example, if a sequencing assay produced 30 Gb of data for a single genome of 3 Gb, the depth would be 10x. Coverage is also used as direct synonym of depth. Increasing the depth of sequencing increases the accuracy, but it also increases the cost. The cost and depth must therefore be balanced, and the appropriate depth depends on the sequencing objective.

Converting the data for individual segments into a full genomic sequence. The fact that individual segments are overlapping allows us to reconstruct the segments into full chromosomes and genomes. This process is accomplished by using sophisticated bioinformatic software, and is based on "alignment" of fragments based on finding the commonalities between them and the reference genome for the species. When no reference genome exists, then, an original genome must be "assembled", which is a more complex processing that usually relies on long read sequencing (see later in the section) and comparison of genomes with related species, when feasible.

SNP discovery. The ideal SNP discovery process for designing and developing a diversity array for a given livestock species involves WGS of a panel of unrelated individuals, preferably from diverse breeds across widely dispersed geographical locations, thus ensuring the detection of as many of the variants present within the targeted species as possible. The low-depth sequencing of large numbers of individuals has proven to be more advantageous than deep sequencing of fewer individuals in improving the power of the SNP discovery process (Buerkle and Gompert, 2013; Homburger *et al.*, 2019). When available,

SNPs identified from previous studies and stored in public databases (e.g. Hapmaps, BAC libraries, dbSNP) can also be utilized to increase the number of candidate SNPs available for the design of the array (Matukumalli *et al.*, 2009).

Selection of SNPs. Errors in genome sequencing can lead to the detection of false SNPs. Hence *de novo* variants need to be validated based on quality parameters like: (i) minimum sequencing read depth; (ii) consensus base ratio; (iii) SNP quality score; and (iv) relative distance of the base from the 3'-end of the read, normalized by read length (Shen *et al.*, 2010). Generally, the SNP discovery process results in the identification of several million SNPs after initial validation. Among these, a subset of loci (~ 2–3 million) needs to be selected for drafting a marker panel (screening array) based on different criteria (Fan *et al.*, 2010; Kranis *et al.*, 2013):

- *in silico* design score for likelihood of success in the genotyping assay;
- type of polymorphism (i.e. transition versus transversion);
- minor allele frequency (MAF), that is, frequency of the less-common allele for biallelic SNP;
- presence of nearby SNPs (e.g. exclusion of SNPs within 10–15 bases);
- linkage disequilibrium (LD) with other SNPs included in the panel;
- physical distribution of SNPs (e.g. equidistant spacing over the genome);
- polymorphism in multiple populations; and
- enrichment of specific regions of the genome (e.g. genomic regions potentially associated with important phenotypic traits).

Validation of SNPs. The screening array with a draft panel of preselected SNPs must be validated to identify a subset of high-performance SNPs that show the potential for various downstream applications, such as diversity analysis, marker-trait association studies and gene mapping. During the process of SNP validation, the following criteria are utilized to identify loci that are placed on routine genotyping arrays (Illumina Inc., 2016; Affymetrix Inc., 2020; Fan *et al.*, 2010; Kranis *et al.*, 2013):

- performance of SNPs in terms of high call rates (i.e. proportion of samples for which the genotype can be resolved);
- accuracy of genotyping (e.g. efficiency in clustering genotypes, to distinguish homozygous and heterozygous classes);
- informativeness of SNPs;
- association with traits of interest;
- tagging other variants based on LD;
- imputation of other variants in the genome;
- spacing and location with respect to known LD blocks (genomic regions with low diversity);
- functional significance of SNPs (e.g. exonic/intronic, synonymous/non-synonymous, coding DNA sequence/untranslated region).

Routine genotyping array and marker density. Arrays of different SNP densities may be used depending on the purpose of downstream applications and the genetic structure of the population investigated. Among the arrays routinely used in livestock species, the marker density ranges from low (< 20 000 SNP), medium (50 000), medium high (150–200 000) to high (> 500 000) SNP density. The cost per array increases with the number of SNPs, but the cost per marker decreases. The best-performing and most informative markers can be utilized to

design smaller, cost-effective arrays for routine genotyping for a specific purpose. Low-density SNP arrays designed with markers having higher MAF and uniform spacing across the genome may be used to “impute” (see specific subsection on this topic later in this section) to a higher SNP density or even a complete sequence, if a large set of reference animals are genotyped at higher density or whole-genome sequenced (Boichard *et al.*, 2012). However, with advancements in array fabrication and automated fluidic technologies, the cost of medium density arrays has significantly decreased, resulting in the phasing out of low-density arrays.

Ascertainment bias (AB)

When SNPs selected for designing and developing an array are discovered by sequencing only a few samples or from only a few selected breeds, an ascertainment bias (AB) may occur, affecting inferences about larger and distinct populations. Two major kinds of AB are associated with SNPs: (i) MAF bias; and (ii) subpopulation bias. MAF bias occurs when the SNPs are preferentially selected based on intermediate frequencies and is nearly impossible to avoid with SNP selection. The MAF bias results in over-representation of common polymorphic loci but under-representation of low frequency SNPs. Consequently, estimates of population genetic parameters, allele frequency distribution and linkage disequilibrium can be biased (Albrechtsen, Nielsen, F. and Nielsen, R., 2010; Heslot *et al.*, 2013). Subpopulation bias occurs when the SNP discovery panel is chosen from individuals belonging to a specific population or a small group of breeds. This results in an overestimation of the variability present in that population or group of breeds but an underestimation of the variability in genetically dissimilar populations. Accordingly, this AB can inflate heterozygosity estimates in populations of breeds that are closely related to the breeds in the SNP discovery panel. The AB can increase or decrease the estimates of various genetic parameters as compared to the expected estimates from unbiased data, thus distorting information on population differentiation (Lachance and Tishkoff, 2013; McTavish and Hillis, 2015).

The phenomenon of AB is not unique to SNP markers and may also occur with morphological and microsatellite markers. The AB in microsatellites occurs when the most polymorphic markers are typically selected (Vowles and Amos, 2006; Vali *et al.*, 2008). Such bias influences estimates of genetic variability of populations, but the influence of biased SNPs is much greater than that of microsatellites, by virtue of their large numbers and biallelic nature. One of the potential approaches to mitigate AB is to adopt SNP filtering strategies, such as LD pruning (see also later in the section). The basic idea is to remove multicollinearity effects by removing SNPs that are highly correlated with other SNPs within a given genome window, thus reducing LD among the SNPs left after pruning. LD pruning has been effective in reducing AB when estimating genetic differentiation measures among populations, including genetic distance, fixation index, inbreeding coefficient, kinship and principal component analysis (PCA – see Section 4) (Malomane *et al.*, 2018). Although LD-based pruning does not fully offset the AB, it does help to reduce its effects. Another potential approach to mitigate AB is to utilize ancestral SNPs (i.e. those SNPs that are also polymorphic in wild relatives of a given livestock species) while comparing highly divergent populations. The ancestral loci have relatively higher heterozygosity and are less likely to show population bias, but fine scale patterns of diversity among closely related breeds may be missed (Malomane *et al.*, 2018; Barbato *et al.*, 2020). If resources are available,

the sequencing of a few samples and comparison of estimates of genetic variation from the sequence data versus the SNP data can help to determine the degree of AB.

How to choose your array?

Choosing a suitable array for genotyping depends on the purpose of the study. Although not exclusive, the following factors may be considered while choosing the array:

- SNP density (e.g. high, medium and low-density arrays);
- potential AB (e.g. genetic/geographic distance between discovery panel and the population to be investigated);
- tagging of specific genomic features (e.g. markers recommended by the International Society for Animal Genetics (ISAG) for parentage testing, copy number variants (CNV), markers for detection of recessive traits);
- SNPs in common with existing genotype data;
- cost effectiveness of genotyping (e.g. marker density vs array cost); and
- performance of the array (genome coverage, genotype clustering).

Selecting a high-density array for genome wide association can help in capturing maximum possible variants that have potential influence on the trait (Tsai *et al.*, 2015). In case of genetic evaluation (genomic selection) studies, where genotypes/sequences of several individuals from the reference population are available and population LD is generally high, choosing a low-density array followed by genotype imputation (see subsequent subsection) may be cost-effective (Boichard *et al.*, 2012; Georges, Charlier and Hayes, 2019). With the rapid growth of sequence/marker information over time, newly updated versions of arrays with special features keep evolving for many livestock species across different platforms (see Appendix 7). While choosing a new array for genotyping, checking the overlap of SNP with other available arrays can improve harmonization with existing data for comparative and meta-analysis across studies.

Merging SNP datasets

Unlike microsatellites, SNP markers facilitate merging and harmonizing datasets across different studies, generated in various laboratories and/or different platforms. The genome wide SNP genotypes can also be merged with the genotypes identified by genome resequencing data to generate information on specific loci of interest. Datasets are merged based on marker identification numbers and genomic positions, after alignment to a standard reference genome assembly of the species investigated. Care needs to be taken on the strand orientation of markers while merging different datasets. Open-source programs, such as PLINK (Chang *et al.*, 2015; Purcell, 2010), are available to facilitate merging genotypes from different datasets and optimize resources available for genomic characterization studies in livestock.

GENOTYPING-BY-SEQUENCING

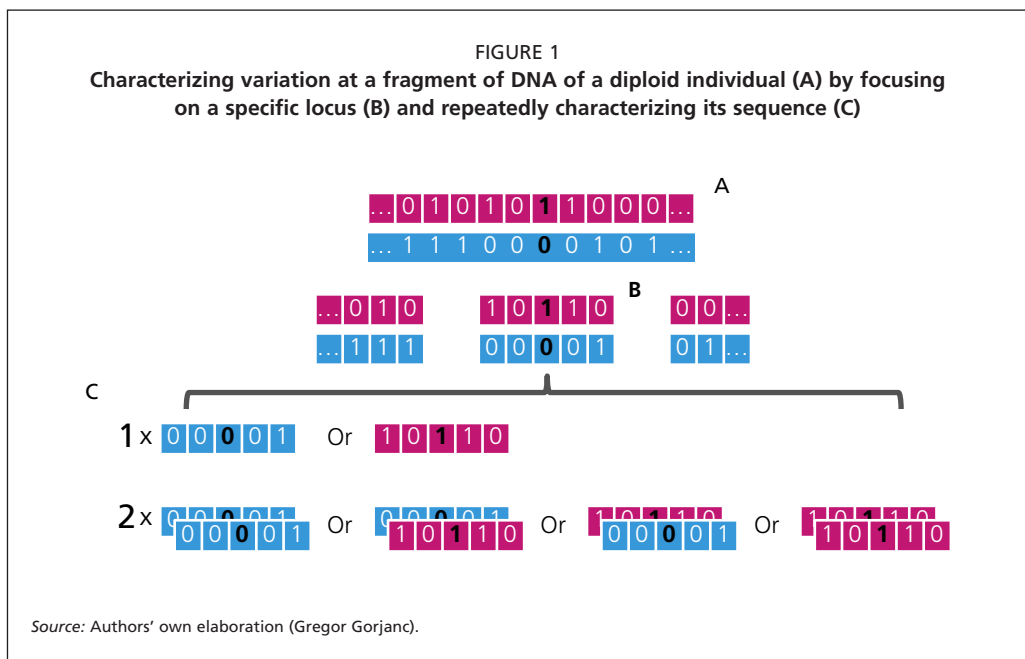
The principle

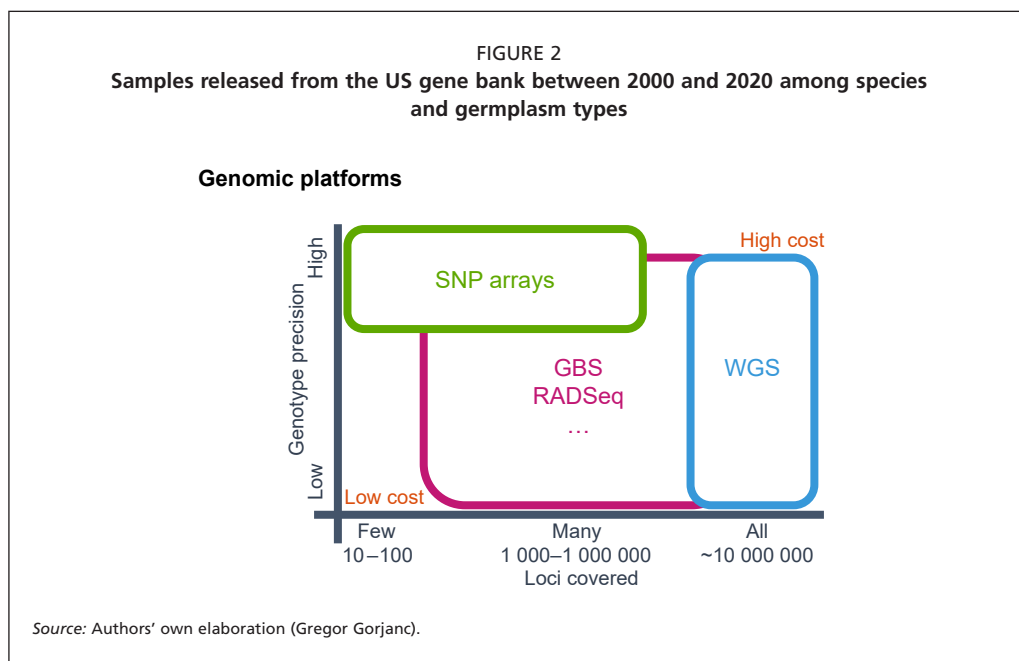
Although SNP arrays are arguably the most popular genotyping platform, methods have been developed to ascertain genotypes from DNA sequence data. In general, these methods obtain genotypes based on one of two approaches: (i) low depth WGS; or (ii) sequencing a specific subset of the genome (also known as “reduced-representation sequencing”).

These two classes of methods are often collectively referred to as “genotyping-by-sequencing” (GBS), although GBS is also used to describe a specific method of reduced-representation sequencing. This subsection emphasizes the latter approach, although many of the statements made apply to all GBS methods. GBS can be a method of choice in certain settings, particularly in *de novo* applications (Scheben, Batley and Edwards, 2017), such as for species with no standard SNP chip. GBS can in a single step both identify SNPs, many of which may be novel, and yield the desired genotypes.

The principle of reduced representation GBS is the sequencing of a fraction of the genome in such a way that the same fraction is sequenced in all individuals. This can be accomplished, for instance, by hybrid-capture or size selection of fragments generated by restriction enzyme digestion. The fragments are then sequenced, sequence reads are mapped against a reference genome and alleles are called from the reads (see Figure 1). There is considerable variation among the various methods in terms of these steps, which ultimately control the number and quality of resulting markers. Fourteen different methods of GBS have been described (Scheben, Batley and Edwards, 2017) and new ones are continually being proposed (e.g. Rowan *et al.*, 2017).

Calling genotypes from alleles is reliable if fragments are sequenced many times. Obviously, the probability of correctly calling a heterozygote increases with the depth of sequencing (where “depth” refers to the number of times each fragment is sequenced, on average): 0.000 for $x = 1$, 0.500 for $x = 2$, 0.750 for $x = 3$, 0.875 for $x = 4$, 0.938 for $x = 5$ and 0.998 for $x = 10$. Generally, sequencing error is low, e.g. 0.01–0.001, but not zero, which makes genotype calling a bit more complex than schematically presented below (Figure 1). Available software tools account for sequencing errors (see Scheben, Batley and Edwards, 2017; Lou *et al.*, 2021 and the Appendix 6).





Advantages and disadvantages

The GBS approach has several main advantages relative to SNP chips. First, GBS does not depend on a previously chosen set of markers or on the availability of whole-genome data, and will reveal genetic variation within any livestock or wildlife population. Consequently, GBS may provide more relevant markers than a commercially available SNP array. Second, because only the partial genome is sequenced, GBS yields novel SNPs much faster than can be done by WGS, and thus, for more immediate information on aspects such as MAFs and phylogeographic patterns within the panel of samples. Third, GBS completely avoids the AB (e.g. Schraiber and Akey, 2015; Simčič *et al.*, 2015; Lou *et al.*, 2021), except for GBS after hybrid capture. Fourth, users can tune GBS to their purpose and budget by choosing the number of sequenced fragments and the depth of sequencing (Figure 2) (see also Section 4). As previously mentioned, the GBS approach also provides an option for less common species that lack commercial SNP arrays.

There are, however, also disadvantages. First, GBS generally requires purified high-molecular weight DNA. Second, unique alleles in a population can be hard to distinguish from sequencing artefacts, whereas presence–absence variation due to deletions or insertions cannot be scored reliably at low sequencing depth. Third, because the number of markers depends on the sample and the output consists of allele calls instead of genotype calls, GBS requires additional bioinformatic attention in downstream analyses. Note that these considerations also apply to SNP calling on the basis of WGS assemblies. However, sequencing methods and tools for downstream analyses are continually improving.

Implementation and applications

Several commercial providers offer continually evolving custom GBS. Interested scientists are recommended to use an internet search engine to identify an appropriate service provider. The GBS approach has been adopted actively in several scientific communities (e.g. Buerkle and Gompert, 2013; Scheben, Batley and Edwards, 2017; Li *et al.*, 2021; Lou *et al.*, 2021). This adoption was fuelled by a lack of genomic resources or SNP arrays in target species, and/or by unacceptably large AB in the available SNP arrays. GBS is less expensive than SNP bead arrays for genotyping a few individuals, but for high numbers of samples the streamlined SNP arrays become cheaper and offer the advantage of a more straightforward data analysis.

Several studies have leveraged GBS for population genetics studies. For example, Dodds *et al.* (2015) and Bilton *et al.* (2018) accounted for sequencing depth when estimating relatedness and LD, respectively. Dodds *et al.* (2019) and Whalen, Gorjanc and Hickey (2019) applied GBS to parentage assignment. Gorjanc *et al.*, 2015, 2017) and Liu (2020) discussed the use of GBS with genomic selection. Interestingly, Lou *et al.* (2021) reviewed a number of standard population genetic analyses, most of which were based on GBS data without imputation (see subsection below) and therefore had to develop specific approaches for standard analyses. An alternative approach is to infer genotypes from GBS allele calls from multiple individuals and loci, and then follow standard analyses for genotypes obtained with SNP arrays.

PREPARING A WORKING MULTILOCUS DATASET

Genotyping and sampling errors can occur when performing genomic assays. The analysis of genetic data requires quality checks to identify such errors. Genotyping errors in SNP arrays can be due to insufficient DNA concentration, low quality of the DNA samples, sample contamination, poorly performing genotype probes, poor hybridization of DNA from very divergent populations or related species; and – in the case of WGS and GBS data – to low sequence coverage. Quality checks are performed at both the individual- and SNP-level, and guide the “filtering” or the removal of the individuals and/or SNPs. The most common filtering approach attempts to preserve the number of individuals rather than the redundancy of SNPs. However, the optimal procedure and cut-off values for discarding of data should always be evaluated according to the dataset and the objectives of the study.

Although the manipulation and analysis of high-throughput genomic data, and interpretation of the results may require specialized bioinformatic competence (FAO, 2015), several well-documented and freeware bioinformatic tools are available, providing user-friendly tools and routines for the quality checking procedures. Among many, the following are the most popular and comprehensive software packages for working with genomic data: PLINK (Chang *et al.*, 2015; Purcell, 2010), GWASTools (Gogarten *et al.*, 2012), GenABEL (Karssen, van Duijn and Aulchenko, 2016) and KING (Manichaikul *et al.*, 2010). Appendix 8 provides a step-by-step example of using PLINK (and R software) to perform quality checks and data editing.

Merging of datasets

As mentioned previously, a substantial advantage of SNPs relative to microsatellite markers is the option to combine data with existing datasets based on the same SNP panel or panels that have many SNPs in common. Since, in livestock species, most DNA arrays are provided by two major companies (i.e. Illumina and Affymetrix) with considerable across firm and backward compatibility of new versions, this is often the case. Merging of data is not trivial, but differences in SNP identification codes and genomic coordinates can be harmonized across the datasets to be merged by using the most updated assembly information for a given species. Chromosomes are composed of complementary strands of DNA, and, as previously mentioned, opposing strands may have been genotyped between the datasets being merged. In such instances a “strand flip” must be undertaken to ensure proper merging. Such differences in strand orientation are identified by four alleles (A, C, G and T) recorded at a given locus, although a strict biallelic locus is generally expected. Such errors can be detected by PLINK during the merging operation and corrected by swapping $A \leftrightarrow T$ and $C \leftrightarrow G$. However, different strand orientations in A/T and G/C SNPs cannot be detected, and it is advisable to remove all A/T and C/G SNPs if a strand flip must be carried out. It is also recommended that the datasets to be combined share at least one breed, to verify that, in the combined dataset, all animals from this breed cluster together and the diversity patterns are not confounded by systematic differences between the datasets that have been merged.

Mode of inheritance

With rare exceptions, mitochondrial DNA (mtDNA) markers are only inherited via the maternal lineages and markers on the male-specific part of the Y chromosome (MSY) only via the paternal lineages. Neither mtDNA nor the MSY participate in meiotic crossing-over, and both constitute a single non-recombining haplotype. As a consequence, they profoundly differ from autosomal markers in terms of mutation rate, evolutionary mechanisms, effect of genetic events and phylogeographic diversity patterns. X-chromosomal markers have a biparental mode of inheritance, but nonetheless differ in several properties from the autosomes:

- They have only one copy in males, and thus a 25 percent lower effective population size (N_e).
- Genes involved in reproduction are overrepresented on the X chromosomes (Vaiman, 2002).
- X chromosomes are more involved in meiotic drives than are autosomes, the reasons for which are only partially understood (Hughes *et al.*, 2020).
- X chromosomes also have a pattern of diversity that differs from the autosomal pattern (Wilson Sayres, 2018; da Fonseca *et al.*, 2019).

For poultry species, the sex chromosomes are referred to as Z and W, and females are the heteromorphic gender (ZW). Therefore, the points made above generally hold true, but for the opposite gender.

As a practical consequence for genetic diversity studies, mtDNA, MSY, sex chromosomes and autosomes should be analysed separately. This happens automatically if LD pruning is applied, since this removes all three types of SNPs with a special mode of inheritance.

For localization of genetic traits by GWAS (see Section 4), marker data from autosomes and X chromosomes can be combined.

Missingness of data

The “missingness” of individual SNP and samples is defined as the percentage of missing genotypes for a given marker or individual, respectively, and is complementary to the “call-rate” (5 percent missingness equates to a 95 percent call-rate). High proportions of SNP missingness can be due to a probe design inadequacy for all or a part of the samples, and, in the latter case, can lead to a bias in estimates of allele frequencies. A high proportion of missing genotypes for a sample indicates either a poor DNA quality, deviating species origin (e.g. *Bos indicus* rather than *Bos taurus*), or low sequence coverage for WGS- and GBS-based datasets.

For DNA array datasets, SNP-level filtering is typically performed first, followed by sample-level filtering, both allowing 5 percent missingness. A more refined approach can reduce the amount of data lost due to filtering by following a two-step approach. In the first step, a more relaxed threshold (such as 20 percent) to remove SNPs and samples with extremely high levels of missingness, followed by an additional filtering step with a more stringent threshold (e.g. 5 percent). Note that the SNP filtering step removes Y-chromosomal SNPs unless males account for at least 95 percent of the samples.

Sex discrepancy

When the sex of the animal providing the samples is known, discrepancies between the recorded and inferred sex may identify mislabelling of samples. Such an occurrence may indicate poor sample management and should be followed by a thorough check of the dataset and available metadata. The gender is derived by PLINK from the classical inbreeding coefficient [$F = (\text{expected homozygosity} - \text{observed heterozygosity}) / \text{expected homozygosity}$] for X-chromosomal SNPs; $F > 0.8$ and < 0.2 are often adequate cut-off values for males and females, respectively. Intermediate values may indicate either a mixed sample origin, or, for male samples, the presence of SNPs from the pseudoautosomal region (PAR), region of commonality between sex chromosomes. The PAR can be localized by comparing the frequency of heterozygotes across the X chromosome in males and females, and can be relabelled as a separate chromosome not to be used for sex assessment. It is also essential to perform the sex-check assessments in PLINK on sets of loci in approximate linkage equilibrium (see subsection LD below). Furthermore, even if the sex inferred from analysis agrees with the sex recorded in the data, the presence of Y-chromosomal genotypes in a sample labelled as female is another clear warning of a problem that demands attention.

Minor allele frequency (MAF)

As explained previously, the MAF for a biallelic SNP is the frequency of the least-commonly occurring allele at a given locus, hence MAF must be ≤ 0.5 . The SNPs with a MAF of a few percentage points or less can represent true low-frequency variants but also be the result of genotyping errors. For this reason, it is common to retain only SNPs with $\text{MAF} \geq 0.05$ for many analyses. Alternatively, with large datasets, the threshold $\text{MAF} \geq 10 / N$, based on the consideration that a larger sample size (N) reduces the likelihood that a small MAF has

resulted from genotyping errors. However, some analyses (such as GWAS, see Section 4) are highly affected by low-frequency variants and thus more stringent thresholds are often applied (e.g. requiring $MAF \geq 0.05$ even for $N \leq 10\,000$).

The MAF is particularly affected by sampling bias and AB. If a dataset has a large degree of homozygosity (hence, a small MAF on average), a larger sample size is necessary to obtain enough SNPs passing the MAF filtering because of the low overall frequency of heterozygotes. The effect of AB is proportional to the degree of divergence between the discovery and the study populations (Helyar *et al.*, 2011), and leads to an underestimation of the genetic diversity of the study population. This applies for instance to zebu cattle analysed by the popular arrays designed mainly for taurine cattle (Barbato *et al.*, 2020). As discussed above, the AB can be mitigated somewhat by filtering loci in high LD and by selection of SNPs that have a high MAF in the ancestral breeds or in the wild ancestors.

Allelic frequencies are used to calculate expected heterozygosity (H_e) and genetic distances between breeds. For optimizing breed comparison analyses, one must keep in mind that a low sample size for a given breed (< 20 animals) can lead to underestimating the H_e and thus to inflating the genetic distances to other breeds.

Hardy-Weinberg equilibrium

The Hardy–Weinberg equilibrium (HWE) implies a direct relationship between allelic frequencies (p and q , with $p + q = 1$, in the case of a biallelic SNP) and the genotypic frequencies (p^2 and q^2 for the homozygotes, and $2pq$ for the heterozygotes), which is supposed to remain constant over generations. The HWE is valid for a Wright-Fisher population of infinite size with random mating, no selection, mutation and migration. Significant departure from HWE for individual SNPs can be caused by population subdivision and substructure (e.g. Wahlund effect) (Garnier-Géré and Chikhi, 2013), recessive lethal mutations, selection, non-random mating, inbreeding and genotyping errors (Chen, Cole and Grond-Ginsbach, 2017). For datasets of larger populations and datasets aimed at GWAS, it is a common practice to explain strong deviations from HWE by genotyping error and remove the corresponding SNPs. However, removal of such SNP must be done judiciously, as such deviations may be an indicator of selection or some other real phenomenon that is of interest in the analysis (e.g. Pausch *et al.*, 2015).

The HWE can be tested by the so-called exact test, which provides a P -value for the significance of the HWE departure and is fairly robust to deviations from the theoretical assumptions. If the downstream analysis focuses on the identification of selection signatures (see Section 4), it is recommended to use a permissive HWE filtering threshold (e.g. $P < 10^{-10}$) to avoid filtering out too many variants. In the case of GWAS applied to binary traits, it is common practice to use more lenient thresholds in cases (e.g. $P < 10^{-10}$) than in controls ($P < 10^{-6}$), because deviations from HWE in such cases can be due to true genetic association with the focal trait. When assessing quantitative traits, stricter thresholds can be used (e.g. 10^{-6}).

Non-random mating, which, in its most extreme form, leads to population subdivision (stratification into different subpopulations), is expected to cause HWE deviations across the whole genome, but such subdivision is commonly tested by comparing H_e and observed heterozygosity (see below) and applying model-free multivariate statistics such as PCA or multidimensional scaling (see Section 4).

Heterozygosity

The observed heterozygosity (H_o) of an individual is the proportion of heterozygous genotypes, that is carrying two different alleles at a given locus, preferably considering only autosomal loci. Within populations, individual heterozygosity tends to be variable, but values for individuals with three standard deviations (SD) above the population average may indicate sample contamination or crossbreds, whereas values three SD below the average can be caused by inbreeding. Filtering H_o outliers is particularly important when the dataset is supposed to be genetically homogeneous, as with data for GWAS.

For checking the genetic constitution of a population, H_o averaged across the individuals of a population is an indicator of the diversity of the breed, although it should be borne in mind that it can be influenced by AB (see above) or be influenced by recent breeding decisions (e.g. subpopulations of inbred individuals within a relatively genetically diverse breed). It is further useful to compare H_o with H_e , calculated on the basis of the allele frequencies of the population. The H_o is generally lower than H_e as a consequence of sporadic non-random mating or sampling bias. Large differences between H_o and H_e indicate significant deviations from the Wright-Fisher ideal population, and, for example, the condition $H_o > H_e$ may suggest recent population admixture or outbreeding, while $H_o < H_e$ can indicate population subdivision or inbreeding. Both conditions may influence analyses that presume a homogeneous population. The extent of inbreeding/outbreeding can be quantified as $F_{IS} = (H_e - H_o) / H_e$, where F_{IS} represents the inbreeding coefficient of the population. F_{IS} is > 0 when inbreeding is present, and < 0 when heterozygosity is greater than expected, such as with outbreeding or crossbreeding.

Linkage disequilibrium (LD)

SNPs are in LD if there is a non-random association of their alleles. This is to be expected for loci that are nearby on the same chromosome, for instance, within or proximal to the same gene, so that recombination between them is a rare event. LD is also normal for the non-recombining mtDNA and the MSY. However, high LD of well separated genomic SNPs, that is not compatible with the expected recombination rate (about 1 percent per 10^6 base pairs (bp) per generation), may be caused by demographic processes such as admixture and genetic drift or by selective processes such as “hitchhiking” or background selection (McVean, 2007). Occasionally LD is observed between SNPs on different chromosomes (expected recombination rate of 50 percent per generation), which is usually an artefact due to the sampling of a limited number of gametes or individuals, but may be a real phenomenon resulting from epistatic effects or unknown sources of population stratification.

A consequence of LD is that nearby markers are not independent. Therefore, it is common to implement LD-based pruning procedures to retain markers that are in approximate linkage equilibrium and are thus largely independent. This procedure is required when an analytical model does not explicitly take LD into consideration (see subsections on MAF and relatedness). A common PLINK implementation of LD-based pruning calculates correlation coefficients (r^2) within sliding windows across the genome, and removes one of each pair of SNPs having LD greater than a given threshold. Typically, markers are selected in 2 Mbp windows that shift 0.2 Mbp forward (~10 percent of the window size) at each iteration while applying a LD threshold corresponding to $r^2 = 0.2$. This method could, however,

induce additional bias if the LD removed is meaningful for a particular population. If the approximate average size of LD blocks is known for the species/breed of interests, another method to remove LD is to randomly select markers at a distance that is greater than the average level of LD specific to the breed. This method would then allow for the detection of extended LD that may be due to selective breeding or other biologically important characteristics of the population.

LD-based pruning can also be used for data size reduction, as it removes redundant SNPs by selecting only those markers that are representative of the genetic haplotype blocks. Under this scenario it is possible to apply more conservative LD thresholds (e.g. $r^2 \geq 0.5$), which will remove the most highly correlated loci while preserving moderately correlated SNPs (e.g. due to low-frequency variants). Such an approach will retain the underlying genetic structure of the dataset while reducing the computational burden.

Note that LD-based pruning removes mtDNA and Y-chromosomal SNPs. It should be carried out after removal of the SNPs with low call rates and bad-scoring or contaminated samples. LD-based pruning is beneficial for calculation of genetic distances, PCA or model-based ancestry analysis (e.g. as implemented in the popular software Admixture (Alexander, Novembre and Lange, 2009), but not for haplotype-based studies, such as runs of homozygosity (ROH) and local ancestry analyses. An additional benefit of LD-based pruning is alleviating the AB in DNA array datasets (see above) (Malomane *et al.*, 2018). These various types of analyses are explained in greater detail in Section 4.

Relatedness

Relatedness or kinship indicates the similarity of a pair of individuals due to common ancestry. Checking the pattern of kinship is essential for several reasons:

- Individuals may be found to be duplicated or be identical twins.
- The presence of highly related individuals introduces a bias towards a specific family in the dataset or reduces the statistical power of GWAS. It may be avoided during sampling by using pedigree information or a well-considered sampling scheme, but this is not always feasible.
- More in general, kinship may lead to population stratification, which confounds several analyses that assume a homogeneous population. For instance, it may easily cause false positives in single-gene association studies.
- Outlier animals with a much lower kinship with the other individuals than the population average should generally be excluded from analyses, as they are likely to be crossbreds or mislabelled.
- If genetic distances between animals within the same breed are practically the same as between animals from different breeds, genetic clusters will no longer correspond to breeds. As a possible consequence, breed definitions may be reconsidered, especially if the lack of breed-level differentiation is due to uncontrolled gene flow between local populations kept under an extensive management regime.

A common method for the identification of related individuals is to compute the pairwise proportion of shared alleles inherited from a common ancestor – identity by descent (IBD) – across all individuals. The magnitude of the resulting IBD score then reflects the relatedness of two individuals. This classic approach requires approximate linkage equilibrium

(see the subsection on LD) and should be performed on autosomal markers. It relies strongly on the assumption of a homogeneous population structure. A matrix of IBD scores may be visualized by plotting the scores as a heat map.

A robust and efficient algorithm for inferring relatedness of individuals has been implemented in the software KING (Manichaikul *et al.*, 2010), which has been recently included in PLINK 2.0. The KING algorithm does not require LD among markers or a homogeneous population structure. The KING relatedness coefficients are scaled to correspond to kinship coefficients (which are $0.5 \times$ the relationships coefficients): monozygotic twins/duplicate samples have kinship = 0.5; first-degree relations (parent-offspring, full siblings) have kinship = 0.25; etc. As a cut-off value, the geometric mean of the values corresponding to two degrees of kinship is used (e.g. a kinship coefficient > 0.354 , which is the geometric mean of 0.5 and 0.25) to identify monozygotic twins and duplicate samples.

Alternatively, a neighbour-joining (NJ) phylogram of allele sharing distances between individuals can be generated to visualize identical samples, variable kinship, outliers, breed-level differentiation and, via the lengths of the terminal branches, the effect of genetic isolation on the diversity within breeds (Cardoso *et al.*, 2018). Such an analysis will also show the clustering of closely related breeds. For more distant breed relationships, genetic distances between breeds instead of between individuals is more suitable.

Identical individuals should be removed from the dataset, and, if possible, also those individuals with first or second-degree kinships (this is particularly important for GWAS), by removing the individual with the lowest mean genotype call rate from a closely related pair. Because one individual can be involved in several pairs, PLINK 2.0 has implemented an algorithm that maximizes sample size while pruning for relatedness. For calculation of relatedness on the basis of GBS datasets, see Dodds *et al.* (2015).

Sample size

For diversity studies involving several breeds, availability of samples may be a limiting factor. Having a few samples of an interesting breed is still valuable, but its heterozygosity may be underestimated. Sample size does not have a large influence on the topology of phylogenetic networks or trees, but empirical evidence suggests that having less than 20 animals inflates the genetic distance to other breeds, as mentioned previously. Moreover, 20 animals may not be sufficient to detect low levels of breed admixture or for obtaining meaningful GWAS results (Ball, 2013).

On the other hand, more is not always better, especially when only some breeds have more data. When comparing multiple populations, breeds that are overrepresented may very well distort the results by dominating the principal components and the inference of clusters, respectively, especially if these breeds are also inbred. Hence, for these analyses, it is recommended to reduce the number of samples from overrepresented breeds to obtain a dataset with a more balanced representation of breeds.

WHOLE-GENOME SEQUENCING

Genotyping arrays are limited to assessing variants that have been preselected and are adaptable to array genotyping (generally, only SNP), such that the same sample may have to be run with multiple different assays to achieve desired goal(s) or to properly assess

and account for AB. In contrast, WGS has the advantages that variants across the entire target genome are assessed, and it is possible to identify all types of variants including SNP, indels (insertions and deletions), CNV, and structural variation (SV) like inversions or large deletions. The success of applying WGS for genome characterization depends on several factors including the type and depth of sequencing used and the analysis method(s). Here, we present some background on the currently available approaches to using WGS for characterizing variation independent of the target species. Characteristics of the population (breed or species) such as N_e , or polyploid genomes, may affect the decision on the best approach to be applied for data generation and analysis.

De novo assembly

The most comprehensive evaluation of a genome comes from an independent, haplotype-resolved *de novo* whole genome assembly of the animal. This is the most expensive and technically demanding approach, although the cost has dropped by more than four orders of magnitude since the first livestock genomes were assembled years ago, and the quality of the assemblies has dramatically increased. The basis of this improvement is the advent of long-read sequencing (LRS) platforms, each of which has particular advantages and drawbacks (Amarasinghe *et al.*, 2020; Logsdon, Vollger and Eichler, 2020). The LRS technology is developing rapidly, but the next paragraphs provide a brief overview of the state of the art and most common technologies. Amarasinghe *et al.* (2020) provides a recent review.

The Pacific Biosciences (2015) platform, often referred to as “PacBio,” can operate in two “modes,” one in which read length is maximized at the expense of read accuracy, and one with relatively shorter reads where read accuracy is maximized (the “HiFi” mode). The former produces contiguous assemblies at lower cost but with higher error rate, whereas the latter produces very high accuracy but somewhat less contiguous assemblies due to the shorter read length. The Oxford Nanopore Technologies (2008) sequencing platforms excel at generating extremely long reads that are very useful for assembling repetitive or duplicated regions of the genome, but require relatively large amounts of input DNA and therefore substantial sample volume. Both LRS platforms are dependent on availability of suitable DNA template that consists mainly of very long fragments (10s to 100s of kb) of undamaged DNA. The characteristics of the two currently available LRS platforms differ so much that a single extraction method has yet to be formulated that is ideal for both platforms.

De novo assemblies of genomes based on short-read sequencing (SRS) (Bentley *et al.*, 2008; Margulies *et al.*, 2005; McKernan *et al.*, 2009; Rothberg *et al.*, 2011) are typically a less expensive option relative to LRS, and can be suitable for population level analyses and transcriptomics. However, their accuracy (in terms of the lengths of contiguous genomic regions) is orders of magnitude lower than with LRS, and all analysis of repetitive content and gene families must be done with consideration of their deficits. For these reasons, LRS has mostly replaced SRS for *de novo* assembly of genomes. There are approaches to improve SRS assemblies through large insert size libraries, or hybrid approaches that combine SRS and LRS. The most effective hybrid approach is linked-read technology (Wang *et al.*, 2019; Zhang *et al.*, 2017; Zheng *et al.*, 2016). In linked-reads, a library production technology is applied that adds barcodes to short fragments derived from a single, longer template molecule. Millions of these independent “micro-libraries” with different barcodes

are then sequenced on standard short-read platforms. Post-sequencing, the barcode information is used to partially reconstruct the longer DNA molecule and provides longer-range assembly continuity compared to standard, whole-genome SRS.

All platforms are limited in their ability to assemble genomic regions that undergo somatic cell rearrangements in blood cells (immune gene complexes like the T-cell receptor or immunoglobulin loci) if blood is the source of DNA.

Sequence data is first assembled into “contigs,” which are stretches of DNA sequence without gaps. The SRS assemblies have N50 size (the length of the contig where the sum of all longer contigs is > 50 percent of the total assembly size) in the range of 100 kb, while the contigs that can be created from LRS data can have N50 size over 70 megabases (Mb), or 700x as long. Generally, the genome sequence depth target is $\geq 100x$ for SRS and in the range of 50x for LRS to provide a quality assembly; however, if a haplotype-resolved assembly is desired, then a greater depth is helpful. Also, if using the HiFi mode of sequencing, coverage can be as low as 20x, although haplotype-resolution is steadily improved up to 40x or so. Contigs from LRS need to be “polished” (i.e., checked and corrected) to increase accuracy, with SRS data being useful for this step (e.g. Zimin and Salzberg, 2020). A second commonly used approach is “trio-binning, which utilizes divergence between two parental species or breeds and LRS of an F1 to create two, almost perfect haploid assemblies (Low *et al.*, 2020).

Y chromosomes remain challenging to assemble due to their highly repetitive nature. Even with LRS they often remain highly fragmented. Methods for the identification of these contigs include using the normalized ratio of female to male alignments (Hall *et al.*, 2013), and k-mer based approaches (Carvalho and Clark, 2013; Rangavittal *et al.*, 2019). Prior to polishing an assembly, it's important to make sure the mitochondrial genome has been assembled. Failure to do so can result in over-polishing of nuclear insertions of mitochondrial sequence (NUMTs) which can lead to difficulties in identifying mitochondrial variants. There are methods to assemble mitochondrial genomes from both SRS and LRS (Al-Nakeeb, Petersen and Sicheritz-Pontén, 2017; Dierckxsens, Mardulyn and Smits, 2017; Formenti *et al.*, 2020; Hahn, Bachmann and Chevreux, 2013; Meng *et al.*, 2019). Polished contigs generated from LRS are sufficient to detect the majority of SNP, indels, CNV and SV in the genome by alignment to reference genome(s). Ideally, a breed- or population-specific reference or a pangenome representation would be used for such alignments, when available, to maximize mapping accuracy and variant detection. Alternatively, a full *de novo* assembly can be achieved by adding scaffolding data, either optical mapping (Hastie *et al.*, 2013; Nagarajan, Read and Pop, 2008) or chromatin conformation contact mapping (Hi-C) (Burton *et al.*, 2013; Kaplan and Dekker, 2013).

Resequencing

De novo assembly will nearly always yield the most detailed information about a single genome; however, the cost and computational effort required is still impractical for analysis of multiple animals and populations in most circumstances. Resequencing, which involves SRS or LRS at much lower coverage than necessary for assembly and depends on mapping the reads to a reference genome to detect variants, is more amenable to application in larger populations. The goals of resequencing and the platform chosen will determine the depth of sequencing required. For discovery of SNP and indels, the high accuracy

and low cost of SRS makes it the common choice. Tenfold mapped genome coverage is typically recommended as the minimum depth, and usually requires about 12-13x raw depth depending on platform. Extensive testing indicates that this is sufficient for accurate genotyping of ≥ 95 percent of SNPs compared to very high coverage sequencing ($\geq 30x$ coverage). At a low genome depth ($< 8x$), some heterozygous genotypes will be scored as homozygous genotypes and some sequencing errors will be recorded as heterozygous genotypes (Nielsen *et al.*, 2012), thus distorting the estimates of allele frequencies and genetic diversity. To address this problem, a genotype-likelihood based method has been developed to estimate nucleotide diversity for low sequencing coverage (Nielsen *et al.*, 2012). Resequencing by SRS can also accurately detect small (1–10 base) indels, but much higher coverage is needed to predict larger indels or CNVs, and some structural variants are very difficult to detect with only SRS data. LRS can also produce quality SNP and indel calls, but except for HiFi, sequencing will need to be done to a greater depth, around 20x. LRS excels when it comes to CNV and SV. With the same 10x coverage required for SNP and indels, HiFi data will yield many high quality CNV and SV calls. Regular LRS and SRS will benefit from a minimum of 20x coverage. LRS can call these larger variants with much greater frequency and accuracy than SRS, and can capture larger variants than HiFi.

Prior to the advent of haplotype-resolved assemblies, it was common to choose female reference animals due to the drop in coverage associated with sequencing the heterologous portions of the sex chromosomes in males. When the sequenced animal is a male and the reference has no Y chromosome to align to, identifying variants becomes more challenging. In this case, reads that do not map to the reference can be collected and aligned to an alternate dataset representing the Y chromosome. This can be the Y chromosome of a closely related animal, or a collection of Y chromosome genes.

The success and accuracy of WGS for detection of variation is also highly dependent on the reference genome to which the sequencing reads are mapped. Currently, most studies use a single representative assembly agreed upon by the community of scientists working with the species in question, which provides a common basis for comparison among studies. However, many livestock species are now seeing high quality assemblies of multiple breeds emerging, and studies comparing the success of using a breed-specific reference as opposed to “the reference” assembly have not been completed. Early results suggest that variant detection will be improved by breed-specific references, but at the cost of easy cross-study comparison (Crysnanto and Pausch, 2020). Recent efforts in cattle have focused on the creation of a “pangenome” reference including all genomic segments globally present among breeds, as a solution to this problem (Heaton *et al.*, 2021).

Preparing a working multilocus dataset from genome resequencing

Regardless of the approach used (LRS or SRS), resequencing of an animal's genome initially yields a large quantity of individually sequenced DNA reads. These reads require substantial processing to finally provide a dataset of genetic variants positions on individual chromosomes. Figure 3 is a flow chart that illustrates the process to be taken to transform raw SRS data from a sample of genomic DNA into a dataset of genetic variants. For each step, the commonly used data formats and software modules are indicated. Other options for formats and software exist and may be used depending on personal preference.

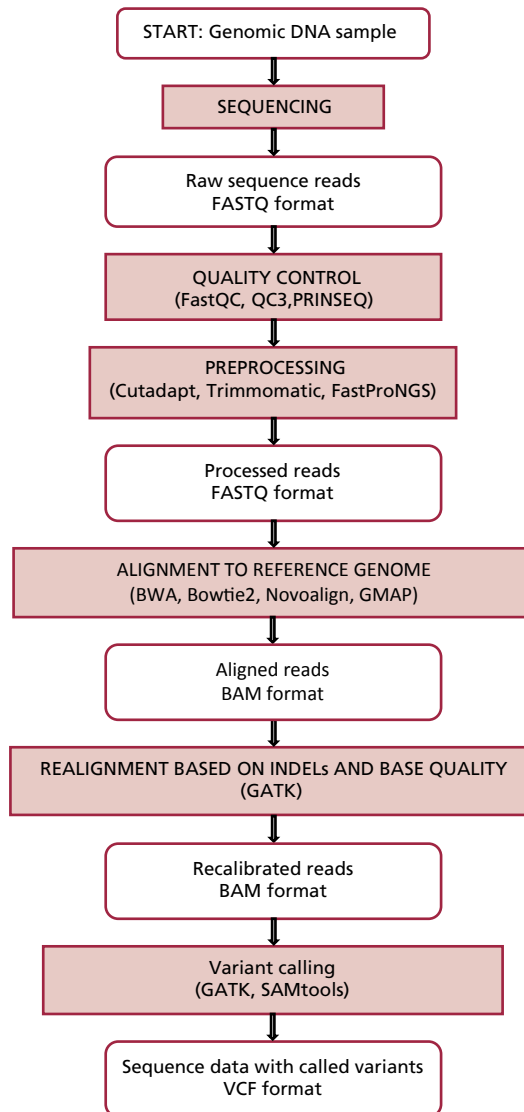
Many sequencing platforms output the raw sequence data for individual reads into a format called FASTQ. The FASTQ format is text based and includes a sequence identifier, the sequence and a quality score. The raw sequence reads then undergo quality control and preprocessing. FASTQC (Andrews, 2010) is a software that undertakes a general overview to help identify problems in the sequencing process. QC3 (Guo *et al.*, 2014) is a software with more features than FASTQC, such as identification of individual “good” and “bad” reads. PRINSEQ (Schmieder and Edwards, 2011) can perform both quality control and preprocessing, the next step in data preparation. Preprocessing often performs filtering and trimming of raw sequences. Individual sequences may be filtered based on quality based on parameters such as quality score, read length and content of guanine and cytosine nucleotides. Trimming removes sequence that corresponds to DNA adapters that may be incorporated during the sequencing process. Preprocessing improves the computing performance and accuracy of subsequent steps. In addition to PRINSEQ, Cutadapt (Martin, 2011), Trimmomatic (Bolger, Lohse and Usadel, 2014) and FastProNGS (Liu *et al.*, 2019) are other software options for preparation of genome sequence data.

Following these steps, the individual reads are ready to be aligned to the reference genome. Several software programmes are available for this step, including BWA (Li and Durbin, 2009); Bowtie2 (Langmead and Salzberg, 2012); Novoalign (Novocraft Technologies, 2020) and GMAP (Wu and Watanabe, 2005). The alignment process yields more information for each read (e.g. chromosomal location) and thus requires a new output data format. The basic format is called SAM for “sequence alignment/map format,” but given the large amount of data, the binary format of these data, “BAM” is more commonly used.

Finally, it is generally recommended to check and realign the sequence data due to indels and base quality. The GATK software (Van der Auwera and O’Connor, 2020) can be used for this task. This leads to the final step, variant calling. This step identifies the genomic locations of differences from the reference sequence. As this results in new information, another data format is used, VCF format, for “Variant Call Format.” GATK can be used for variant calling, along with other software, including SAMtools (Li *et al.*, 2009). The resulting data serves as the basis for further analyses.

As is the case for SNP assays, the resulting dataset should be subject to quality control. In particular, checks for the estimated level of relatedness of animals is important, both for the identification of putative duplicate samples and for possible removal of closely related animals that may distort the analysis. The quality control software described in Appendix 6 are generally applicable to sequence as well as SNP data.

FIGURE 3
Steps to be taken in the processing of a sample of genomic DNA
into a dataset of genetic variants



Note: The white boxes indicate the material (DNA sample or data), whereas the shaded boxes indicate the action undertaken. Common software for each action is shown in parentheses

Source: adapted from Carter, T.C. & He, M.M. 2016. Challenges of identifying clinically actionable genetic variants for precision medicine. *Journal of Healthcare Engineering*, 2016: 3617572. <https://doi.org/10.1155/2016/3617572>

GENOTYPE IMPUTATION

Imputation: filling up missing values

Missing value imputation is the name given to the process of predicting incomplete values in a set of data. In many instances, missing values cannot be processed in an analysis and thus should be either removed or replaced by a guess beforehand. Although the first approach is simpler (e.g. the missing data could be handled by ignoring the missing information during computations), the second approach may help retain more statistical power in the analysis since it attempts to minimize data loss.

Imputation consists of taking advantage of correlations between variables in order to fill up the empty data. This approach involves the use of prediction methods adapted from statistics and machine learning, or even, use of deterministic approaches based on heuristics. As for any type of prediction, the imputation of missing values comes with uncertainty, and the accuracy of that prediction is dependent on several factors that are inherent to the data.

The number of errors that can be tolerated in an imputation procedure is dependent on the missingness rate. If a data set contains very few missing values, imputation with low accuracy could be performed with little impact on subsequent analyses, since the small number of affected values would be diluted over the rest of the data. This is frequently referred to as “minor imputation.” Conversely, if the number of missing values to be imputed is large, accuracy must be high in order to avoid the “garbage in, garbage out” effect - that is, poor quality input leading to faulty output.

If high missingness rates suggest poor quality data, it's logical to question whether imputing the subsequent large numbers of missing values will be of value. However, in specific situations, large numbers of missing data are not caused by systematic errors, but are rather deliberately planned during experimental design. It may be logical to “plan” to have missing data if a smaller subset of data can predict the missing data with a high accuracy, and at a substantially decreased cost. Genomic data are a logical target for imputation, because DNA markers are inherited in linear sequences on chromosomes that remain intact from generation to generation (if recombination does not occur). Because of this, having data for the first and last nucleotide in a DNA sequence often allows accurate prediction of the entire DNA sequence between those nucleotides, based on full genome sequences of genetically related animals.

In a WGS experiment, for example, millions of variants are detected across samples. Despite drastic reductions in sequencing costs in the past decades, sequencing a whole genome (10x coverage depth) may still cost from USD 300 to more than USD 1 000, depending on one's location, the facilities and commercial providers available. In parallel, microarray technologies have matured substantially, making it possible to generate reliable data for a subset of these variants in a cost-effective manner. As discussed previously, the number of variants in these microarrays typically ranges from tens to hundreds of thousands. This is substantial data, but still a tiny fraction of the overall number of variants that could be captured by WGS. Therefore, one may design an experiment where a smaller number of animals have their whole genome sequenced, thus generating a reference panel of millions of variants. In turn, this reference panel is used as a guide to impute the bulk of the data that was genotyped using a microarray or by low-depth sequencing (Li *et al.*, 2021).

The same rationale can be applied in imputing genotypes between microarrays, where a low-density panel (LDP) of markers is imputed to a high-density panel (HDP), or even to a full genomic sequence.

Imputation workflow

Genotype imputation is a straightforward procedure, albeit the mathematical and algorithmic mechanics behind it can be rather complex. First, the HDP (or WGS data) and LDP are selected such that most – if not all – of the variants in the LDP are included in the HDP. Second, a set of animals is genotyped in the HDP in order to compose the reference panel. Third, animals to be imputed are genotyped in the LDP. Fourth, data quality control is applied to both the HDP and LDP sets. Finally, LDP data is imputed to the HDP using one of many methods available for genotype imputation.

Building a reference haplotype library

The selection of animals to be included in the reference panel must be optimized for improved imputation performance. If no genotypic data is available, one can use pedigree relationships to select key ancestors capturing a large proportion of the genetic variation in the population (Goddard and Hayes, 2009). Otherwise, reference animals could be selected based on the LDP data such that haplotype diversity is maximized. This is achieved by iteratively selecting individuals carrying large numbers of high frequency haplotypes (Butty *et al.*, 2019). Of note, if high imputation accuracy must be achieved for low frequency variants, selection of animals carrying rare haplotypes should also be considered (Bickhart *et al.*, 2016).

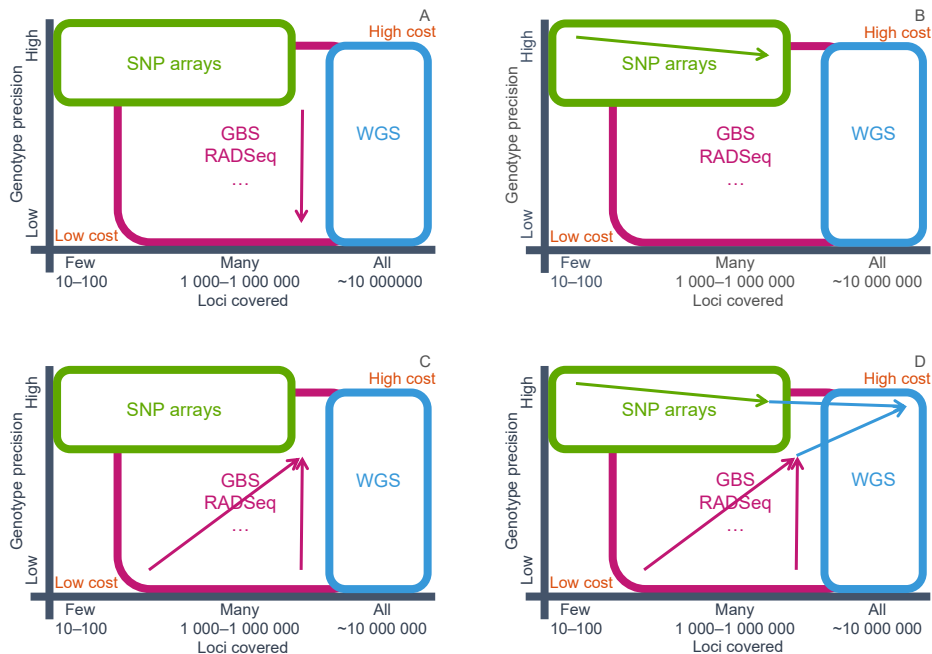
Imputation methods

Genotype imputation is divided into family-based and population-based methods. In the first, haplotypes from close relatives, typically identified via pedigree data, are used to impute the unobserved genotypes of LDP samples. In the second, pairs of individuals are assumed to share a common ancestor, such that LDP samples are interpreted as mosaics of haplotypes that are present in the HDP samples. Furthermore, there are methods that take advantage of both paradigms in order to achieve improved performance. Importantly, some methods require genotypes to be phased (i.e., the parental haplotypes are separated) before imputation, while most phasing algorithms perform minor imputation on the fly.

Imputation of GBS datasets

The availability of GBS and other genotyping platforms has created the need for multiple types of imputation (see Figure 4). Imputation of genome-wide data is a large and a fast-evolving field, and is particularly suitable for populations with a known pedigree and a small number of parents genotyped by routine SNP array genotyping. For such populations, GBS strategies have been designed that enable the imputation of whole-genome sequences in hundreds of thousands of individuals (Ros-Freixedes *et al.*, 2020a, 2020b). Whalen *et al.* (2018) and Whalen, Gorjanc and Hickey (2020) have developed methods, implemented in the programs ALPHAPEEL and ALPHAFAM, that quickly and accurately calls genotypes, carries out phasing and imputes whole-genome sequence data of any sequencing depth

FIGURE 4
Strategies to increase volume of genomic data per unit of cost are indicated by arrows – to enable generating genomic data in the first place or genotyping more individuals at higher densities: (A) reduction of the depth in genotyping-by-sequencing; (B) imputation from low-density to high-density SNP arrays; (C) imputation from low-density and low-depth to high-density and high-depth in genotyping-by-sequencing; and (D) imputation to whole-genome sequence



Source: Authors' own elaboration (Gregor Gorjanc).

in pedigrees. GBS data can also be imputed without pedigrees by using software such as BEAGLE (Browning, B. and Browning, S., 2016); STITCH (Davies *et al.*, 2016); and GLIMPSE (Rubinacci *et al.*, 2021). Whereas the imputation of SNP array datasets to a higher density or to a WGS is at the expense of accuracy, imputation of GBS datasets improves the accuracy of genotypes relative to those derived from low-depth datasets, which can have some inaccuracies due to missed alleles (see Figure 4B, Figure 4C and Figure 4D).

The decision on imputation

Imputation of data can be attractive due to its potential to decrease costs, but accuracy will be sacrificed. When making the decision to impute data, the main factors to consider are the price difference between the low-density genotyping method and the high-density approach, and the ramifications of possible errors. The accuracy of imputation will also rely heavily on the population to which it is applied. Accuracy is increased when a large

amount of reference data is available, and when the animals making up the reference data are generally more closely related to the sample of animals to which imputation is to be applied. Accuracy of imputation is also increased by greater LD between the loci with known genotypes and the loci to be imputed. Increased mean LD of the breed will also increase imputation accuracy. For a small group of animals from one or more local breeds being genomically characterized for the first time, the conditions for imputation will generally not be favourable. Imputation may however be quite attractive for further studies (such as GWAS) for a well-characterized population.

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SECTION 4

Applications of genomics

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This section will describe how genetic and genomic information can be used to describe patterns (such as genetic diversity, differentiation, classification, and genetic clines) or to infer historical processes (expansions, contractions, admixture and gene flow) affecting animal genetic diversity. In the first case, one can quantify how genetic diversity changes per individual, location or environment or by domestication. The second group of approaches aims to identify, quantify and determine the date of ancient demographic events, possibly including direct or indirect selection acting on specific genomic regions. The principles and limits of several methods that have become available in the last couple of decades are noted. This section provides the underlying genetic theory behind the most common analyses undertaken for genomic characterization, and describes the methods used. The seminal research associated with each approach is cited. This section is complemented by Appendix 9, which provides a summary of the main steps and analyses that are frequently undertaken in genomic characterization, and provides examples of the commonly used software for each step.

Developments from the genomics era allow novel options to address previously unanswerable questions with increasingly sophisticated methods, including modelling. In some cases, the resulting patterns can only be interpreted by understanding the underlying models and processes. In addition, the analysis of patterns and processes should be integrated. For instance, most available approaches to identify selection typically make strong assumptions about the demographic history that generate “neutral” patterns, while methods reconstructing demographic events often ignore selection. Inferring selection and demography simultaneously, in order to explain spatial patterns, is one of the greatest challenges of evolutionary genomics.

ASSESSMENT OF GENOMIC VARIATION WITHIN POPULATIONS

Measures of genetic diversity

Local well-adapted breeds are considered to be reservoirs of genetic diversity that are recognized to contribute to future traits of interest (Bruford *et al.*, 2015; Groeneveld *et al.*, 2010). Assessment of the within-breed component of genomic variation is essential for the effective management of breeds (Boettcher *et al.*, 2010; Caballero *et al.*, 2010; Ginja *et al.*, 2013;

Lenstra *et al.*, 2012; Ruane, 2000; Toro and Caballero, 2005; Toro, Fernandez and Caballero, 2009). Assessment allows for monitoring, on one hand, the effect of isolation on the genetic diversity and the ensuing risk of inbreeding depression, and on the other hand, the effect of crossbreeding on the genetic constitution of breeds. In addition, a comparison of the diversity of domestic and wild populations, and the geographic pattern of within-bred diversity may reveal the serial population bottlenecks that occurred in the past in association with domestication and migration, respectively.

The genetic diversity can be quantified via the observed (H_o) and expected (H_e) heterozygosities, nucleotide diversity (π) and effective population size (N_e , see below). The H_o is based on the actual distribution of genotypes, whereas H_e is based on allele frequencies (Nei, 1973; 1978). Both of these measures are easily obtained from datasets of genetic markers. For nucleotide sequence data, the estimate of π gives the average number of nucleotide differences per site between two DNA sequences chosen randomly from the studied population (Nei and Li, 1979), and is equivalent to H_o if all nucleotides are considered as loci. The parameter N_e represents the number of reproducing individuals given a number of conditions (see subsection on “Effective population size” that appears later in this document).

Inbreeding and runs of homozygosity

Inbreeding results from the mating of related individuals, and it is estimated by the probability (F) that two alleles at a locus are identical. Classically, inbreeding was calculated using pedigree records, but genomic data and H_o and H_e values allow for a more accurate quantification of inbreeding without using pedigree data (Curik, Ferenčaković and Sölkner, 2014; Druet and Gautier, 2017; Howrigan, Simonson and Keller, 2011; Purfield *et al.*, 2012). Inbreeding increases the homozygosity in individuals and thus decreases H_o , but it does not change H_e based on the allele frequencies, resulting in a heterozygote deficit and a departure from the Hardy-Weinberg equilibrium (HWE) of homozygous and heterozygous genotypes. Thus, Wright’s F -statistics based on comparing H_o and H_e can be used to infer inbreeding (F_{IS}) from molecular data (see Box 6 for more details on F -statistics). Because F_{IS} is insensitive to inbreeding in more ancient generations, the genomic coverage by runs of homozygosities (ROHs) has become a commonly used measure for inbreeding if single nucleotide polymorphism (SNP) and whole-genome sequencing (WGS) data are available.

Inferring the frequency and length of ROHs in the genomes of farm animals helps to understand population histories including the occurrence of bottlenecks, estimate inbreeding and identify signatures of selection (Bruford *et al.*, 2015; Ceballos *et al.*, 2018; Meyermans *et al.*, 2020; Peripolli *et al.*, 2017). For example, long ROH segments suggest recent inbreeding (consanguinity) and low genetic diversity. This has been observed for Holstein-Friesian cattle (Doekes *et al.*, 2019; Peripolli *et al.*, 2017; Purfield *et al.*, 2012; Upadhyay *et al.*, 2019) and is possibly associated with deleterious homozygous variants (Pryce *et al.*, 2014). In contrast, relatively many short ROHs with only a few long ROHs indicate a reduced population size in the past and little recent inbreeding, as found for wild boars (Peripolli *et al.*, 2017). Conversely, hybridization reduces the ROH coverage and increases genetic diversity, as in cattle of admixed taurine–zebu ancestry (Kim *et al.*, 2017; Purfield *et al.*, 2012).

BOX 6

F-statistics

Wright's¹ fixation indices can be defined in at least three hierarchical levels that measure the probability of two randomly sampled alleles being the same in a set of subpopulations (e.g. farms) that together form a population (e.g. breed). For instance, the probability of sampling the same allele twice in a subpopulation (a measure of inbreeding) can be obtained from the difference between the average H_o of the individuals in the subpopulation (\bar{H}_o) and the HWE expectation of the heterozygosity for the subpopulation (H_e):

$$F_{IS} = \frac{H_e - \bar{H}_o}{H_e} = 1 - \frac{\bar{H}_o}{H_e}.$$

F_{IS} measures deviations in random mating within a subpopulation from one generation to the next, with negative values (minimum is -1) indicating an excess of heterozygotes in the subpopulation, while positive values (maximum is +1) indicate a deficit of heterozygotes. It is worth noting, that in a subpopulation deviating from HWE, a single generation of random mating would restore the HWE proportions, rendering F_{IS} equal to zero. Similarly, the correlation between allele frequencies in a pair of subpopulations can be expressed as the difference in the average H_e of the two subpopulations (\bar{H}_e) and the expected heterozygosity estimated for the two subpopulations together (H_T , with $H_T = 2\bar{p}(\bar{1}-\bar{p})$ and \bar{p} being the average frequency of one of the two alleles in a biallelic locus):

$$F_{ST} = \frac{H_T - \bar{H}_e}{H_T} = 1 - \frac{\bar{H}_e}{H_T}.$$

F_{ST} varies between 0 (indicating no differences in allelic frequencies between the two subpopulation) and 1 (indicating that the allelic frequencies in the two subpopulations are completely different), with the latter indicating that each of the two populations is fixed for a different allele, thus, within population, diversity must be zero. F_{ST} was originally defined as a measure of fixation, but it is often seen as a measure of genetic differentiation, and some authors even use it as a measurement of divergence that works like a genetic distance. In practice, it cannot be used as a distance since it has a maximum value of 1, however, Reynolds, Weir and Cockerham² suggested a distance measure that can be

seen as measuring drift accumulating between populations in the absence of mutations contributing new genetic variation to the population. For small F_{ST} values, Reynolds distance is approximately the same as F_{ST} and linearly increases with time since separation.^{2,3}

Lastly, the probability of sampling the same allele twice can also be obtained for two samples randomly collected from the total population (i.e. ignoring the breakdown of groups of samples in subpopulations). F_{IT} thus measures deviations between the average H_o across all individuals in the population (H_o) and the HWE expected heterozygosity for the population (H_T):

$$F_{IT} = \frac{H_T - \bar{H}_o}{H_T} = 1 - \frac{\bar{H}_o}{H_T}.$$

As for F_{IS} , F_{IT} varies from -1 to +1, with the obtained value representing the deviation from HWE expected genotypic frequencies due to non-random mating and divergence in allele frequencies between subpopulations. The three fixation indices shown above relate to each other by following: $1 - F_{IT} = (1 - F_{ST}) \times (1 - F_{IS})$. This relationship enables for additional hierarchical levels to be included as required by the study design, e.g. should there be colonies within subpopulations, the relationship between the fixation indices would become: $1 - F_{IT} = (1 - F_{ST}) \times (1 - F_{IS}) \times (1 - F_{IC}) \times (1 - F_{CS})$, with the corresponding F_{IC} and F_{CS} being:

$$F_{IC} = \frac{H_C - \bar{H}_o}{H_C} = 1 - \frac{\bar{H}_o}{H_C}, \text{ and}$$

$$F_{CS} = \frac{H_e - \bar{H}_C}{H_e} = 1 - \frac{\bar{H}_C}{H_e},$$

with H_C being the HWE expected heterozygosity for the colony, and with F_{IC} varying between -1 and +1, while F_{CS} varies between 0 and 1.

¹ Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics*, 15(1): 323–354.

<https://doi.org/10.1111/j.1469-1809.1949.tb02451.x>

² Reynolds, J., Weir, B.S. & Cockerham, C.C. 1983, Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, 105(3): 767–779.

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³ Weir, B.S. & Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, 38(6): 1358–1370.

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A high frequency of ROHs detected around a specific locus in several individuals can be indicative of recent positive selection (Curik, Ferenčaković and Sölkner, 2014; Zhang *et al.*, 2015). ROHs fixed in a population indicate a selective sweep by rapid selection for a beneficial mutation. However, appropriate analyses and demographic modelling are needed to disentangle the processes behind specific genomic patterns and the associated parameters (MacLeod *et al.*, 2013) (see subsections below on “genetic distances among breeds”, and “reconstruction of population history and demographic modelling”).

Effective population size

The effective size of a population (N_e) corresponds to the number of individuals in an idealized Wright-Fisher population (i.e. reproducing with random mating, even sex ratio and non-overlapping generations) that would become inbred or lose diversity at the same rate as this population (Frankham *et al.*, 2002). The concept of N_e is central to population genetics, as it quantifies genetic drift and allows for comparisons across species or populations, but it is difficult to estimate. The census size of a population and the number of breeding animals (N_b) are usually larger than N_e , because actual populations do not follow the assumptions of an idealized population. Non-random mating and uneven sex-ratios are common in domestic animal populations that are under strong artificial selection, especially with the use of reproductive biotechnologies, and may also result from admixture events. Thus, N_e can be small even for a large population, and through fixation of alleles, it implies loss of genetic variability, fast genetic drift, a high level of inbreeding and possibly decreased viability (Kristensen *et al.*, 2015; Peripolli *et al.*, 2017).

Estimation of N_e depends substantially on the indicators of genetic diversity considered, such as inbreeding, and on variation of genetic diversity through time. For instance, from a dataset of sequences N_e can be estimated as θ/μ (where θ is the population mutation rate estimated on the basis of the number of segregating sites, and μ is the individual mutation rate). Whole-genome data provide the least biased estimation of diversity (and thus N_e). Linkage disequilibrium (LD) based methods can account for sample size, mutation, phasing (assigning alleles to the paternal and maternal chromosomes), and recombination rate (Barbato *et al.*, 2015; Orozco-terWengel *et al.*, 2015). ROH may also serve as a robust genome-scale N_e estimator, although interpretation and scaling depend on local recombination (Bruford *et al.*, 2015).

However, computed values should be treated as estimates that are prone to biases, especially if the population is subdivided. For instance, Wakeley (1999) has shown that a structured population in which all subpopulations increase in size, while exchanging more migrants, will exhibit a signal of decrease of N_e despite the increase in the actual size of the population. Similar results were obtained by Mazet *et al.* (2016) for genomic data from single individuals analysed using the pairwise sequentially Markov coalescent (PSMC) method (see below). If N_e is used for translating various measures of genetic diversity within a specific model, it should be interpreted within that model. Various complex coalescent-based methods have been developed, that allow to infer the population demography over time including the variation of N_e or connectivity, such as the PSMC, the multiple sequentially Markov coalescent (MSMC) method and the site frequency spectrum (see subsection on “reconstruction of population history and demographic modelling”).

Increasing the N_e of a breed or population is challenging, as it may require a balanced number of breeding males and females contributing to subsequent generations. Although this type of breeding scheme would help to avoid inbreeding, it would also limit opportunities for directional selection (Curik, Ferenčaković and Sölkner, 2014).

Several software packages available for the analysis of WGS data allow for the estimation of within-breed genetic diversity summary statistics. These software packages include the following: PLINK (Chang *et al.*, 2015; Purcell *et al.*, 2007); VCF Tools (Danecek *et al.*, 2011); R package *diveRsity* (Keenan *et al.*, 2013); BEAGLE – specifically for detecting IBD (Browning, S. and Browning, B., 2010); SNeP – specifically to estimate N_e (Barbato *et al.*, 2015); ANGSD (Korneliussen, Albrechtsen and Nielsen, 2014) useful for low coverage WGS data. For more information, see Appendix 6.

The take home message is that estimation of parameters related to within-breed genetic diversity such as N_e is important, but this information needs to be interpreted properly and placed in the proper context. Estimation of such parameters should be complemented with clustering methods and demographic modelling (see subsequent subsections) to better understand evolutionary processes. By combining different approaches, it will be possible to use genomics to make a more informed management of breeds and other populations of livestock by monitoring N_e and practices such as crossbreeding, while avoiding high levels of inbreeding and ultimately preserving adaptive variation.

ASSESSMENT OF POPULATION STRUCTURE AND BETWEEN-BREED GENOMIC VARIATION

Principal component analysis (PCA)

Assessment of the genetic variation within breeds is usually an important objective for all genomic characterization studies. Such studies often involve multiple breeds or populations, however, so, the analysis of the structure of the multiple populations and how they relate genetically to each other is also a common research application and provides information that is useful for management of the populations. The foremost way of visualizing genetic structure of a multi-population (multi-breed) sample is to perform a principal component analysis (PCA) on the individual multilocus genotypes. PCA is a statistical method to capture the variability associated with many variables (such as marker genotypes) into a much smaller number of variables that still contain most of the original variation. The resulting variables – the principal components (PCs) – are ranked in descending order according to the amount of variation they explain. Thus, PC1 explains the most variation, PC2 the second most, and so forth.

The theoretical basis of the PCA approach has been described by several studies. In a sample made of two distinct genetic groups, the PC1 separates these two groups, and the proportion of variance explained by this first component corresponds exactly to the F_{ST} of the sample (McVean, 2009). Distinguishing the two groups is possible if (and only if) the product of the number of samples and markers is larger than $(1 / F_{ST})^2$ (Patterson, Price and Reich, 2006). Similar arguments suggest that more complex patterns of population structure also will be detected if the dataset is large enough. For instance, for a sample made of n_g distinct genetic groups, the number of PCs with significant contributions to the total observed variance is equal to $n_g - 1$ (Patterson, Price and Reich, 2006). The PCA may also

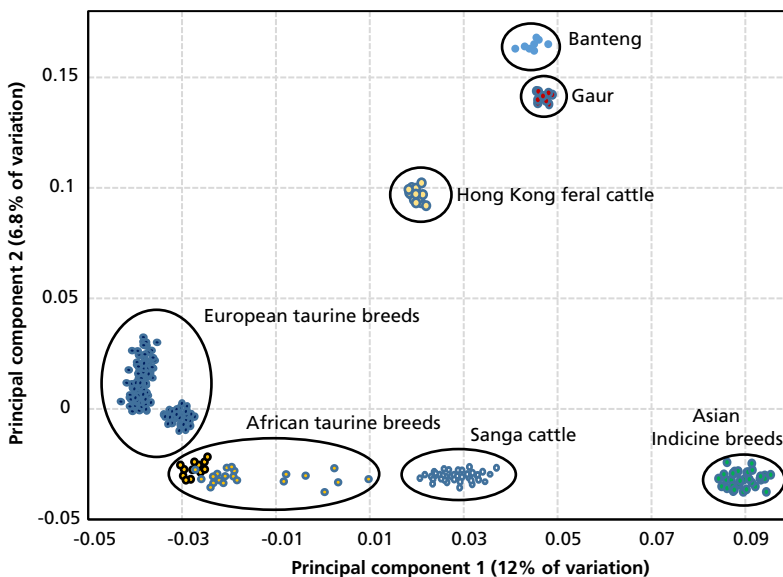
suggest about past admixture events: admixed individuals are on the PCA located between the two parental populations while their distances to these parental populations reflect the percentage of admixture (McVean, 2009; Patterson, Price and Reich, 2006). When population structure arises from a continuous isolation-by-distance model, the PC often show genetic clines that correlate with geographical clines, and the two-dimensional patterns may even resemble the geographic map of the area under study (Novembre *et al.*, 2008).

A standard procedure is to plot projections of the samples on the plane defined by two PCs, usually PC1 and PC2 or PC1 and PC3, thus providing a useful survey of the dataset. Although the individual PC has no biological or other significance *a priori*, plotting of the data frequently reveals patterns that suggest a logical interpretation. Figure 5 shows an example of a PCA plot of cattle breeds and wild relatives (P. Ajmone-Marsan, personal communication, 2021). The wild relatives (Banteng and Gaur) appear at the top of the graph, separated from cattle breeds along the lower part. The Hong Kong feral cattle, which seems to include some common ancestry with wild relatives, falls closest to the middle of the plot. The other cattle breeds are separated horizontally according to subspecies. European taurine breeds group on the far lower left, whereas Asian Indicine breeds are on the far right. African taurine breeds are just to the right of the European taurine group, whereas Sanga cattle, which are considered to be a composite of African taurine and indicine breeds, are grouped in the bottom centre of the graph, between their two ancestral groups. Therefore, a plausible interpretation in this case is that PC1 captures the variation between taurine and indicine cattle, whereas PC2 seems to distinguish domesticated cattle from their wild relatives.

However, several limitations, potential sources of bias and associated misinterpretations should be avoided. Because the PCA plots are two-dimensional projections of samples from a multidimensional space, they visualize only a small proportion of the total variation. For instance, they do not unambiguously indicate a close relationship of samples and do not clearly indicate duplicates. Published plots are often too dense, and thus only allow a superficial survey of the dataset. In addition, the use of many different symbols can be confusing. This can be remedied by plotting breed averages instead of individuals and by positioning the breed codes within the plot, similar to what is done with geographical names on a road map. Unbalanced sample sizes between genetic groups may strongly affect the position of these groups on the plot; those with large sample sizes are typically pushed towards the centre of the plot (McVean, 2009). If the dataset contains a breed that, by genetic isolation, differs substantially from all others, the major PCs mainly reflect this difference, which corresponds to the bias due to inbreeding (Lenstra *et al.*, 2012). This result can be prevented by excluding such breeds while computing the PCs but not from the plot, an approach referred to as “supervised” PCA (Ciani *et al.*, 2020). Another notable feature is that several higher-order PCs typically each correspond to the contrast of one breed with the others (Kijas *et al.*, 2012).

Menozi, Piazza and Cavalli-Sforza (1978) introduced PCA for the study of human genetic variation across continental regions. It has since become a standard of population genetic data analysis since the 2000s, especially with the advent of high-density genotyping and WGS technologies. It can be applied using generic R functions or population genetics software such as PLINK (Chang *et al.*, 2015) or eigensoft (Price *et al.*, 2006). See Appendix 6.

FIGURE 5
A principal components analysis plot of groups of bovine populations, including Banteng, Gaur, Hong Kong feral cattle and groups of European taurine, African taurine, Sanga and Asian Indicine breeds



Source: Author's own elaboration (Paolo Ajmone-Marsan).

While genetic clines that correlate with geographic clines often result from past migration waves, alternative explanations should be explored, such as isolation by distance (Novembre and Stephens, 2008). Ancient DNA (aDNA) samples with heterogeneous sample ages may also distort PCA representations, which can be amended by factor analysis (FA) instead of PCA (François and Jay, 2020). Another common method for comparing ancient and modern DNA samples is a supervised PCA, in which low-coverage aDNA samples are projected in a plot of coordinates computed on the basis of high coverage depth DNA samples (Daly *et al.*, 2018), but this does not correct all potential biases related to sample age heterogeneity (François and Jay, 2020). For more details on the analysis of aDNA specimens, see Box 7. Another mode of supervised PCA is the calculation of PCs that show the difference between geographical (or genetic) extremes, such as the northern-most and the southern-most breeds, in order to test the clines between these extremes (Ciani *et al.*, 2020).

Multidimensional scaling (MDS) is an alternative method to survey the dataset by reducing the number of dimensions (Tzeng, Lu and Li, 2008). In practice, MDS and PCA patterns are largely identical, but MDS cannot be run in a supervised mode.

BOX 7

Archaeogenetics applied to farm animals

Daniel Bradley

The recovery of genome variation from archaeological livestock samples is now eminently feasible, thanks to methods for DNA extraction and high throughput sequencing, particularly using instruments that give high read numbers from small fragments. Application of these technologies has helped solve the problems of contamination in the field; that is, the presence of modern livestock DNA within an extract is now measurable through bioinformatic analysis of the resulting data and problem samples can simply be excluded. Also, it is possible to identify damage patterns to authenticate aDNA sequences and validate results.

Ancient genomics is an invaluable tool for understanding modern diversity and its origins. Archaeological bones from the cradle of domestication in the Near East have yielded genome data for both cattle and goats,^{1,2} showing that the initial domestication of both species must have been followed by secondary input from additional wild populations. A more extreme example is clear in pig ancestry, where the Near Eastern genome of the first pigs that entered Europe was eventually almost completely replaced by introgression from the local wild boar.³ Because DNA degrades with time, more recent genomes will be more accessible, and the early development of modern breeds will become visible. For example, in the horse, a sharp drop in genome heterozygosity within the last 250 years has been shown.⁴ Ancient genomics can also reveal past domestic animal diversity that has since disappeared; for example, ancient Iberian horse genomes from more than four millennia ago have revealed the presence of a now-extinct lineage that is not ancestral to modern horse populations.⁴

One enduring aim is to understand genome variation in terms of impact and function, and ancient genomics has the potential to add to this in two ways: in giving temporal resolution to the search for genome regions which have been under selection and in ancient epigenomics. A group of 8 000-year-old goat populations from Serbia and Iran showed evidence for selection of genetic variation involved in pigmentation and some production traits; the earliest discovered direct evidence of human manipulation of the genomes of herded animals.¹ Inference of the methylation patterns of ancient molecules is also possible, although requiring high coverage sequencing, a route to directly mapping epigenetic markers in the past.⁵

Because of inherent properties of ancient DNA (particularly low concentration, short fragments

and chemical damage) the number of methods that are useful is limited. Some suggested guidelines are as follows:

- Only high throughput sequencing is regarded as reliable and the current state of the art; PCR-based assays including microsatellite size calls and Sanger sequences are no longer deemed to be reliable data.
- The best assay is the highest-possible coverage genome retrieved by shotgun high-throughput sequencing. This is a standard that the field should aim for, particularly with rare irreplaceable ancient specimens.
- However, many successful population genetic analyses can also be performed using low coverage depth genomes (i.e. < 1x), and some samples may not feasibly be sequenced to higher density because of poor preservation.
- Methods such as RNA bait capture⁶ may also be useful (particularly for less well-preserved samples) for targeted read enrichment prior to sequencing. Examples of useful and achievable bait targets are either whole mitochondrial genomes or specific autosomal loci such as selected genes or panels of thousands of informative SNPs.
- Additionally, it is highly desirable to use uracil–DNA glycosylase (UDG) treatment of sequencing libraries to remove C/G→ T/A misincorporations that have occurred as a result of age-related damage to ancient molecules.

¹ Daly, K.G., Delsler, P.M., Mullin, V.E., Scheu, A., Mattiangeli, V., Teasdale, M.D., Hare, A.J., et al. 2018. Ancient goat genomes reveal mosaic domestication in the Fertile Crescent. *Science*, 361(6397): 85–88. <https://doi.org/10.1126/science.aas9411>

² Verdugo, M.P., Mullin, V.E., Scheu, A., Mattiangeli, V., Daly, K.G., Maisano Delsler, P., Hare, A.J., et al. 2019. Ancient cattle genomics, origins, and rapid turnover in the Fertile Crescent. *Science*, 365(6449): 173–176. <https://doi.org/10.1126/science.aav1002>

³ Frantz, L.A.F., Haile, J., Lin, A.T., Scheu, A., Geörg, C., Benecke, N., Alexander, M., et al. 2019. Ancient pigs reveal a near-complete genomic turnover following their introduction to Europe. *Proceedings of the National Academy of Sciences of the United States of America*, 116(35): 17231–17238. <https://doi.org/10.1073/pnas.1901169116>

⁴ Fages, A., Hanghøj, K., Khan, N., Gaunitz, C., Seguin-Orlando, A., Leonardi, M., Constantz, C. M., et al. 2019. Tracking five millennia of horse management with extensive ancient genome time series. *Cell*, 177(6): 1419–1435.e31. <https://doi.org/10.1016/j.cell.2019.03.049>

⁵ Hanghøj, K., Renaud, G., Albrechtsen, A. & Orlando, L. DamMet: Ancient methylome mapping accounting for errors, true variants, and post-mortem DNA damage. *GigaScience*, 8(4): giz025. <https://doi.org/10.1093/gigascience/giz025>

⁶ Soares, A.E.R. 2019. Hybridization capture of ancient DNA using RNA baits. *Methods in Molecular Biology*, 1963: 121–128. https://doi.org/10.1007/978-1-4939-9176-1_13

Model-based clustering

Another common approach to summarize the genetic structure observed in a set of individuals is the clustering method first developed by Pritchard, Stephens and Donnelly (2000) and implemented in the software *Structure*. This method assumes there are K genetic groups (or clusters) and all polymorphic sites are at HWE and linkage equilibrium within these groups. Each individual i is modelled as a mixture of these K groups with specific proportions q_{ij} with $j = 1$ to K (i.e., $\sum_{j=1}^K q_{ij} = 1.0$). *Structure* estimates these proportions from the observed individual genotypes using a Monte Carlo Markov Chain (MCMC) algorithm. In the most standard application (“unsupervised” clustering), the K clusters and their associated allele frequencies are inferred, and the biological meaning of cluster number j is deduced *a posteriori* from the set of individuals. For an individual i , a value of q_{ij} close to 1.0 indicates that the individual is most likely a member of cluster j . Ideally, this reveals the major ancestral populations and the composition of admixed individuals or populations. “Supervised” clustering allows one to define *a priori* the ancestral clusters, and to infer directly the admixture proportions of the other individuals, similar to assignment methods like *GeneClass* (Piry *et al.*, 2004). The original approach of Pritchard, Stephens and Donnelly (2000) was later extended to account for sampling location (Hubisz *et al.*, 2009), linked loci and shared ancestry between clusters (Falush, Stephens and Pritchard, 2003). More efficient inference approaches of the same model were also developed in the software *Frappe* (Tang *et al.*, 2006) and *Admixture* (Alexander, Novembre and Lange, 2009), allowing the analysis of much larger datasets and larger values of K , albeit with the constraint that loci are independent from each other, and that they are in linkage equilibrium.

Again, there are caveats and pitfalls to be avoided. Many scientists choose to interpret the results of model-based clustering in terms of population history. For instance, the separation of one cluster into two subclusters at high K values is often interpreted as a past divergence event. More generally, inferred clusters are equated to ancestor populations and any breed that is not assigned to one cluster is assumed to be admixed. However, *Structure* is essentially a clustering approach for genetic data, but does not model evolutionary processes such as drift, mutation, migration or divergence (see subsections on “genetic distances among breeds” and “reconstruction of population history and demographic modelling” for such methods). The implicit assumption that the ancestral populations are present in the dataset is often not met. Thus, the inferred clusters do not necessarily correspond to real past or present populations and strongly depend on the composition of the dataset. Applying *Structure* to populations along a genetic cline formed by preferential mating of proximate parents (isolation-by-distance) may infer homogeneous clusters that contain the extreme populations and mixed compositions for the central populations (Engelhardt and Stephens, 2010). This may be remedied by modelling continuous spatial variation between (Corander, Waldmann and Sillanpää, 2003; François, Ancelet and Guillot, 2006; Guillot, Mortier and Estoup, 2005) or within clusters (Bradburd, Coop and Ralph, 2018), and the latter option allows the analysis showing both discrete and continuous genetic structure. By the sampling and the inbreeding bias, inferred clusters tend to correspond to overrepresented and inbred breeds, respectively (Lenstra *et al.*, 2012).

However, despite the risk of mis- or over-interpretation of results, model-based clustering does contribute to the data analysis by showing graphically: (i) groups of related individuals from one or more breeds; (ii) ancestral proportions, provided inferred clusters correspond to

ancestral populations, which may be accomplished in the supervised mode; and (iii) subdivision of breeds not inferred by PCA or genetic distances.

As an alternative to the supervised mode, breed-specific admixture analysis (BSAA) is based on the selection of 300 ancestry informative markers (AIM) specific for a breed suspected to be introgressed, for instance, by markers that have the highest F_{ST} between the putatively introgressed breed and a group of other breeds. It was demonstrated that *Structure* analysis with 300 to 400 AIM markers provides a more sensitive detection of (pre)historic admixture than f_4 analysis (see the subsection below on “detection of admixture using f_3 , f_4 and D statistics”).

Both *Structure* and PCA attempt to summarize observed genetic diversity by using a reduced number of K latent factors, namely the clusters for *Structure* and the PCs for the PCA (Engelhardt and Stephens, 2010). More specifically, both methods decompose the genotype matrix X as $X = QF$, where Q is a matrix of n individuals \times K factors that quantify the individual compositions. The two methods impose different constraints to the matrices Q and F ; the coefficients of Q are positive and must sum per individual to one with *Structure*, but not with PCA. Other latent factor approaches were proposed to analyse genetic data (Engelhardt and Stephens, 2010; Frichot *et al.*, 2014) and represent interesting alternatives to PCA and *Structure*. For instance, the sparse Nonnegative Matrix Factorization (sNMF) developed by Frichot *et al.* (2014) and implemented in the *LEA* R package estimates admixture proportions with the same accuracy as the *Admixture* software, while reducing computation time by a factor of 3 to 30 and being less affected by the inbreeding of the analysed populations.

Genetic distances

Genetic distances between individuals

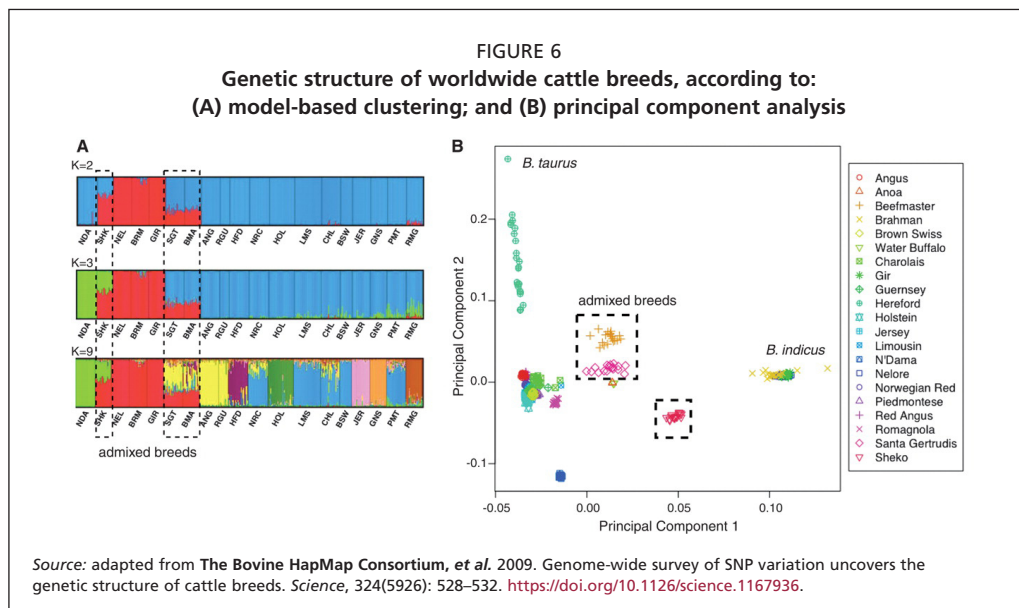
The third common approach to visualize genetic structure uses a matrix of genetic distances between all pairs of individuals. For SNPs of a diploid species, distances are based either on the identity by state (IBS) of markers or on genomic relationships of individuals (Grünwald *et al.*, 2017; Yang *et al.*, 2011), both of which can be computed using *PLINK*. These distances can be visualized via MDS analysis, the alternative to PCA described earlier (Tzeng, Lu and Li, 2008), or via neighbour-joining trees (Saitou and Nei, 1987) (see subsection on “genetic distances among breeds”). Such trees unambiguously show the identity or close relationship of samples and are recommended for quality control of SNP datasets (see Section 3). They also show the level of inbreeding of breeds via short terminal branches. However, individual-based trees can be misleading on the deeper phylogenetic relationships of the breeds, which are better explored by genetic distances based on allele frequencies of breeds (see subsection on “reconstruction of population history and demographic modelling”). A representation of the genetic distances in a network that connect each individual to its ten nearest neighbours (“supermagnetic clustering”) shows genetic structure within populations (Neuditschko, Khatkar and Raadsma, 2012), which may be related to the uneven use of breeding sires (Neuditschko *et al.*, 2017).

So far, the approaches described assume markers to be independent, but several recent studies take advantage of haplotype information of phased datasets, that is, data with information about which alleles of different markers are on the same haploid chromosome copy (maternal or paternal). Such information is not provided by dideoxy-Sanger or short-read sequencing (SRS). Without data from the (expensive) PacBio or Oxford Nanopore long-read

sequencing (see Section 3), phasing must be inferred statistically by exploiting observed LD patterns in the population or genotype information from close relatives (Al Bkhetan *et al.*, 2019; Browning, S. and Browning, B., 2007; Delaneau, Marchini and Zagury, 2012).

The *ChromoPainter* approach implemented in the program *FineSTRUCTURE* (Lawson *et al.*, 2012) builds a co-ancestry matrix of individuals based on local haplotype similarity along the genome, and uses this matrix for PCA and clustering (similar to *Structure*). For both applications, the use of haplotype information significantly improved the resolution of the inferred population structure. The method was developed for high-density SNP datasets, and its performance with low-density (< 50 000 SNPs) datasets is not yet clear. Therefore, when using this method, it is essential to show that the linked mode, using haplotypes, gives better results than the unlinked mode (using separate SNPs). Going beyond this genome-wide description, the *Relate* (Speidel *et al.*, 2019) and *tsinfer* (Kelleher *et al.*, 2019) methods reconstruct the phylogeny of observed haplotypes, i.e. the most-probable inheritance at every SNP position in the genome, accounting for the haplotype diversity observed among sampled individuals around this position. All these methods are either based on or inspired by the model of Li and Stephens (2003), which iteratively considers one sampled haplotype and tries to reconstruct it as a mixture of the others.

In a study by The Bovine HapMap Consortium, *et al.* (2009), genotype data at 37 470 SNPs were available for 497 cattle from 19 geographically and biologically diverse breeds. This dataset was analysed using a *Structure*-like (Figure 6A) and a PCA approach (Figure 6B). PC1 separates zebu and taurine breeds, similar to the *Structure* analysis with $K = 2$ clusters. PC2 further separates African versus European taurine breeds, similar to the *Structure* analysis with $K = 3$ clusters. Breeds resulting from admixture between these three main groups are indicated by dashed rectangles, and clearly stand out in both analyses. The *Structure* analysis with $K = 9$ identifies several clusters corresponding to single breeds.



Genetic distances among breeds

Since the development of selective breeding in the eighteenth century, the mating of animals with similar desired characteristics has resulted in the establishment of breeds, which are usually defined by a range of morphological, economic and behavioural traits (Taberlet *et al.*, 2008). Depending on the degree of genetic isolation, animals from the same breed are genetically more similar to each other than to animals from different breeds. These differences among animals from different breeds can be quantified using genetic distances between individuals, and also by determining the components of the genetic variation within and between breeds using an Analysis of Molecular Variance (AMOVA) (Excoffier, Smouse and Quattro, 1992; Michalakis and Excoffier, 1996).

There are two important differences between phylogenies at the species (macroevolution) and breed (microevolution) levels. First, the bulk of molecular differences between species are quantified via fixed differences in the DNA sequence. However, the mammalian substitution rate, approximately 1.0 to 1.25×10^{-8} /bases/generation (The 1000 Genomes Project Consortium, 2010; Kong *et al.*, 2012; Venn *et al.*, 2014), is too slow to generate many of the DNA differences observed between breeds that diverged at most 10 000 years ago and, in most cases, only 50 to 200 years ago (Laval, SanCristobal and Chevalet, 2002). Instead, the major DNA-based differences between breeds depend on genetic drift, the mechanism behind the change of allele frequencies in genetic markers as a function of the N_e . Second, many species do not interbreed and hybrids of related species are often infertile. As a consequence, the phylogeny of species can be largely represented by a tree topology without connections between branches. In contrast, fertile crossbreeding of breeds is a rule rather than an exception, and trees of breeds are at best a practical visualization of an estimation of the true phylogeny of breeds.

The extent of genetic drift separating two hypothetical populations A and B can be quantified as the additive statistic f_2 , or the mean of $(P_A - P_B)^2$ with P_A and P_B corresponding to the frequencies of allele P for a SNP in populations A and B, respectively. The f_2 is the average of the squared differences across all genetic markers studied in the two populations (Patterson *et al.*, 2012; Peter, 2016). Several genetic distances have been derived from f_2 , such as the Reynolds distance or D_R (Reynolds, Weir and Cockerham, 1983), which is the preferred genetic distance for breed comparisons (Laval, SanCristobal and Chevalet, 2002) and which correlates very well with the F_{ST} fixation index (Wright, 1951). Because it approximates the average inbreeding coefficient of the two breeds compared, D_R trees may represent the loss of within-group genetic diversity (Laval, SanCristobal and Chevalet, 2002). However, with SNP panels, this is likely to be confounded by the ascertainment bias (AB), which may increase the estimates of diversity of the common international trans-boundary breeds.

Note that the accuracy of allele frequencies depends on an adequate sample size per breed. In practice, 20 animals per breed are considered to be sufficient for biallelic markers; in fact, a sample size of 20 diploid animals has a probability of 95 percent of including the whole genealogy of the breed (Hein, Schierup and Wiuf, 2003). As with any statistical test, larger amounts of data will increase precision. If at least 20 samples cannot be obtained, a lower sample size tends to increase the proportion of homozygote genotypes and thus the genetic distances to other breeds, but usually does not alter the topology of a tree

(see the following subsection on “phylogenetic trees and networks”). The F_{ST} distance as estimated by Weir and Cockerham (1984) accounts for low and uneven sample sizes. Using medium-density array datasets, Ramljak *et al.* (2018) calculated genetic distances between short haplotypes consisting of the alleles of up to four SNPs located within a stretch of 150 kilobases.

Phylogenetic trees and networks. The neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) is the most common and one of the simplest methods to reconstruct phylogenetic trees on the basis of a matrix of $n \times n$ pairwise genetic distances between n breeds. It works by choosing the pair of breeds that, after being combined, gives the largest reduction of the total branch length of the tree, after which the distance matrix is recalculated and reduced to $(n - 1) \times (n - 1)$ breeds, continuing iteratively until all breeds have been paired. Such a tree complements PCA and model-based methods and represents a hierarchical clustering of breeds, with the length of the terminal branches indicating the degree of genetic isolation of a breed. In contrast to the (not recommended) unweighted pair group method with arithmetic mean (UPGMA) approach, the NJ algorithm does not assume an even rate of change across breeds, which agrees with the effect of genetic drift being breed specific. The effect of genetic drift can be observed when the NJ tree is visualized like a phylogram where the tree’s branch lengths are proportional to the amount of change in each breed. Other visualizations such as cladograms are not suitable to visualize the effect of genetic drift, as the branches in such methods are not proportional to the amount of change.

Conceptually, the topology of a tree directly shows genetic events as breed divergence, genetic drift and breed isolation. In practice, this is realized mainly for the most closely related breeds, but is not shown by PCA or model-based clustering. Trees of individuals based on genetic distances also show the genetic drift, but do not show very well the relationships between breeds. However, phylogenetic trees of breeds have a few inherent limitations:

- Testing the significance of clusters is problematic and bootstrapping values are low.
- The deeper bifurcations are supported by short branch lengths and may not be realistic.
- As shown by their hierarchical topology, trees cannot visualize reticulations and are thus confounded by gene flow between breeds leading to admixture. Breeds that have been derived by crossbreeding, such as taurine–indicine composites, tend to cluster in the NJ tree in between the source breeds (Ginja *et al.*, 2019; Kopelman *et al.*, 2013; Mei *et al.*, 2018). Whereas this still may be a useful visualization of a genetic cline with again the terminal branches showing the breed-specific genetic drift (Decker *et al.*, 2014), it should be realized that the genetic differences are dominated by the degree of crossbreeding. Thus, bifurcations and branch lengths no longer correspond to divergence events and genetic drift, respectively. If crossbreeding occurred recently, the genealogy of the alleles depends on the position of the alleles in the genome.

Various methods have been developed to introduce reticulation in a tree by construction of an admixture graph. The most popular methods are the *NeighbourNet* and *Treemix* algorithms. *NeighbourNet* graphs (Bandelt and Dress, 1992; Dopazo, Dress and von Haeseler,

1993; Dress, Huson and Moulton, 1996) can be constructed via the easy-to-use program *Splitstree* (Huson, 1998) with many graphical options. It is based on the NJ algorithm, but considers three instead of two breeds before reducing the difference matrix. *NeighborNet* graphs share the main advantages of NJ trees by reproducing the terminal branches and unambiguous clusters of breeds, but replaces the often meaningless bifurcations of a tree by networks (Ciani *et al.*, 2020; Kijas *et al.*, 2012; Pitt *et al.*, 2019). These network trees are not to be interpreted in terms of specific genetic events and may correspond to ancestral breed pools, and at least visualize the uncertainty of the phylogenetic reconstruction. However, *NeighborNet* graphs rarely show reticulations corresponding to specific cases of crossbreeding. Kijas *et al.* (2012) and Ciani *et al.* (2020) showed that deeper phylogenetic splits, i.e. between breed clusters rather than between breeds, are resolved better if: (i) the most recent and documented crossbreds are removed; and (ii) the unambiguous monophyletic breed clusters are combined in one taxon. All this may contribute to the formulation of specific hypotheses to be tested statistically or by modelling.

The sophisticated program *Treemix* (Pickrell and Pritchard, 2012) constructs a maximum-likelihood tree based on the covariances of the allele frequencies, which overall agrees with NJ trees. Subsequently, the agreement between the covariances explained by the tree and the real covariances can be improved by adding vectors between branches that represent gene flow (migration) between those branches. Taking a user-defined number of migrations, *Treemix* optimizes a new tree topology, the start and endpoint of the migrations and their weights (Decker *et al.*, 2014; da Fonseca *et al.*, 2019; Orozco-terWengel *et al.*, 2015). However, the graphical output has a poor quality that cannot be adapted easily. More importantly, we recommend that the migrations inferred on the basis of an overall match of graph-based and real covariances are tested, for instance, using the f_3 or f_4 statistics, which is rarely done. Ciani *et al.* (2020) found that plausible admixture confirmed by both f_4 analysis and BSAA were only partially reconstructed by *Treemix*. Other methods to construct admixture graphs are discussed in the next section.

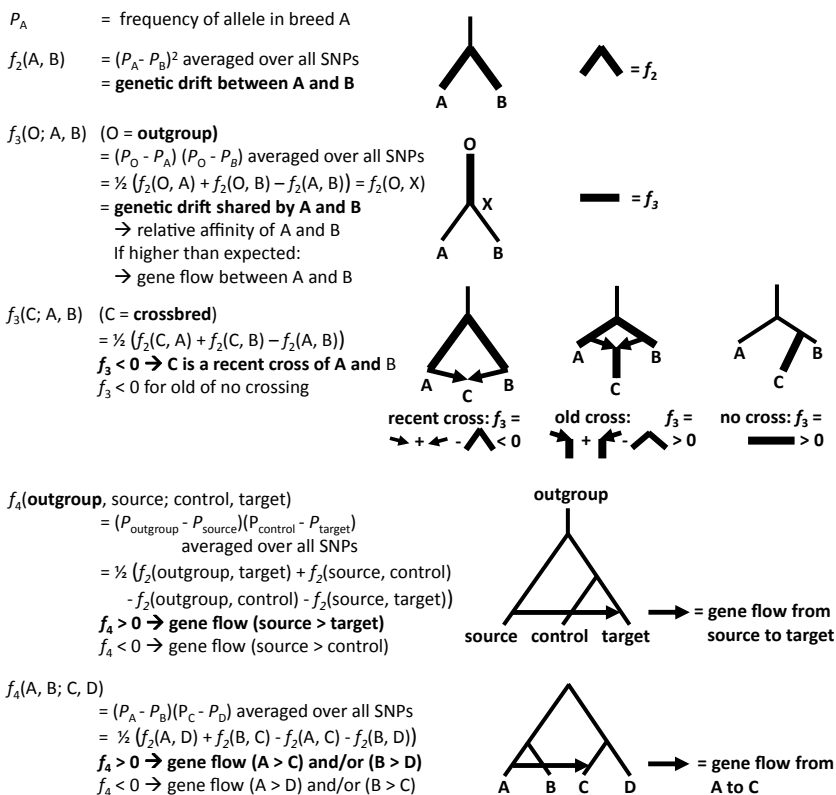
Detection of admixture using f_3 , f_4 and D statistics. The availability of genome-wide datasets obtained by SNP arrays (The Bovine HapMap Consortium, 2009; Kijas *et al.*, 2012) and WGS (Alberto *et al.*, 2018; Bosse *et al.*, 2014; Fan *et al.*, 2020) led to the understanding that hybridization and admixture among breeds are more common than previously thought. The marker density of SNP data now allows for an accurate estimation of new statistics for the detection of gene flow between well-diverged breeds (Patterson *et al.*, 2012; Peter, 2016). These statistics can be used with both individuals and breeds. Figure 7 shows the most common applications of the f_3 and f_4 statistics based on allele frequencies of three (f_3) or four (f_4) breeds. As is shown in Figure 7, they can be decomposed as the sum or difference of distances between nodes as quantified via the f_2 genetic drift statistic. For a more extensive theoretical treatment involving path lengths and admixture proportions, we refer to Patterson *et al.* (2012) and Peter (2016).

Calculation of the f_3 statistic while using an outgroup (a reference population that is distantly related to the populations of interest in a genetic study) is useful for quantifying the relative affinity of individual animals or breeds, for instance, of ancient and modern DNA samples (Daly *et al.*, 2018). The properties of f_3 for detection of admixture can be understood by considering that the average of $(P_C - P_A) \times (P_C - P_B)$ becomes negative if P_C

is intermediate between P_A and P_B for many SNPs, which indeed reveals recent admixture. However, a positive value of f_3 does not exclude admixture, which limits its use.

The statistic f_4 is more predictable since admixture follows directly from systematic differences from zero. These deviations can be understood by considering that sharing of characteristic alleles by source and target or, for a more general case, by A and C (Figure 7), but not by the other possible pairs of breeds, leads to a correlation of P_A and P_C , which makes the average of $(P_A - P_B) \times (P_C - P_D)$ positive; decomposing f_4 into a function of four f_2 -scaled distances as indicated in Figure 7 results in a total length of zero without gene flow, but becomes greater than zero if gene flow shortens the $f_2(A, C)$ distance.

FIGURE 7
Definitions of the f_2 statistic, the outgroup f_3 statistic, the admixture f_3 statistic and the admixture f_4 statistic with an outgroup and the more general case with four breeds



Note: The figure shows how the statistics correspond to branch lengths and how they are used as test statistics for the occurrence of gene flow.

Source: Author's own elaboration (Johannes A. Lenstra).

The f_4 statistic is related to the D statistic from the ABBA/BABA algorithm. This method considers the sister taxa one and two, the donor three and the outgroup. For a given SNP, A is the ancestral allele fixed in the outgroup, while B is the derived allele fixed in the donor three. For a haploid genome, the possible allele combinations for one, two, three and the outgroup are AABA, ABBA, BABA and BBBA. Ignoring the non-informative AABA and BBBA, a higher degree of admixture from three into two than from three into one follows from a majority of SNPs with the ABBA pattern:

$$D = [n(ABBA) - n(BABA)]/[n(ABBA) + n(BABA)],$$
 in which $n(ABBA)$ and $n(BABA)$ are the counts of SNPs with the ABBA and BABA patterns, respectively. For a collection of samples, the use of allele frequencies instead of counts and an adapted formula allow to consider also SNPs for which A and B are not fixed in the outgroup and the donor, respectively (Patterson *et al.*, 2012). For statistical testing, D is preferred above f_4 (Patterson *et al.*, 2012). However, we stress that both methods strictly depend on a tree-like evolution of the breeds being considered

The F -statistics can be used to construct more realistic admixture graphs than a *NeighborNet* or *Treemix* graph. However, because this procedure is very specialized and only partially automated by software, the methods described in the literature are not generally used. The programs *QPTOOLS* from the *AdmixTools* package (Patterson *et al.*, 2012) and *ADMIXTUREGRAPH* (Leppälä, Nielsen and Mailund, 2017) take as input a user-specified admixture graph and all possible f_2 , f_3 and f_4 values and then optimize the branch lengths and admixture proportions. *MIXMAPPER* (Lipson *et al.*, 2013) selects breeds for which a hierarchical tree can be constructed (i.e. no negative f_3 values, additivity of f_2 values) and then sequentially and interactively adds admixed breeds while optimizing topology, sources of gene flow, branch lengths and admixture proportions.

Phylogeny across the genome. So far, we compared individuals or groups on the basis of genome-wide genetic variation. However, as a consequence of genetic recombination, the genome of a given animal may consist of segments with widely different genealogical histories (Li and Durbin, 2011). For instance, within crossbred individuals, portions of the genome originate from different breeds (Fan *et al.*, 2020), which have resulted from various demographic processes such as migration, inbreeding and selection. Software products have been developed that attempt to overcome the deviating phylogenetic signals from markers and thus establish the genealogy that truly reflects the breeds' evolutionary processes. Among these, *SNAPP* (Bryant *et al.*, 2012) uses a likelihood-based approach to estimate the groups' true tree based on the genetic variation of markers across the genome. Hobolth *et al.* (2007) established a framework that enables simultaneous modelling of divergence or speciation times, and demographic parameters such as the ancestral N_e , which has been successfully applied to model the divergence history of the European bison, *Bison bonasus* (Gautier *et al.*, 2016).

RECONSTRUCTION OF POPULATION HISTORY AND DEMOGRAPHIC MODELLING

Several methods presented above can identify evolutionary processes in the history of species, populations and breeds, while representing patterns of differentiation. The subsequent subsections will focus on methods that explicitly aim at detecting, dating and quantifying population size changes and other events such as admixture or population splits. Because the complexity of the evolutionary history of species and breeds probably precludes its full

reconstruction, even with genomic data, the interpretation of an inferred demographic history should thus always be done with caution. Also, in the case of domesticated animals one must keep in mind that this evolutionary history integrates the recent artificial selection, the domestication process, and the history of the wild species from which they were derived. Genetic and genomic data enable testing alternative interpretations and models by comparing the genetic consequences of alternative evolutionary scenarios with observed data. This is particularly true with the approximate Bayesian computation (ABC) framework (Beaumont, 1999; Beaumont, Zhang and Balding, 2002).

An initial step is to describe the coalescent theory, which is at the heart of modern population genetics (Hudson, 1991, 2002; Wakeley, 1999). It was developed in the 1980s and 1990s to study the statistical properties of gene trees for non-recombining DNA fragments (Kingman, 1982) evolving under a simple demographic model that assumes panmixia and isolation from other populations (i.e. no population structure), non-overlapping generations, neutrality, and constant population size (e.g. the Wright-Fisher model). It has since been extended to account for population size changes (Griffiths and Tavaré, 1994), population structure (Herbots, 1994), recombination (Griffiths and Marjoram, 1997) and different sampling times to integrate aDNA or viral samples. Some types of selection can also be integrated. The coalescent theory takes a retrospective approach that differs from modelling or simulation approaches used before the 1980s. Coalescence and classical simulation are now commonly denoted as backward and forward simulation, respectively. A coalescent approach works backward in time and focuses on the statistical properties of the gene trees of the samples. If a population is large, the probability that lineages coalesce (have a common ancestor) is low, whereas in a small population this probability is high. As a consequence, starting from a particular sample size the length of the branches connecting these samples will depend on the changes in population size, as one goes backward in time, and as the number of remaining lineages decreases. In a more general way, the coalescent theory studies how gene trees depend on the parameters of the demographic models of interest (past population sizes, migration rates, age of a bottleneck or an admixture event). Coalescent-based methods are sample-based and thus computationally less expensive than the population-based forward methods, which require the simulation of the whole population. The development of software such as *ms* (Hudson, 2002), allowed the development of simulation-based inference methods including ABC. Forward simulations are still used, for instance, for selection and are being revived after recent improvements in efficiency, e.g. *Slim* (Haller and Messer, 2019).

The coalescent theory played a central role in the development of inferential methods in the 1990s and 2000s. It was shown that bottlenecks and expansions have different effects on the shape of trees, hence allowing the use of coalescence-based inference to distinguish between the two types of events. This led to the development of likelihood-based methods to compute the conditional probability $P_M(Data | Param)$ of generating the observed *Data* under a predefined demographical model *M*, defined by a set of parameters $Param = (Par_1, \dots, Par_k)$, which may correspond to divergence times, population sizes or migration rates. Likelihood methods apply optimization algorithms, often via a sophisticated search of the parameter space, to find parameter values that best explain the observed *Data*, i.e. the maximum likelihood estimates of the parameters. A Bayesian perspective can also be used to estimate probability density functions for the parameters, assuming prior distributions for these parameters, $P_M(Param)$,

and multiplying them by the likelihoods to obtain posterior distributions using Bayes formula: $P_M(\text{Params} \mid \text{Data}) = P_M(\text{Param}) \times P_M(\text{Data} \mid \text{Param}) / P_M(\text{Data})$. Likelihood-based methods were developed between the mid-1990s and mid-2000s and applied to different demographic models to consider: population size change (Beaumont, 1999); population structure and gene flow (Beerli and Felsenstein, 2001); population split and post-split migration rates (Nielsen and Wakeley, 2001); or admixture (Chikhi, Bruford and Beaumont, 2001).

In the last decade several important methods have been developed that allow users to infer either simple or complex demographic models with genomic data. One of the most popular is the PSMC method of Li and Durbin (2011), which uses the genome of a single diploid individual (or two haploid genomes) and infers a histogram or “skyline plot” usually interpreted as a history of population size changes. The PSMC uses information on mutation and recombination rates to analyse and interpret the distribution of heterozygous sites along the genome. Under panmixia (i.e. absence of genetic subdivision) the method estimates the history of population size changes that best fits the distribution of heterozygous sites. Schiffels and Durbin (2014) extended the method by allowing the use of multiple phased genome sequences simultaneously (MSMC). Under panmixia the MSMC also estimates the history of population size changes. It is important to note that both the PSMC and MSMC only use information from the first coalescence time, T_k , where k is the number of haploid genomes and thus ignores most of the information contained in the whole coalescent tree. These methods provide only information on events that took place within the time frame defined by the T_k . Thus, the more haploid genomes one uses with MSMC, the earlier the start and end of the reconstructed curves.

Other methods use the site or allele frequency spectrum (AFS) as a way to summarize genomic data across many independent loci. For instance, the *Fastsimcoal2* (Excoffier *et al.*, 2013) and *dadi* (Gutenkunst *et al.*, 2009) software allow the user to infer the parameters of tree models in which populations are allowed to have different population sizes and may be connected by gene flow. For computational reasons, the number of populations is limited, typically two to five populations depending on the methods. *Fastsimcoal2* uses a likelihood approach and can thus be used to compare alternative tree models, with or without gene flow or admixture.

A general inferential framework for comparing alternative models is ABC, which is particularly well adapted to study complex demographic datasets and evolutionary models for which the likelihood may be difficult or impossible to compute. In such cases, the principle of ABC is to use large numbers of simulations under different demographic models and parameter values together with two approximations. First, the data are summarized by a limited number of summary statistics (such as n_A , Tajima's D or H_e), and the objective is to estimate $P_M(\text{SummData} \mid \text{Param})$, where *SummData* is a vector of the summary statistics. Second, simulated datasets are selected with a *SummData* that, according to a tolerance threshold, are close enough to the *SummData* of the real dataset. The parameter values corresponding to the selected simulations (usually the best 0.1 or 1 percent) are kept for inference. The number of simulations used to estimate the posterior distributions of the parameters of a particular model is typically $> 10^5$ - 10^6 . The coalescent theory played a central role in the development of ABC methods by enabling an efficient simulation of data. The simplest ABC algorithm is usually called “rejection” because it only requires filtering the best simulations according to the tolerance threshold and rejecting the others.

The ABC framework is extremely flexible, and can handle complex datasets and other situations for which classical models do not perform well. For instance, Boitard *et al.* (2016) used the AFS together with summary statistics measuring LD at different distances to reconstruct a history of population size changes in large samples obtained from a set of four cattle breeds (Angus, Fleckvieh, Holstein and Jersey). ABC can also be used to compare alternative demographic models by computing the relative proportions, among alternative models, of simulated *SummData* closer to the observed *SummData*. It can also test the quality of the inference process by generation of pods (pseudo observed data) on the basis of the different models, and check if an ABC analysis of the pods indicates the corresponding model and generates a meaningful posterior distribution of parameters. However, the large number of simulations required by ABC methods can become computationally intensive with genomic data and complex alternative models.

Beaumont, Zhang and Balding (2002) demonstrated that a regression algorithm achieved the same quality of the inference as the rejection algorithm but with ten times fewer simulations, allowing a much lower tolerance threshold. Other approaches have been proposed to improve the regression step by using non-linear regression and neural networks (Csilléry *et al.*, 2010). More recently, a machine-learning approach called “random forests” has been proposed as a way to avoid the selection step of summary statistics, and the need to define a tolerance threshold (Raynal *et al.*, 2019). One powerful and versatile software package for ABC analyses is the *ABCtoolbox* (Wegmann *et al.*, 2010)

MITOCHONDRIAL DNA AND THE SEX CHROMOSOMES

The genetic diversity of autosomes, sex chromosomes and mtDNA is affected in different ways by demography, and their combined analysis provides insight into the various historical processes that shaped present-day populations. Because of their lack of recombination, the evolution of mtDNA and Y-chromosomal DNA is less complex than the evolution of autosomes, and can be reconstructed largely by obtaining a phylogenetic tree and coalescence analysis. Generally, it reveals links between the domestic species and their wild ancestors, and shows ancient bottlenecks that contributed to differentiated haplogroup distributions, e.g. in (sub)continents (Lenstra *et al.*, 2014), but is considerably less informative than autosomal DNA for reconstructing breed histories. In addition, mito-nuclear discordances occur frequently simply due to incomplete lineage sorting or by sex-biased introgression, such as for the European bison carrying cattle-like mtDNA (Wang *et al.*, 2018), zebu outside Asia carrying taurine mtDNA (Ginja *et al.*, 2019), or possibly the Grey jungle fowl that carries a domestic/Red jungle fowl mitochondrial haplotype (Lawal *et al.*, 2020).

Before the availability of WGS data, the analysis of Y-chromosomal variation was limited by a lack of informative markers. It shows the paternal history, which by the small male population size for most domestic species has led to a considerable breed-level differentiation (see Box 8). For many species, in current genome assemblies the Y chromosomes are partially represented by unplaced scaffolds that are only present in males, and for a large part consist of Y-chromosomal repeats and multicopy genes. Note that single-copy male-specific SNPs are hemizygous, that is, they have one allele per individual without heterozygosity scores (H_o and H_e) and should not have scores in females.

BOX 8

Y-chromosomal variation

Barbara Wallner

Due to its complex DNA structure, it is often omitted from genome assemblies. But the Y chromosome has a powerful property: its male-specific, non-recombining part (MSY) is transmitted as a tight linkage group (a haplotype) from the father to the son. Hence, genetic variation on the MSY reflects the paternal history of populations.¹

The MSY diversity is best studied in the humans, where deep insights derive from several decades of steady progress in variant discovery and haplotype analyses. A diverse spectrum of polymorphisms on the human MSY, ranging from small- and large-scale rearrangements, SNPs, indels and CNVs as single tandem repeats, now presents a clear picture of the human male genealogy (reviewed by Jobling and Tyler-Smith in 2017²). The wealth of MSY markers available in humans enables the study of evolution over different time scales. The work in humans has also demonstrated that a community accepted MSY phylogeny, based on defined haplotype determining variants, is pivotal to consolidate results from different studies.³

The tremendous amount of WGS data becoming available is now empowering comprehensive fine-scaled MSY haplotype analysis in domestic species. To infer the MSY pattern in a population of interest, proper MSY variant discovery is a prerequisite. As a first step, one usually needs to define MSY sequences that can be used as the reference for variant calling from NGS data. The potential of MSY draft assemblies, combined with an approach to define MSY regions appropriate for reliable variant calling, has been recently demonstrated in the horse,⁴ camel⁵ and sheep.⁶ After mapping WGS data to the reference and variant ascertainment, the allelic states of MSY variants can be concatenated into haplotypes, and a robust haplotype phylogeny can be built under the principle of maximum parsimony. Once the MSY phylogeny is defined, haplotype dispersal should be screened by genotyping haplotype determining markers in larger sample collections.⁶

Defining the MSY haplotype signatures that are diagnostic for a given breed or even a single influential individual enables to univocally trace their influence. This process has been conducted

in the horse to assess the influence of the Thoroughbred breed.^{7,8} From horse studies the importance of an appropriate ascertainment panel has become evident, as haplotypes that are private in rural populations are still not fully defined⁹ and ancient remains enlighten significant turnover of MSY haplotype spectra through time and space.¹⁰

Establishing MSY phylogenies is computationally demanding, and obtaining inferences on haplotype distribution is labour intensive. The building of international consortia to coordinate MSY research in livestock would be extremely beneficial.

¹ Jobling, M.A. & Tyler-Smith, C. 1995. Fathers and sons: The Y chromosome and human evolution. *Trends in Genetics*, 11(11): 449–456. [https://doi.org/10.1016/S0168-9525\(00\)89144-1](https://doi.org/10.1016/S0168-9525(00)89144-1)

² Ve2. Jobling, M.A. & Tyler-Smith, C. 2017. Human Y-chromosome variation in the genome-sequencing era. *Nature Reviews Genetics*, 18(8): 485–497. <https://doi.org/10.1038/nrg.2017.36>

³ Van Oven, M., Van Geystelen, A., Kayser, M., Decorte, R. & Larmuseau, M.H. 2014. Seeing the wood for the trees: A minimal reference phylogeny for the human Y chromosome. *Human Mutation*, 35(2): 187–191. <https://doi.org/10.1002/humu.22468>

⁴ Wallner, B., Palmieri, N., Vogl, C., Rigler, D., Bozlak, E., Druml, T., Jagannathan, V., et al. 2017. Y chromosome uncovers the recent oriental origin of modern stallions. *Current Biology*, 27(13): 2029–2035.e5. <https://doi.org/10.1016/j.cub.2017.05.086>

⁵ Felkel, S., Wallner, B., Chuluunbat, B., Yadamsuren, A., Faye, B., Brem, G., Walzer, C. & Burger, P.A. 2019. A first Y-chromosomal haplotype network to investigate male-driven population dynamics in domestic and wild bactrian camels. *Frontiers in Genetics*, 10: 423. <https://doi.org/10.3389/fgene.2019.00423>

⁶ Deng, J., Xie, X-L., Wang, D-F., Zhao, C., Lv, F-H., Li, X., Yang, J., et al. 2020. Paternal origins and migratory episodes of domestic sheep. *Current Biology*, 30(20): 4085–4095. <https://doi.org/10.1016/j.cub.2020.07.077>

⁷ Wallner, B., Vogl, C., Shukla, P., Burgstaller, J.P., Druml, T. & Brem, G. 2013. Identification of genetic variation on the horse Y chromosome and the tracing of male founder lineages in modern breeds. *PLoS ONE*, 8(4): e60015. <https://doi.org/10.1371/journal.pone.0060015>

⁸ Cosgrove, E.J., Sadeghi, R., Schlamp, F., Holl, H.M., Moradi-Shahrabak, M., Miraei-Ashtiani, Salma Abdalla, S.R., et al. 2020. Genome diversity and the origin of the arabian horse. *Scientific Reports*, 10(1): 9702. <https://doi.org/10.1038/s41598-020-66232-1>

⁹ Han, H., Wallner, B., Rigler, D., MacHugh, D.E., Manglai, D. & Hill, E.W. 2019. Chinese Mongolian horses may retain early domestic male genetic lineages yet to be discovered. *Animal Genetics*, 50(4): 399–402. <https://doi.org/10.1111/age.12780>

¹⁰ Fages, A., Hanghoj, K., Khan, N., Gaunitz, C., Seguin-Orlando, A., Leonardi, M., Constantz, C. M., et al. 2019. Tracking five millennia of horse management with extensive ancient genome time series. *Cell*, 177(6): 1419–1435.e31. <https://doi.org/10.1016/j.cell.2019.03.049>

Deviations from the expected levels of genetic diversity and divergence of the X chromosomes have been observed in several species. For mammals, the X chromosome is present in two copies in females and one copy in males, therefore, the expected ratio between the genetic diversities of chromosome X and the autosomes is 0.75 (Ellegren, 2009; Wilson Sayres, 2018). However, this ratio has varied widely in several breeds of European and African cattle (da Fonseca *et al.*, 2019). Increased levels of X-chromosomal diversity have been shown to result from growth in population size (Van Belleghem *et al.*, 2018; Pool and Nielsen, 2007). Conversely, a reduction in relative diversity follows an overall reduction in N_e (Ellegren, 2009; Pool and Nielsen, 2007). In livestock populations, this is often a consequence of breeding practices that involve the selection of a few individuals with desirable phenotypes. A reduction in population size would result in relatively strong genetic drift of X-chromosomal alleles, which yield smaller estimates of N_e than do marker data from autosomes. In the case of cattle, molecular signatures of a bottleneck have been detected in several cattle breeds (Boitard *et al.*, 2016; da Fonseca *et al.*, 2019; Ginja, Telo Da Gama and Penedo, 2010; Kim *et al.*, 2017; Martín-Burriel *et al.*, 2011).

Another process that is expected to impact the relative diversity of the sex chromosomes is sex-biased gene flow (Ellegren, 2009; Wilson Sayres, 2018). This is especially relevant for cattle, for which artificial insemination of many cows with semen of very few sires is a widespread practice. Furthermore, it is known that historically female populations were more likely to be geographically constrained and human-driven hybridization/crossbreeding may have been carried out mainly using males (Lenstra *et al.*, 2014). Generally, introgression is more efficient in the autosomes than in the sex chromosomes, as observed for the domestic chicken (Lawal *et al.*, 2020). This pattern can be explained by stronger species barriers on the sex chromosomes (Meiklejohn *et al.*, 2018).

As shown by da Fonseca *et al.* (2019), the use of whole-genome sequences led to two observations that are probably related and may very well be of fundamental significance. First, a ratio of F_{ST} values for autosomal and X-chromosomal sequences depends dramatically on the cattle being compared. For taurine vs indicine cattle, the X-chromosomal F_{ST} values were ~25 percent larger than the autosomal values. In contrast, within taurine cattle, the autosomal F_{ST} values were about twice those of the X chromosome, and for Iberian cattle with autosomal F_{ST} values ranged from 0.1 to 0.26, whereas the X-chromosomal range was 0 to 0.04. Second, in model-based clustering at $K = 2$, the clusters corresponded to taurine and indicine cattle. In this case, the autosomes of African taurine cattle showed an indicine component of 18 to 36 percent, whereas the X chromosomes remained purely taurine. It is not yet clear if these effects can be explained by a relatively ineffective X-chromosomal introgression; there may well be consequences of genetic conflicts underlying the meiotic drive (Meiklejohn *et al.*, 2018). Since LD-based pruning removes X-chromosomal SNPs, this was never noticed with bead-arrays, but it is certainly worth following up.

IDENTIFICATION OF GENOMIC REGIONS SUBJECT TO SELECTION

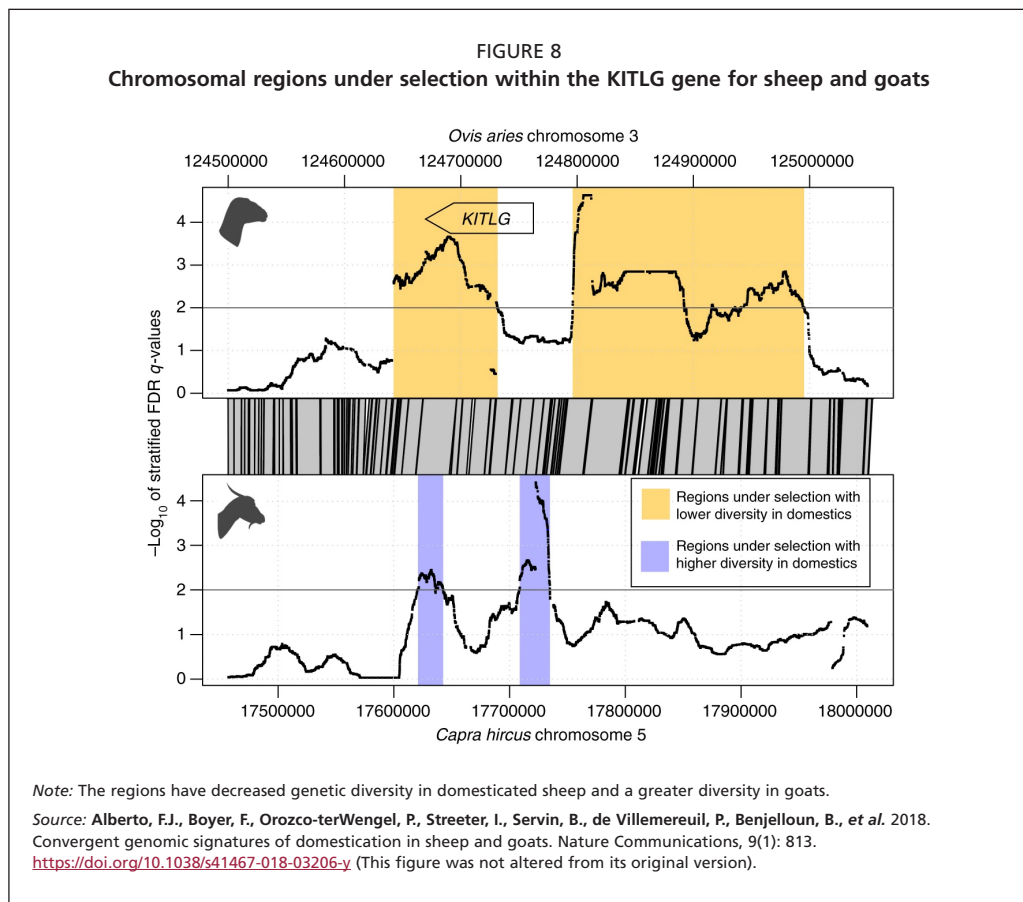
Domestication, adaptation, breed formation and selective breeding have left specific genetic patterns, “selection signatures,” in genomic regions of farm animal breeds (e.g. Raudsepp *et al.*, 2019). The identification of selection signatures is a central goal in evolutionary and population genetic studies, but also has importance in characterization of

animal genetic resources (AnGR) for food and agriculture. With methods for the detection of selection signatures it may be possible to identify, for example, genes related to economically important traits or for adaptation to challenging environments (Saravanan *et al.*, 2020; Weldenegodguad *et al.*, 2019). Breeds displaying special adaptive traits may merit high priority in conservation strategies. Selection signature studies can also provide knowledge of quantitative trait loci (QTL) for production characters and important causal mutations (Saravanan *et al.*, 2020). Moreover, genome-wide searches for regions associated with phenotypic traits may promote our understanding of the function of genomes. For example, several whole-genome datasets have shown that a large proportion of SNPs exhibiting selection signatures correspond to non-coding genomic regions, indicating that selection occurs specifically via the regulatory elements of genomes (Librado *et al.*, 2015).

Types of selection

During the domestication process, genomes of animals have been shaped by natural selection and artificial selection. Human-initiated artificial selection (or selective breeding) conducted over generations for productive, morphological, fertility and other economically important traits has had remarkable effects on genetic and phenotypic variation within and between breeds of farm animal species (Feliuss, 1995; Forutan *et al.*, 2018). Natural selection driven by environmental circumstances involves differential reproduction of genetically diverse types, such that some types leave more offspring than others (e.g. Falconer and MacKay, 1996). Three forms of natural selection are typically classified: (i) positive selection; (ii) purifying selection (negative or background selection); and (iii) balancing selection.

The various forms of selection have specific effects on allelic and genotypic frequencies, as well as on genomic structural variation in animal populations (de Simoni Gouveia *et al.*, 2014). When artificial selection or natural positive selection increases frequency of an advantageous allele, the frequencies of neutral variants that are physically linked to the advantage allele tend to change (de Simoni Gouveia *et al.*, 2014, and references therein). This process is termed “genetic hitchhiking.” When a substantial (or complete) reduction of genetic variation at the target of selection and its surrounding genomic regions occurs, it’s called a “selective sweep” (Maynard-Smith and Haigh, 1974). Moreover, in these chromosomal regions an increase in the average LD is expected to occur, leading to long haplotypes. These changes can typically be observed in statistical parameters related to genetic diversity within and between populations (de Simoni Gouveia *et al.*, 2014, and the references therein). The within-population diversity is typically decreased in these regions, while between-population diversity tends to increase. In negative (purifying) selection, the selective pressure operates against disadvantageous alleles at the target of selection, thus not always affecting the surrounding genetic variation, especially when there are multiple advantageous or disadvantageous alleles. However, negative selection can also result in loss of genetic variation at the region of the genome which, although neutral, is linked to the negatively selected sites; this process is known as “background selection” (Charlesworth, B., Morgan and Charlesworth, D. *et al.*, 1993). The third main form of natural selection, balancing selection, maintains multiple alleles in a locus and high genetic diversity (Oleksyk, Smith and O’Brien, 2010). Major histocompatibility complex genes are classical examples



of genes shaped by balancing selection (Hedrick, 1998). More information on various forms of selection and their effects on nonneutral and linked neutral genetic variation is provided in the literature (de Simoni Gouveia *et al.*, 2014; Horscroft *et al.*, 2019; Saravanan *et al.*, 2020). Figure 8 is a graph showing trends in diversity of the KITLG genic region of sheep and goats (Alberto *et al.*, 2018). This region includes subregion that shows decreased diversity in domesticated sheep but decreased diversity in goats, presumably due to species-specific selection processes associated with domestication.

Methods of detecting selected loci

Methods to detect selection signatures have been recently reviewed by Utsunomiya *et al.* (2015), Horscroft *et al.* (2019) and Saravanan *et al.* (2020). The methods commonly used in livestock studies can be classified into two main groups: (i) methods based on intra-population statistics; and (ii) those on inter-population statistics (Saravanan *et al.*, 2020). In intra-population methods, genomic or DNA-marker data are compared within populations. In this group, methods are based on the site frequency spectrum, LD or the identification of genomic regions with reduced variation compared to the genome average (de Simoni Gouveia *et al.*, 2014; Alachiotis and Pavlids, 2018; Saravanan *et al.*, 2020). Alachiotis and

Pavlidis (2018) introduced a composite evaluation test, μ statistic for detection of positive selection, which examines genomic regions by quantifying the site frequency spectrum, the levels of LD and the amount of genetic diversity along a chromosome. *PCAdmix* (Brisbin *et al.*, 2012) is a sophisticated and popular software program that can be used to detect adaptive introgression. It uses phased genotypes and applies PCA and a Hidden Markov Model on sliding windows along the genome in order to detect the ancestry of the individuals as this varies across the genome. Local deviations of the ancestry shared by all individuals of a breed then may indicate that the region is involved in adaptation

The inter-population approaches are based on allele frequency differences and the degree of differentiation among the populations (Saravanan *et al.*, 2020). The level of genetic differentiation can be analysed using single site differentiation or haplotype-based differentiation (Saravanan *et al.*, 2020). Coding sequences of orthologous genes among animal species can also be compared and tested based on synonymous and non-synonymous substitution rates applied to detect the neutrality or the form of selection (positive or negative) (de Simoni Gouveia *et al.*, 2014). In addition, “landscape” genetics and genomics (Manel *et al.*, 2003, 2010) approaches may reveal associations between genomic data and environmental variables, and thereby environmental adaptive signatures (e.g. Mdladla, Dzomba and Muchadeyi, 2018; Vajana *et al.*, 2018). See Box 9 for more information on landscape genomics.

Appendix 6 provides examples of software and includes a section for applications that have been used in domestic animal studies to detect selection signatures. Because various methods have different strengths and weaknesses, it is typically recommended that more than one method be used for analyses of selection signatures (de Simoni Gouveia *et al.*, 2014; Utsunomiya *et al.*, 2015; Yurchenko *et al.*, 2018; Horscroft *et al.*, 2019; Saravanan *et al.*, 2020). In animal populations, demographic events such as population expansions, genetic bottlenecks and population subdivision can lead to false selection signals that mimic signatures of selection (de Simoni Gouveia *et al.*, 2014; Utsunomiya *et al.*, 2015). de Simoni Gouveia *et al.* (2014), Utsunomiya *et al.* (2015), Ahrens *et al.* (2018) and Horscroft *et al.* (2019) have presented approaches to avoid effects of demography or other factors causing bias in selection signature analyses.

GENOME-WIDE ASSOCIATION STUDIES

As mentioned previously, the development and application of dense marker panels that can be genotyped at a high-throughput frequency by using microarray technology (see Section 3) has facilitated the generation of large-scale genotype data for many domestic animal species. The most widely applied microarrays provide genotypes for between 50 000 and 800 000 SNPs that are evenly spread across the genome. Millions of domestic animals have been genotyped with these arrays in the past decade with the primary goal to implement genomic selection and predict genomic breeding values (Georges, Charlier and Hayes, 2019). The availability of dense genotypes and a diverse array of phenotypes, often available over multiple generations, makes domestic animal populations amenable to genome-wide association testing. In genome-wide association studies (GWAS), statistical tests are applied to examine if molecular markers are associated with the expression of phenotypes. Because such analyses effectively involve the testing of multiple hypotheses

BOX 9

Landscape genomics*Stephane Joost*

Landscape genomics is a research field that combines population genomics, landscape ecology, computer science and bio-geoinformatics to explicitly quantify the effects of environmental heterogeneity on neutral and adaptive genetic variation and underlying processes. Landscape genomics has potential for addressing questions in various research fields, including ecology, evolution, and conservation of livestock breeds. The discipline is based on the idea that environmental conditions can substantially affect the genetic variation of local populations, with important consequences for evolutionary processes.

The landscape genomics approach consists of linking genome-wide information to environmental variables to identify potentially valuable genetic material, like genomic regions conferring adaptive advantages. Typically, data obtained from genome-wide scans carried out on a number of animals from populations living in different habitats or across ecological clines will be compared to geo-environmental information characterizing these habitats (such as yearly amount of precipitation, monthly temperatures, number of days with ground frost, etc.). Comparisons are carried out by means of association models calculated between allele or genotype frequencies and the values of the environmental variables. The parallel and simultaneous processing of up to billions of statistical equations (i.e. hundreds of environmental variables times millions of SNPs) makes it possible to identify genomic regions of interest, for instance, linked to genes involved in adaptation to heat, or genes involved in processes of resistance against parasites.

In developing countries, small ruminants play an important role in the livelihood of a large proportion of farmers. In these countries,

conserving traditional breeds is essential since they are able to survive in harsh conditions and often changing habitats, to which their wide genomic diversity makes it possible to adapt. Therefore, landscape genomics is an important tool to identify the key genetic features of local adaptation and to support sustainable breeding of low-input livestock.

In the current context of global warming, this genetic adaptive potential can be directly integrated into conservation management using spatial areas of genotype probability (SPAGs). This tool is able to transpose landscape genomic results into an adaptive potential spatial prediction framework. SPAGs can then be integrated with climate change projections to forecast the future spatial distribution of genotypes. The analysis of the mismatch between current and future SPAGs (“genomic offset”) makes it possible to identify vulnerable populations lacking the adaptive genotypes necessary for future survival.

Environmental data are necessary to compute landscape genomics models. The WorldClim¹ database contains monthly minimum, maximum and average temperatures and total precipitation together with a series of bioclimatic variables computed from these variables. The Climatic Research Unit² in Norwich, United Kingdom, one of the world's leading institutions concerned with the study of natural and anthropogenic climate change, also produces several datasets that can be used in the context of landscape genomics studies.

For computation, several open-source software packages implement landscape genomics algorithms. These are provided in Appendix 6.

¹ WorldClim. 2020. *WorldClim*. Cited 20 March 2021. www.worldclim.org

² CRU (Climate Research Unit). 2021. Climatic Research Unit. University of East Anglia, Norwich, United Kingdom. Cited 18 March 2021. www.uea.ac.uk/groups-and-centres/climatic-research-unit

simultaneously, the markers need to pass stringent significance thresholds to be considered as significantly associated with the trait of interest. Various statistical methods have been developed to account for multiple testing (Rice, Schork and Rao, 2008; Joo *et al.*, 2016). Marker panels typically do not contain causal mutations, so the primary aim of GWAS is to identify regions likely to harbour causal variants through an association with a physically linked genotyped variant. The precision to localize causal variants through GWAS with anonymous marker panels depends on the marker density. Sparse marker panels may result in large confidence intervals for QTL, rendering the identification of causal variants a difficult task. Due to extensive LD between adjacent markers in populations with small N_e , QTL confidence intervals may also be large even when dense marker panels are employed. Functional investigations are required to differentiate between causal variants and anonymous markers in LD with the causal variants (Karim *et al.*, 2011).

Apart from the density of the marker panel, the power of the GWAS, that is, the probability to detect trait-associated markers, depends on: (i) the LD between molecular markers and QTL; (ii) the heritability of the trait and/or size of the single locus effect; and (iii) the size of the mapping cohort (Goddard and Hayes, 2009). The most widely applied genotyping arrays in domestic animal species have been developed for cosmopolitan breeds (Matukumalli *et al.*, 2009), thus they are less informative for local breeds that are greatly diverged from the breeds used to develop the marker panels. Moreover, the genotyped markers are often depleted for low-frequency variants. This may result in AB (see Section 3), which can compromise the power of GWAS, particularly in local breeds. While large mapping cohorts may be established easily in cosmopolitan breeds, the small census size of local breeds may also be a limiting factor for GWAS to detect markers associated with within-breed variation. Across-breed GWAS may reveal variants that are associated with breed differences (Schoenebeck and Ostrander, 2013). However, even in large cosmopolitan breeds, the large sample size required to detect trait-associated variants may not be readily available for novel traits, thus requiring efforts to coordinate collaborative projects to establish powerful mapping cohorts (Lu *et al.*, 2018).

Both binary (discretely distributed) and complex (continuously distributed) traits may be subjected to GWAS testing. Association testing for binary traits requires genotypes for a cohort of individuals that expresses a particular phenotype (“cases”) and for a cohort of control individuals that don’t express the phenotype (“controls”). Chi-square tests and Fisher tests of allelic association may be implemented to test the association between markers and binary traits (Balding, 2006). It is important though, that both cohorts are matched because systematic differences in allele frequencies between cases and controls due to stratification may confound the GWAS and lead to false-positive associations. The highest-ranking PCs of a genomic relationship matrix can be effectively used to account for population structure and cryptic relatedness in case-control GWAS (Price *et al.*, 2006; Nosková *et al.*, 2020). Case-control association testing of genome-wide markers has been successfully used to reveal causal variants for many Mendelian traits in domestic animals, and most of them are curated at the Online Mendelian Inheritance in Animals (OMIA) database (OMIA, 2021).

Most continuously distributed traits are highly polygenic and recent empirical evidence suggests that they are predominantly determined by additive effects (Hayes *et al.*, 2010;

Hivert *et al.*, 2021). Each causal variant explains only a small fraction of the phenotypic variation of these complex traits. To avoid an inflation of false-positive association signals arising due to population stratification (see previous paragraph), software (see Appendix 6) for complex trait GWAS typically rely on mixed model-based approaches that fit a (genomic) relationship matrix. While QTL that explain a relatively large fraction of the trait variation can be detected in small mapping cohorts (Pausch *et al.*, 2011; Signer-Hasler *et al.*, 2012), the discovery of QTL with small effects usually requires tens of thousands of genotyped animals (Bouwman *et al.*, 2018).

The first GWAS were performed in 2005 for monogenic traits in human populations (DeWan *et al.*, 2006; The Wellcome Trust Case Control Consortium, 2007). Likewise, the first SNP-based GWAS in domestic animals were performed for monogenic traits using genotypes for approximately 25 000 SNPs (Karlsson *et al.*, 2007; Charlier *et al.*, 2008). Since then, both the size of the mapping cohorts and the marker density have increased continually. The largest GWASs in domestic animals have been performed in cattle, which included the genotypes at more than 25 million variants for more than 94 000 samples (van den Berg *et al.*, 2020). With an ever-increasing size of mapping cohorts and more markers being tested, efficient software tools are required to undertake marker quality control and the association analyses. Since its initial release, the *PLINK* software has undergone a major overhaul and has become a practically indispensable tool for genotypic data analysis at the population scale (Purcell *et al.*, 2007; Chang *et al.*, 2015). Moreover, the *.bed/.bim/.bam*-format and its derivatives used by *PLINK* to store genotypic data are accepted as an input for most downstream analyses. Basic association analyses may be performed with *PLINK*. However, more sophisticated statistical methods that are based on Bayesian or mixed model-based approaches may be conducted efficiently with the *EMMAX* (Kang *et al.*, 2010), *GCTA* (Yang *et al.*, 2011) and *BayesR* (Erbe *et al.*, 2012; Moser *et al.*, 2015) and *LFMM2* (Caye *et al.*, 2019) software tools (see Appendix 6). Latent factor mixed models (LFMMs) are, in particular, currently gaining popularity. This method has been designed for detection of gene-environment associations and identifies SNPs with allele frequencies that correlate with environmental clines or with phenotypes.

With an increasing number of animals being sequenced, GWAS nowadays often rely on imputed sequence variant genotypes (see Section 3). Informative haplotype reference panels are required to accurately infer sequence variant genotypes for mapping cohorts that have array-derived genotypes. Such reference panels have been established for a variety of animals, including cattle (Daetwyler *et al.*, 2014; Hayes and Daetwyler, 2019), sheep (ISGC, 2014), goats (IGGC, 2013) and dogs (Plassais *et al.*, 2019). Genotypes imputed from informative haplotype reference panels may readily reveal causal variants in GWAS (Pausch *et al.*, 2017). The integration of functional data is required to fine-map QTL regions and reveal causal variants (Xiang *et al.*, 2019). Cross-species approaches where information from humans is used to prioritize causal variants in livestock has also been explored (Costilla *et al.*, 2020; Raymond *et al.*, 2020). Novel approaches that are based on low-pass sequencing and imputation-based genotype refinement offer a cost-effective opportunity to perform sequence-based GWAS also in populations that either lack informative haplotype reference panels or for which dense microarrays are not available (Li *et al.*, 2021; Rubinacci *et al.*, 2021; Fuller *et al.*, 2020), which offers opportunities for less common livestock species.

APPLICATION OF GENOMICS IN SUSTAINABLE USE AND CONSERVATION

This document emphasizes the use of genomics for the characterization of AnGR, and therefore are designed to facilitate the implementation of Strategic Priority Area (SPA) 1 of the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007) on Characterization, Inventory and Monitoring of Trends and Associated Risks. Genomics can also play an important role in the sustainable use and development (SPA2) and conservation (SPA3) of AnGR. Those roles for genomics are somewhat out of the scope of this documents, so they will not be addressed in detail. The subsequent subsections will briefly introduce some of the opportunities that genomics provide for these SPAs. Oldenbroek (2017) addresses the contributions of genomics to the management of AnGR in more detail.

Every breed has a history

Never the same; livestock breeds are not static entities but have continuously evolved. Changes have been substantial after domestication (Larson and Fuller, 2014; Zeder, 2015), but perhaps the most consequential changes have taken place during the last 200 years through the formation of breeds and the systematic breeding. This can now be reconstructed by the analysis methods outlined herein. The following are a few examples showing how the evolution of breeds is directly or indirectly relevant for their management and conservation.

Local and transboundary breeds

Most breeds originate from local populations. Thus, they potentially harbour unique adaptive traits and often belong to the local cultural heritage. However, influence from other populations has often been undocumented. The influence from other breeds may not necessarily affect their adaptation and traditional status, but reconstruction of their local and non-local ancestry by genomic analysis will be most revealing. A recent study in Brazil revealed some unexpected information about Latin American sheep breeds (see Box 10). The genomic characterization of groups of breeds also may reveal the sources of adaptive introgression, which contributes to both resilience and uniqueness (Barbato *et al.*, 2017; Chen *et al.*, 2018). However, the introduction of exotic highly productive breeds, such as the Holstein dairy cattle or the Merino wool sheep, does replace robust unique animals by more productive uniform populations that require intensive management.

For goats, Colli *et al.* (2018) observed on the basis of genome-wide SNP genotypes reported a strong geographic partitioning of goats. In addition, several ancient goat DNA samples are related to modern goats from the same location (Daly *et al.*, 2019), indicating a high degree of local ancestry. In contrast, The International Sheep Genomics Consortium (ISGC, 2012) reports a high level of geneflow among global sheep populations, which was presumably driven by the desire for increased wool or mutton production and has eroded the geographic differentiation.

BOX 10

Application of basic genomic diversity studies: A case study of Brazilian hair sheep breeds

Samuel Paiva

A recent study in Brazil¹ provides an example of how genetic diversity data may advise national conservation and breeding programmes. Genotypes (50 000 SNPs) from seven Brazilian sheep breeds (five hair and two coarse wool types) and 87 worldwide breeds were used to evaluate population structure and phylogenetic origin and quantify genetic diversity. The main result shows that Brazilian hair sheep, contrary to other breeds, have shared alleles with breeds from both African and European continents (Admixture and PCA). This mixture of genetic sources, plus adaptation to local conditions has resulted in high genetic variability (neutral and functional) that will be considered in conservation and breeding programmes. For example, Brazilian hair sheep breeds have high frequencies of a specific allele (FecGE) in the GDF9 gene² linked to litter size, which has not been commonly reported among breeds outside the Americas. This trait has already been used to enrich the collection of material in the Brazilian National Gene Bank and has provided farmers with an additional tool for in-herd selection. The study also helped to identify rare

or genetically distant national breeds for which germplasm collection will be a priority. The Brazilian Somali was the first breed prioritized for germplasm collection, and the gene bank has already begun cryopreservation of semen and embryos. The endangered Brazilian Fat-tail sheep was also identified as a priority for cryo-conservation. Analyses of genetic diversity and population structure were also used to identify the breeds with more potential for breeding programmes. The number of animals in the Santa Ines (hair) breed has expanded over the last decade, in part due to crossbreeding with other populations, and is currently the primary commercial hair breed in Brazil.³ With high genetic diversity and low inbreeding compared to other Brazilian breeds, it has potential to achieve high genetic gains in production traits within a well-designed breeding programme.

¹ Paim, T.P., Paiva, S.R., de Toledo, N.M., Yamagishi, M.B., Carneiro, P.L.S., Facó, O., de Araújo, A.M., et al. 2021. Origin and population structure of Brazilian hair sheep breeds. *Animal Genetics*, 52(4): 492–504. <https://doi.org/10.1111/age.13093>

² Silva, B.D., Castro, E.A., Souza, C.J., Paiva, S.R., Sartori, R., Franco, M.M., Azevedo, H.C., et al. 2011. A new polymorphism in the Growth and Differentiation Factor 9 (GDF9) gene is associated with increased ovulation rate and prolificacy in homozygous sheep. *Animal Genetics*, 42(1): 89–92. <http://doi.org/10.1111/j.1365-2052.2010.02078.x>

³ McManus, C., Hermuche, P., Paiva, S.R., Melo, C.B. & Mendes, C.Q. Geographical distribution of sheep breeds in Brazil and their relationship with climatic and environmental factors as risk classification for conservation. *Brazilian Journal of Science and Technology*, 1: 3. <https://doi.org/10.1186/2196-288X-1-3>

What's in a name?

Many breeds have been exported to other countries, most notably from Europe into the Americas and Australia. Distinct populations of these “transboundary” breeds often have been further developed separately on different continents. This has led, for instance, to the creation in America of black varieties of European beef breeds, and genomic analyses show that several American breeds form a cluster separate from their European namesakes (Davenport *et al.*, 2020). In France, this divergence of a breed into distinct units has even occurred with the national population of the Alpine goat breed, discernible by genomic characterization (see Box 11). Conversely, closely related breeds with different herdbooks, but regularly exchanging sires, may be different in name only.

BOX 11

Genomic characterization as tool in the breed recognition process*Coralie Danchin-Burge*

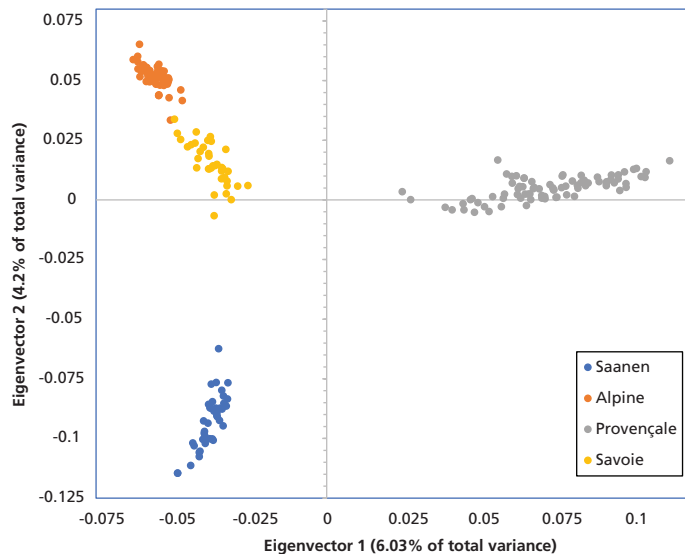
In France, a ruminant breed is officially recognized by the Ministry of Agriculture if it fits a set of criteria validated by a commission of experts. The items to provide to the commission include a description of the breed's history, its phenotypic description, the existence of a breed register, a list of breeders, and the common set of rules for organizing the selection or conservation process, etc.

The most prominent French goat breed is the Alpine, which originated in the northern part of the French Alps. For 50 years the breed has been intensively selected for dairy traits. The main selection nuclei are now located in the west and centre of France. Meanwhile, some farmers kept the traditional population in the Alps and claimed to have limited exchange of animals or germ plasm with the rest of the breed. A breeders'

association was created 20 years ago to preserve this population which they called "Savoie." The official recognition was complicated to achieve because the experts doubted that the population was significantly different from the Alpine breed.

In the mid-2010s, a medium SNP goat chip (by Illumina Inc.) became available in France for a reasonable price (about USD 40/genotype). The breeders' association, in collaboration with a research and development institute (IDELE), took this opportunity to sample and genotype about 40 goats to compare it with available Alpine genotypes. IDELE performed a PCA based on a kinship genomic matrix that showed that the Savoie breed is indeed a close relative to the Alpine breed, but that the population differs now from the Alpine (see Figure 9). The analysis was also an opportunity to detect animals that were clearly crossbred recently with the Alpine, leading to their withdrawal from the conservation programme. The results of the genomic analysis were provided to the expert commission and were one of the factors that led to the official recognition of the Savoie breed in 2020.

FIGURE 9
First two principal components using four French selected goat populations from the Alps



Source: Author's own elaboration.

Valuable DNA or valuable breeds?

Genome analysis allows an accurate assessment of the diversity, both across the genome or at the level of individual genes. However, it is not straightforward to link this diversity to the value of a breed. Differences between breeds are quantified via allele frequencies instead of the number of mutations, as is frequently done for discriminating between species. A unique allele frequency profile by genetic drift does not in itself make a breed valuable (European Cattle Genetic Diversity Consortium, 2005), but may be relevant for breed management in different ways. First, genetic distances on the basis of the allele frequencies may reveal that a breed underwent a long separate history in a given environment and thus is expected to possess unique adaptive traits. Second, levels and patterns of homozygosity as influenced by small population sizes and/or assortative mating are warning signs of inbreeding depression affecting health and fertility. Third, as mentioned, it may indicate a unique adaptive influence of cross-fertile species (Chen *et al.*, 2018). On the other hand, short genetic distances do not exclude large differences in phenotype. For instance, the unique Merino wool sheep are closely related to the coarse-wool Churra breed (ISGC, 2012) despite their vastly different fibre-quality characteristics.

It may be argued that livestock breeds carry largely overlapping portions of the available adaptive variation. Thus, it may be questioned if, in general, breeds are to be considered as unique units of conservation (Felius, Theunissen and Lenstra, 2015). On the other hand, the diversity of livestock species is primarily managed on the level of breeds, which may include conservation measures, adjustment of breeding objectives and crossbreeding. Thus, livestock breeds remain the units for management of diversity, a practice that can now more and more be supported by genomic analysis.

Sustainable use and development

The long-term sustainability of a given breed will be enhanced if that breed has greater survival and longevity, is more productive and profitable, or otherwise contributes competitively to the livelihood of its keepers (FAO, 2013). Improvement through selective breeding is one opportunity to increase the average genetic merit of breeds for heritable traits associated with sustainability. Although it is technically not “genomic characterization” and will thus not be covered in depth herein, “genomic selection” (Meuwissen, Hayes and Goddard, 2001) is an approach that statistically associates the phenotypes of animals within a population to their genotypes, as defined by large panels (typically thousands) of SNPs. Operationally, genomic selection can be performed either by predicting the substitution value of each of the alleles of the panels of SNPs, or by using multi-locus genotypes to obtain more accurate relationship matrices than can be obtained by using pedigrees and replacing the matrices in typical methods based on best linear unbiased predictions (BLUP). When large amounts of historical performance and pedigree data are available, the breeding values of animals without phenotypes can be predicted much more accurately than with pedigrees alone. These “genomic breeding values” may also allow selection to occur prior to sexual maturity, thus increasing response to selection by decreasing the generation interval. Genomic selection is currently being routinely practiced in several livestock species (Georges *et al.*, 2019).

Alas, because the accuracy of genomic selection increases with the amount data available, breeds with small population sizes and/or a lack of historical performance and

pedigree data may have less opportunity to benefit than do larger breeds (Mészáros *et al.*, 2015; Obšteter *et al.*, 2019). Breeds with small population sizes tend to also have small N_b and small N_e , meaning that intense selection to maximize genetic response may quickly decrease genetic variation to unacceptably low levels. Genomic data provides the possibility to balance selection response and maintenance of genetic variation at the molecular level, such as by applying genomic-based optimum contribution selection (Obšteter *et al.*, 2019; Sánchez-Molano, Pong-Wong and Banos, 2016).

Genomics can also contribute to the management of genetic diversity as it relates to one or a few loci. Methods for detecting selection signatures and GWAS can be used to identify targets for introgression or purging of deleterious loci. Genomic or marker-assisted selection can then be applied to increase/decrease the frequencies of the alleles associated with the trait in the population (Gaspa *et al.*, 2015). Genomics may also be used to decrease the proportion of “foreign” genes from a population that had been historically subjected to crossbreeding (Wellmann, Hartwig and Bennewitz, 2012). Finally, genomic characterization can be applied to individual crossbred animals to determine their genetic composition, potentially leading to improved management (see Box 12).

BOX 12

Estimation of ancestral breed proportions to improve management of admixed dairy cattle in developing countries

In many developing countries, imported semen has been used for decades to try to increase the milk production of local cattle populations. This process has increased productivity in many cases but has also tended to erode the genetic background of the original breeds.¹ In many instances, the livestock keepers are unsure about the breed composition of their cattle.² Assessment of the admixture among the breeds contributing to these populations allows the estimation of the proportion of ancestry from local and imported genetics for populations and individual animals. The technology can also be applied for determining parentage and evaluation of genetic diversity of the populations. Knowledge about the

breed composition of individual animals can be used by farmers and other stakeholders to identify the best-performing genotypes in the local production environment, and to inform the management and mating of individual animals. Recent studies have applied this approach in Africa³ and India.⁴

¹ Leroy, G., Boettcher, P., Besbes, B., Peña, C.R., Jaffrezic, F. & Baumung, R. 2020. Food securers or invasive aliens? Trends and consequences of non-native livestock introgression in developing countries. *Global Food Security*, 26: 100420. <http://doi.org/10.1016/j.gfs.2020.100420>

² Manirakiza, J., Hatungumukama, G., Thévenon, S., Gautier, M., Besbes, B., Flori, L. & Deltilleux, J. 2017. Effect of genetic European taurine ancestry on milk yield of Ankole-Holstein crossbred dairy cattle in mixed smallholders system of Burundi highlands. *Animal Genetics*, 48(5): 544–550. <https://doi.org/10.1111/age.12578>

³ Gebrehiwot, N.Z., Strucken, E.M., Marshall, K., Aliloo, H. & Gibson, J. 2021. SNP panels for the estimation of dairy breed proportion and parentage assignment in African crossbred dairy cattle. *Genetics Selection Evolution*, 53(1): 21. <https://doi.org/10.1186/s12711-021-00615-4>

⁴ Strucken, E.M., Gebrehiwot, N.Z., Swaminathan, M., Joshi, S., Al Kalaldehy, M. & Gibson, J. 2021. Genetic diversity and effective population sizes of thirteen Indian cattle breeds. *Genetics Selection Evolution*, 53(1): 47. <https://doi.org/10.1186/s12711-021-00640-3>

Conservation

Genomics may also contribute to reaching conservation objectives. As a first step, genomic estimates of breed-wise genetic distances (distinctiveness) or kinship (diversity) can be used to prioritize genetically valuable breeds for conservation (e.g. Ginja *et al.*, 2013). The methodology for these approaches was originally developed for simpler markers, such as microsatellites, but in general can be applied to genomic data. For a review of these approaches, see Boettcher *et al.* (2010). The genetic assessments may then be considered together with other factors such as risk of extinction and cultural significance for a comprehensive assessment of conservation priority (FAO, 2013).

Toro, Villanueva and Fernández (2014) reviewed the possible contributions of genomics for management of livestock conservation programmes. They discuss genomics-based approaches to evaluate genetic diversity of populations according to identity by descent IBD, rather than IBS. Approaches applied to *in vivo* populations may also be applied for the management of gene bank collections. Ideally, *in vivo* and cryoconserved populations should be managed together. Genomics are currently being used to evaluate and manage populations within the Chinese national conservation programme for chickens, as described in Box 13.

BOX 13

Application of genome-wide SNPs in monitoring the impact of long-term *in situ-in vivo* conservation scheme on the genetic diversity of Chinese indigenous chicken breeds

Han Jianlin

Based on the latest nation-wide survey on animal genetic resources,¹ 114 indigenous chicken breeds, 81 improved chicken breeds/lines and 35 exotic chicken breeds/lines were identified and recorded in China.² Twenty-eight of the indigenous chicken breeds have been recognized and included into the national directory for conservation of livestock and poultry genetic resources.³ Currently, two comprehensive national *ex situ-in vivo* conservation gene banks, one national cryoconservation gene bank for somatic cells and DNAs, and 13 national *in situ-in vivo* conservation farms for individual breeds have been established and approved for the management and sustainable utilization of Chinese indigenous chicken genetic resources.⁴ To establish a conservation farm for an indigenous chicken breed, at least 300 breeding hens and 30 breeding cocks (each from a different family) are recommended to be maintained over generations.⁵ Twenty-one indigenous chicken breeds were found to be at risk of extinction due to their very small breeding population sizes;⁶ fortunately, at least four such breeds have been successfully rescued by the conservation measures.⁷

At one of the national *ex situ-in vivo* conservation gene banks – the National Chicken Genetics Resources program established in Jiangsu, three breeds, Baier Yellow chicken (BEC), Beijing You chicken (BYC) and Langshan chicken (LSC), have been maintained since 1976, 1998 and 1998, respectively, along with other Chinese indigenous chicken genetic resources. The conservation programmes began with a recommended flock size including 300 hens and 30 cocks, which have been continuously maintained with a family rotational mating regime. To evaluate the effectiveness and impact of this scheme on the genetic diversity of these populations,⁸ performed an investigation using genome-wide SNPs for 270 birds from three generations per breed and 30 birds per generation (10 cocks and 20 hens). Up to 716 373 SNPs were screened. Results are shown in Figure 10. Both H_o and H_e were generally lower in these nine populations than other populations of chicken from around the world.^{9,10,11,12,13} Estimates of F_{IS} based on either pedigree information or genome-wide SNPs have been gradually increasing over generations. The proportions of polymorphic SNPs have decreased in BEC and BYC. The loss of genetic

variability and the accumulation of inbreeding over generations was probably due to the rapid reduction of population sizes when the conservation lines were established. Therefore, a combination of both *ex situ* and *in situ-in vivo* conservation populations was recommended to maintain acceptable neutral and functional genetic diversity in the indigenous chicken breeds in the future.

¹ CNCAGR (China National Commission of Animal Genetic Resources). 2011. *Animal Genetic Resources in China – Poultry*. China Agriculture Press, Beijing.

² China. 2020. *Ministry of Agriculture and Rural Affairs publishes National Directory of Genetic Resources for Livestock and Poultry*. (In Chinese). Beijing. Cited 25 July 2021. www.gov.cn/xinwen/2020-05/29/content_5515954.htm

³ China. 2020. *The list of breeds to be conserved*. (In Chinese) Beijing. Cited 25 July 2021. www.nahs.org.cn/gk/tz/202101/P020210115497616621650.pdf

⁴ China. 2014. *The General Office of the Ministry of Agriculture issued the "National Broiler Genetics Notice of Improvement Plan (2014–2025)*. (In Chinese). Beijing. Cited 25 July 2021. www.moa.gov.cn/nybg/2014/dsiq/201712/t20171219_6110244.htm

⁵ China. 2006. *Departmental Regulations-Management Measures for the Protection Areas and Gene Banks of Livestock and Poultry Genetic Resources*. (In Chinese). Beijing. Cited 25 July 2021. www.zjzj.moa.gov.cn/flfg/202009/t20200903_6351442.htm

⁶ CNCAGR. 2011b. *National "Twelfth Five-Year Plan" for the Protection and Utilization of Livestock and Poultry Genetic Resources*. (In Chinese). Beijing. Cited 25 July 2021. www.moa.gov.cn/govpublic/XMYS/201611/t20161111_5360757.htm

⁷ CNCAGR. 2016. *National "Thirteenth Five-Year Plan" for the Protection and Utilization of Livestock and Poultry Genetic Resources*. (In Chinese). Beijing. Cited 25 July 2021. www.nahs.org.cn/xxcm/zybhlly/201905/t20190516_339678.htm

⁸ Zhang, M., Han, W., Tang, H., Li, G., Zhang, M., Xu, R., Liu, Y., Yang, T., Li, W., Zou, J. & Wu, K. 2018. Genomic diversity dynamics in conserved chicken populations are revealed by genome-wide SNPs. *BMC Genomics*, 19: 598. <https://doi.org/10.1186/s12864-018-4973-6>

⁹ Cendron, F., Perini, F., Mastrangelo, S., Tolone, M., Criscione, A., Bordonaro, S., Iaffaldano, N., et al. 2020. Genome-wide SNP analysis reveals the population structure and the conservation status of 23 Italian chicken breeds. *Animals*, 10(8): 1441. <https://doi.org/10.3390/ani10081441>

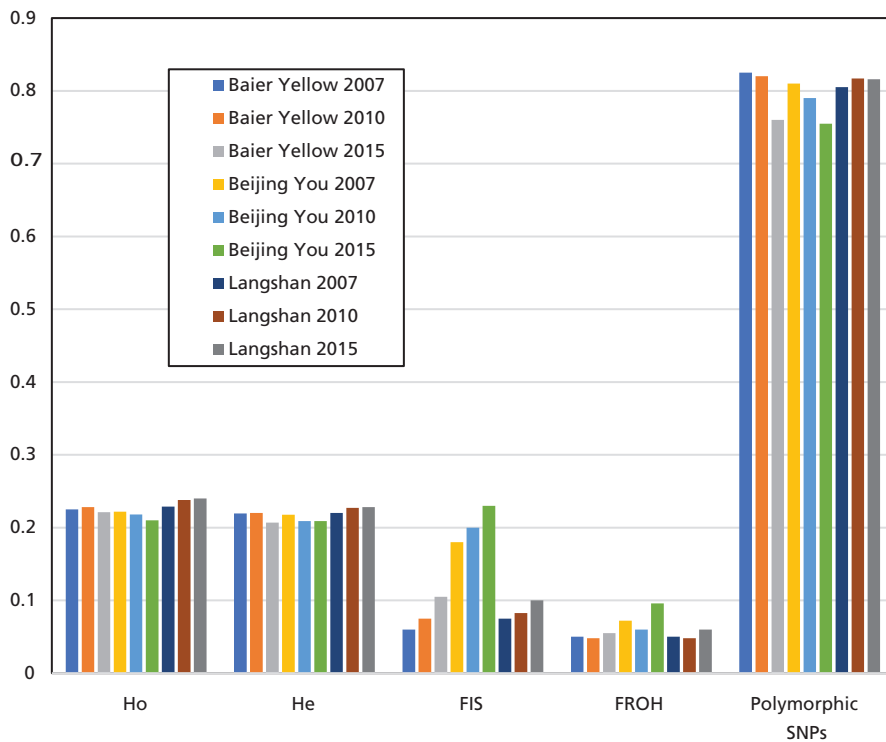
¹⁰ Luo, W., Luo, C., Wang, M., Guo, L., Chen, X., Li, Z., Zheng, M., et al. 2020. Genome diversity of Chinese indigenous chicken and the selective signatures in Chinese gamecock chicken. *Scientific Reports*, 10: 14532. <https://doi.org/10.1038/s41598-020-71421-z>

¹¹ Malomane, D.K., Weigend, S., Schmitt, A.O., Weigend, A., Reimer, C. & Simianer, H. 2021. Genetic diversity in global chicken breeds in relation to their genetic distances to wild populations. *Genetics Selection Evolution*, 53: 36. <https://doi.org/10.1186/s12711-021-00628-z>

¹² Talebi, R., Szmatoła, T., Mészáros, G. & Qanbari, S. 2020. Runs of homozygosity in modern chicken revealed by sequence data. *G3*, 10(12): 4615–4623. <https://doi.org/10.1534/g3.120.401860>

¹³ Zhang, J., Nie, C., Li, X., Ning, Z., Chen, Y., Jia, Y., Han, J., et al. 2020. Genome-wide population genetic analysis of commercial, indigenous, game, and wild chickens using 600K SNP microarray data. *Frontiers in Genetics*, 11: 543294. <https://doi.org/10.3389/fgene.2020.543294>

FIGURE 10
Changes in population genetic diversity parameters over generations/years
of three *in situ-in vivo* conserved Chinese indigenous chicken breeds
(Baier Yellow, Beijing You and Langshan)



Source: Han Jianlin (adapted from Zhang *et al.* (2018). Genomic diversity dynamics in conserved chicken populations are revealed by genome-wide SNPs. *BMC Genomics*, 19: 598. <https://doi.org/10.1186/s12864-018-4973-6>

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SECTION 5

Conclusions and recommendations

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The FAO and the ISAG/FAO Advisory Group on Animal Genetic Diversity recommend that:

1. Countries continue to characterize their animal genetic resources (AnGR) to provide critical information for their improved management and to contribute to implementation of the *Global Plan of Action for Animal Genetic Resources*. Genetic characterization should apply the most advanced methods feasible, currently single nucleotide polymorphism (SNP) and whole-genome sequencing (WGS). For most current applications in genomic characterization, medium density SNP panels will provide sufficient information. Genomic data should be complemented by phenotypic characterization, description of the production environment and recording of geographic coordinates.
2. National genetic resources are investigated by or in close collaboration with researchers from the same country while: (i) respecting the wishes and interests of the livestock keepers; and (ii) complying with legislative, administrative and policy measures addressing access to genetic resources and the sharing of benefits derived from their utilization, as applicable; (iii) complying with measures addressing access to traditional knowledge associated with genetic resources and the sharing of benefits arising from the utilization of such knowledge with indigenous peoples and local communities; and (iv) complying with measures addressing access to genetic resources that are held by indigenous peoples and local communities and the sharing of benefits arising from the utilization of such resources with the peoples and communities concerned.
3. FAO, National Coordinators and any National Advisory Committees for AnGR are made aware of all diversity projects at any geographic level, so that results can contribute to the planning and development of national conservation and sustainable use activities, and so that FAO can help facilitate coordination among projects, exchange information and promote funding. Contact information for National Coordinators can be found at www.fao.org/dad-is/national-coordinators/en.
4. Locally adapted genomic resources are considered within an international context, which, for marker-based molecular genetic studies, imply the use of the same or overlapping sets of genetic markers employed in previous studies, and that, if appropriate, data collected from characterization studies are placed in open-access repositories, including raw data, associated metadata and analysis pipelines and procedures.

5. Studies are guided by one or more specific questions to be answered, recognizing that these may be expanded during data analysis. In addition to establishing the genetic relationships of populations on the basis of neutral genetic markers, the molecular variation involved in phenotypic variation should be searched for, with special emphasis on environmental adaptation and disease susceptibility or resistance.
6. Studies of autosomal markers are complemented by the characterization of mitochondrial DNA sequences as markers representing the maternal lineages, and of Y-chromosomal variation as markers of paternal lineages.
7. Studies of the present patterns of genetic diversity of livestock are complemented, where relevant, by the analysis of ancient DNA samples in order to provide a historic context.
8. Genotype data are subjected to a thorough quality check and filtering in order to identify and remove unreliable data, including duplicates, mislabelled samples and outliers associated with breed-level differentiation or undocumented crossbreeding.
9. For all breeds studied, heterozygosities or other indicators of overall genetic variability are calculated to identify breeds with low diversity and anticipate the potential for bias towards these breeds in multi-breed analyses.
10. Researchers understand the statistical methods being applied in data analysis and critically evaluate the results obtained from computer software.
11. Parameters related to within-breed genetic diversity are not simply estimated, but that this information be complemented with that of clustering methods and demographic modelling to better understand evolutionary processes. By combining different approaches, genomics can provide more informed management of populations by monitoring of within-breed genetic variation and crossbreeding, avoiding high levels of inbreeding and ultimately preserving adaptive variation.
12. Publications of molecular diversity studies are freely accessible.
13. The scientific progress is communicated to breeders, livestock industry, the relevant government agencies and the general public.
14. Results from genomic characterization studies are actively utilized in the monitoring and management of AnGR. Standardized estimates of effective population sizes or other measures of genetic variation could complement data on population size for informing countries on the genetic status and risk of extinction of their national breed populations. More research is, however, required to determine the most appropriate measure of genetic variation. Management of AnGR may include purebred genetic improvement and conservation measures, as well as controlled crossbreeding that preserves the original populations and maintains environmental adaptation.
15. The advances in molecular technology and bioinformatics continue to be closely monitored and relevant innovations be communicated to the AnGR community.

Appendices

Appendix 1

Glossary of technical terms

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Approximate Bayesian Calculation (ABC) Modelling approach based on optimizing the agreement between simulated and measured values of summary statistics.

Admixture The presence of genetic influence of multiple breeds in the genome of an individual or a population, due to historical interbreeding.

Allelic richness Measure of genetic diversity obtained by simply counting the number of different alleles within a breed or other population of interest. Allelic richness among populations is only directly comparable if the number of animals genotyped per population is equal.

Analysis of molecular variance (AMOVA) Estimation of the portioning of diversity over different hierarchical levels: within breeds, among breeds within regions, between regions, etc.

Amplification refractory mutation system (ARMS) A polymerase chain reaction (PCR) during which one of the primer covers a single nucleotide polymorphism (SNP) site in such a way that the amplification depend on the SNP allele in the template DNA.

Ascertainment bias (AB) Systematic distortion in estimates of molecular genetic parameters (such as allelic frequencies) due to irregularities in the process used to identify the markers. For instance, many SNP in large panels were selected according to their high minor allele frequency in international transboundary breeds and can thus underestimate the relative diversity in other breeds.

Assembly The process of arranging overlapping individual DNA sequence reads into a contiguous whole-genome sequence.

Autosomes All chromosomes with the exception of the mammalian X or Y or the avian W and Z sex chromosomes.

Bayesian analysis Estimation of a likelihood distribution of model parameters on the basis of the likelihoods of parameter values in the absence of data (the prior) and the likelihoods of the observed data given different values of the model parameters. These estimations depend on a specific model and are often achieved by a strategy (like the Multiple Chain Monte Carlo simulations) to explore different plausible values of the parameters (the "parameter space").

Bead array Silica bead-based microarray for high-density SNP genotyping.

Coalescence analysis Estimation of the divergence times of individual DNA sequences since their descent from a hypothetical most recent common ancestor, often used to infer present and past effective population sizes.

Copy number variation (CNV) A type of structural variation in the genome resulting from differences in the copy number of chromosomal fragments of up to several megabases in length. CNV can be used as a genetic marker and has been associated with differences in human phenotypes.

Contig Uninterrupted (“contiguous”) DNA sequence assembled by combining separate experimental sequence reads.

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated (Cas) endonuclease 9) Method for highly specific and rapid modification of DNA in a genome, leading to a new biotechnology revolution called “genome editing.”

Crossing-over Mechanism of creating new genetic variability through recombination of chromosomes during meiosis. The newly formed oocyte or sperm chromosome combines segments of the two homologous chromosomes originating from the mother and the father of the individual in which crossing-over takes place.

Denaturing gradient gel electrophoresis (DGGE) Method to discern alternative SNP alleles (within a strand of PCR amplified nucleic acid) according to differences in their melting points while passing across an acrylamide gel.

Depth Approximate number of times that a chromosomal region will be sequenced through a whole-genome procedure that involves sequencing and subsequent assembly of segments.

Diploid Characteristic of a species defined by its genome containing of two copies of all autosomes, the maternal and the paternal copies.

Effective population size (N_e) Hypothetical population size that would generate observed values of diversity parameters for a given population if mated randomly and not subject to forces such as selection and migration. The N_e corresponds to the number of breeding animals per generation and is usually smaller than the actual population count. It may be calculated separately for males and females.

Fixation Retainment within a populations of only one allele for a give genetic marker which thus becomes homozygous, typically the effect of inbreeding reducing the genetic diversity.

Fixation index (F_{ST}) The proportional increase in homozygosity that occurs through population subdivision (for example, the creation of breeds).

Genetic distance A measure of the genetic differences between two populations (or species) calculated on the basis of allelic frequencies in both populations/species, or between two individuals based either on the identity by state (IBS) of markers or on genomic relationships.

Genetic marker Site in the genome that is variable (polymorphic) within a species. The different variants are called alleles, such as the two (usually) different nucleotides of a SNP.

Genome-wide association study (GWAS) Statistical analysis to detect a correlation between the phenotypes of a group of individuals and their genotypes of a given SNP, calculated for 50 000 to 1 000 000 SNPs spanning the whole genome; values exceeding a significance threshold may indicate that the SNP is near the causative gene.

Genotyping by sequencing (GBS) Sequencing of a subset (reduced representation) of the genome, either by a given class of restriction-enzyme fragments or fragments captured by hybridization to a set of DNA fragments. Sequencing of, more or less, the same fragments in a panel of animals yields SNPs and at the same time their genotypes. This allows genome-wide SNP genotyping for species for which no bead-array has been developed, such as wild species.

Haplotype Combination of alleles of two or more genetic markers on the same DNA segment. Haplotypes defined by alleles of markers on mitochondrial DNA and the non-recombining part of the Y chromosome are transmitted to offspring. Autosomal and X-chromosomal haplotypes, typically defined by markers in or near the same gene (in which case a haplotype represents a gene variant), are transmitted to the offspring only if there is no recombination between the markers.

Haplogroup Group of closely related haplotypes.

Haploid Genetic material containing a single copy of the genome, such as sperm cells and oocytes.

Hardy-Weinberg equilibrium (HWE) Ratio for a given marker and population of the numbers of homozygote and heterozygote genotypes as predicted by random mating in a large population in the absence of selection, migration and mutation.

Heterogametic sex The gender that carries two different sex chromosomes, such as XY mammalian males and WZ avian females.

Heterozygosity State of a genetic marker (or any locus) at which both alleles are different.

High-density panel (HDP) SNPs on a bead array, typically containing > 500 000 SNPs.

Homogametic sex The gender that carries two identical sex chromosomes, such as XX mammalian females and ZZ avian males.

Homozygosity State of a marker (or any locus) at which both alleles are identical.

Identity by descent (IBD) Identical genotypes of a given marker in two or more individuals because the individuals descend from the same ancestor.

Identity by state (IBS) Identical genotypes of a given marker in two or more individuals of a population.

Imputation Interpolation of greater-density genotypes (or full sequences) for a given (group of) individual(s) on the basis of low-density data for the same individuals and greater-density data for a panel of related individuals.

indels Acronym for “inserts and/or deletions” of small amounts of sequence (< 50 base pairs) in the genome. Larger insertions and deletions are usually considered to be “structural variation.”

Introgression Movement of a particular allele or set of alleles from one population (i.e. breed) to another, usually by either deliberate crossbreeding or casual contact between neighbouring populations.

Competitive allele specific polymerase chain reaction (KASP) A unique competitive allele-specific PCR combined with a fluorescence-based reporting system for the identification of genetic variation at the nucleotide level, including SNPs or insertions/deletions.

Kinship The probability that a randomly selected allele from two individuals (at the same locus) is identical by descent from a common ancestor (also known as the “coancestry” or “coefficient of coancestry”).

Linkage disequilibrium (LD) Distribution of multi-locus genotype combinations in a population for a given pair of markers that is incompatible with independent inheritance, thus indicating genetic linkage of the loci.

Linkage disequilibrium (LD) pruning Removal of SNP genotypes from a genome-wide SNP dataset in such a way that there is no LD between any pair of SNPs that surpasses a user-given threshold.

Linkage disequilibrium (LD) block Region of the genome with high linkage disequilibrium (also known as a “haplotype block”).

Locus A distinct region of DNA (often a gene) in the genome.

Meiosis Cell division in the germline leading to haploid sperm or oocyte cells.

Microsatellite Tandem DNA repeat of a 2 to 5 base pair unit. In most cases, the repeat unit is the dinucleotide CA. The number of repeats of a given microsatellite locus is often polymorphic within populations, in which case the microsatellite may serve as a genetic marker. Also known as STR (simple tandem repeat) or SSR (simple sequence repeat).

Minor allele frequency (MAF) A metric primarily used to evaluate SNPs, corresponding to the frequency of the less common of the two alleles (SNPs are usually biallelic). A threshold of $MAF \geq 0.01$ is sometimes considered to define a SNP. Genetic variability and information content of a SNP increases as MAF approaches 0.50 (i.e. the maximum value), and MAF is typically among the criteria for the selection of SNPs in commercial panels.

Mitochondrial DNA (mtDNA) The DNA contained in mitochondria, which is widely used in phylogenetic studies because of its variability, lack of recombination and maternal inheritance.

Neighbour-joining (NJ) tree Phylogenetic tree constructed on the basis of a genetic-distance matrix following the principle of identifying pairs of operational taxonomic units (OTUs = neighbours) to minimize the total branch length at each stage of clustering of the OTUs starting with a starlike tree.

Nucleotide Any of the four types of molecules that make up the structural units of DNA (and RNA). For DNA, these molecules are adenine, cytosine, guanine and thymine and are often denoted by their first letter (A, C, G, and T, respectively).

Oligonucleotide A short strand of DNA (commonly referred to as an “oligo”).

Outgroup A more distantly related populations of animals that serves as a reference group for a comparison of similar populations.

Phylogeny Evolutionary history of a taxonomic group in terms of successive divergence events and mutations or genetic drift.

PLINK A popular and powerful software package (an open-source C/C++ WGAS tool set) for handling and analysing large SNP datasets.

Polymorphism The presence of at least two different genetic variants or alleles at a given locus.

Primer An oligonucleotide that serves as a starting point for DNA synthesis in PCR. The sequences of the primers define the start and the end of the DNA segment to be amplified (based on complementarity of the base pair sequences) and during the PCR reaction bind on the template DNA (often genomic DNA).

Principal component analysis (PCA) A method for analysis of a set of variables, such as allele frequencies, by calculation of a new set of statistically independent coordinates that each corresponds to a weighted combination of the original variables in such a way that each coordinate captures as much variation in the original variables as possible. In many datasets, a small number of coordinates may explain a large proportion of the initial variability, thus increasing efficiency. Plotting the distribution of individuals or breeds in a graph of the first two or three coordinates allows for simple visualization of the pattern of diversity.

Quantitative trait locus (QTL) Locus contributing to the variation in a multigenic quantitative trait (such as milk production or carcass weight).

Read The sequence of an individual segment of chromosomal DNA that is obtained through a next generation sequencing procedure.

Resequencing Whole-genome sequencing approach assembled by mapping the reads to the genome sequence of a reference genome of the same species.

Runs of homozygosity (ROH) Stretches of DNA, typically of 1 to 15 Mb, that are (almost) completely homozygous.

Scaffold Non-contiguous combination of contigs that are positioned related to each other on the basis of paired ends, mate pairs, optical mapping or other long-range assembly method.

Selection signature Locus with a deviating pattern of diversity, such as a local high fixation index value in comparison of breeds or a high frequency of a homozygous haplotype within a breed, that can be explained by selection acting on the locus.

Selective sweep Rapid reduction of variability in the DNA sequence flanking a given locus, due to selection for one particular favourable allele at that locus.

Sex chromosomes Chromosomes for which the number of copies per cell (in diploid cells, normally two) depends on the sex, such as the mammalian X chromosome (two in females, one on males), the mammalian Y chromosome (one in males, zero in females), the avian W chromosome (zero in males, one in females) and the avian Z chromosome (two in males, one in females).

Single nucleotide polymorphism (SNP) Variation resulting from a point mutation and most often corresponding to a biallelic (having two different alleles) marker.

Sliding window A concept associated with the application of a given statistical test to a fixed-length segment of the genome (typically measured in either number of SNP or base pairs), which is repeatedly applied across the entire genome. The location of the “window” is progressively advanced by a chosen number of SNP or base pairs, and the location yielding the highest test of significance is assumed to contain the genes responsible the effects for which the test is being applied.

Structural variation DNA sequence variation based on copy number variations (CNV), that is, deletions, duplications and large-scale copy number variants, and on insertions, inversions and translocations.

TaqMan assay A PCR-based SNP genotyping assay that uses TaqMan® 5'-nuclease chemistry for amplifying and detecting specific polymorphisms in purified genomic DNA samples. Each assay allows genotyping of individuals for a SNP. The PCR includes two probes, each specific for one of the two SNP alleles and each carrying a different fluorescent reporter (e.g. VIC or 6-FAM dye), bind to one of the strands of the PCR product depending on the alleles during amplification. A bound probe is degraded by the exonuclease activity of Taq polymerase to generate an allele-dependent fluorescent signal, allowing the discrimination of two different homozygous alleles, and also the differentiation of homozygous from heterozygous alleles.

Transcriptomics The study of all RNA molecules present in a given sample. This may include only the messenger RNA (mRNA) which are translated to proteins and non-coding RNA.

Appendix 2

Example Material Transfer Agreement¹

MATERIAL TRANSFER AGREEMENT (MTA) for genetic material for genotyping

This Material Transfer Agreement is made by and between,

Name of provider of genetic material ("Provider")

Mailing Address

Other contact information – i.e. telephone and fax numbers, email address

and

Name of recipient of genetic material ("Recipient").

Mailing Address

Other contact information – i.e. telephone and fax numbers, email address

The parties have agreed as follows:

1. Provider agrees to transfer to Recipient the following (biological) material ("Material"):
Description of the genetic material including type (e.g. DNA, blood, tissue) amount (i.e. number of samples) and other information (e.g. means of preservation)

¹ Example kindly provided by the International Livestock Research Institute (ILRI), Kenya, Nairobi.

2. This Material will be used by Recipient solely in connection with the project described as follows:
Description of the project ("Research Project"), including assays to be performed (e.g. genomic characterization using a panel of SNPs), use of the data, context in a larger project and project sponsors.
3. This Material will only be used for research purposes by the Recipient in its laboratory. By requesting the material and signing this agreement, the Recipient is considered responsible for appropriate handling of the material and guarantees that suitable containment conditions are available and will be applied in the Recipient's laboratory. This Material will not be used for commercial purposes, such as the production or sale of products or services. Recipient will promptly, after termination of the Research Project, inform Provider of the results of the Research Project.
4. To the extent permitted by law, Recipient agrees to treat in confidence, for a period of XXXX years from the date of its disclosure, any of the Provider's written information about this Material that is stamped "CONFIDENTIAL" (hereinafter "Confidential Information"), except for information that was previously known to Recipient or that is or becomes publicly available through no fault of Recipient or which is lawfully disclosed to Recipient without a confidentiality obligation or that is independently developed by Recipient or its affiliated entities without the benefit of any disclosure by Provider. Recipient may publish or otherwise publicly disclose the results of the Research Project, provided that in all such oral presentations or written publications concerning the Research Project, Recipient will acknowledge Provider's contribution of this Material unless otherwise requested by Provider.
5. This Material is considered proprietary to Provider. Recipient therefore agrees to retain control over this Material, and further agrees not to transfer the Material to other parties not under its supervision without prior written consent of Provider. Provider reserves the right to distribute the Material to others and to use it for its own purposes. When the Research Project is completed, the Material will be disposed of as mutually agreed upon by Provider and Recipient.
6. This Material IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the Material will not infringe any patent or proprietary rights of third parties. Recipient agrees to hold harmless and indemnify Provider for all liabilities, demands, damages, expenses and losses arising out of or as a result of Recipient's use of the Material for any purpose.
7. Nothing in this Material Transfer Agreement shall or may be construed as granting Recipient any right or license to the Material for any use other or further than the evaluation described here above.

8. This Agreement shall be governed and construed in accordance with the laws of *(the country where the 'Research Project' was conducted)*. All disputes arising out of or in connection with this Agreement shall be settled in first instance by the relevant court of *(the country where the 'Research Project' was conducted)*.

RECIPIENT

Place: _____ Date: _____

By: _____ Title: _____

PROVIDER

Place: _____ Date: _____

By: _____ Title: _____

Appendix 3

Sampling of blood for DNA

DESCRIPTION

This protocol describes how the collection of blood from the jugular vein into a vacutainer EDTA containing tubes should be organized. The actual sampling should be done by a veterinarian or someone with comparable qualifications valid in the country where the sampling is done.

PERSONNEL

Qualified sampler (such as a veterinarian), and ideally at least two assistants (one to hold the animal and another to assist with blood sampling and recording)

NON-VETERINARY EQUIPMENT

Labelled tubes for blood samples

Marker pen with permanent ink

Box or other container to store and transport the samples

Notebook, a pen and a permanent marker for documentation

Digital camera

TO BE DONE BY THE ASSISTANTS

- Obey strictly instructions of the owner or his representative regarding hygienic precautions and allowable distance to the animals.
- Photograph the animal, especially relevant morphological traits and, if present, the labels with registration number.
- After receiving the tube, gently invert the tube 4–5 times to mix blood with EDTA and use the marker pen to write the animal ID, breed and date on the tube. Keep the tube at ambient temperature, but do not expose to the sun or other strong light. Preferably, extract DNA within a few days.

Remember to collect as much information about the animal as possible: owner of the animal, animal ID, breed, sampling site and date, age of the animal, animal origin and pedigree information as well as known short description of appearance, major diseases (or lack of them) or other observations by the owner (see Appendix 4).

Appendix 4

Example questionnaire to be filled during sampling

Questions in **bold** are generally considered to be mandatory

Animal code _____

Farm ID _____

Species code _____

AA = *Anser anser domesiticus* (greylag goose)

AC = *Anser cygnoides* (swan goose)

AP = *Anas platyrhynchos* (mallard duck)

BB = *Bubalus bubalis* (water buffalo)

BF = *Bos frontalis* (gayal)

BG = *Bos grunniens* (yak)

BI = *Bos indicus* (zebu)

BJ = *Bos javanicus* (banteng, Bali cattle)

BT = *Bos taurus* (taurine cattle)

CB = *Camelus bactrianus* (Bactrian camel, two-humped)

CD = *Camelus dromedarius* (dromedary, one-humped camel)

CH = *Capra hircus* (goat)

CM = *Cairina moschata* (muscovy duck)

EA = *Equus asinus* (donkey)

EC = *Equus caballus* (horse)

GG = *Gallus gallus* (chicken)

LG = *Lama glama* (llama)

MG = *Melea gallopavo* (turkey)

OA = *Ovis aries* (sheep)

SS = *Sus Scrofa* (pig)

VP = *Vicugna pacos* (alpaca)

VV = *Vicugna vicugna* (vicuña)

Species name _____

Country _____

Number of the sample _____

Official Animal ID (if available) _____

Animal and sampling information

Sex of animal: female male

Year of birth of the animal: _____ (YYYY)

Place (locality) of birth of the animal: _____

Date of collection: _____ (DD.MM.YYYY)

Breed's full name: _____

Collector's name: _____

Collector's institution: _____

Address of the farm and telephone number (if available)

Country of the farm: _____

Province/county of the farm: _____

Region of the farm: _____

Closest town to the farm: _____

International phone code: _____ (4 digits – e.g.: 0033, 0041, ...)

Area phone code: _____

Phone number: _____

Type of biological material: blood tissue hair

other (specify) _____

GPS coordinates _____

Appendix 5

Breed questionnaire

To be completed once per breed – complete all questions that are relevant and for which information is available)

Common name of breed: _____

Name of species: _____

Transboundary or brand name			
Local breed name			
Main location			
Breed society?	Circle: <input type="checkbox"/> Yes <input type="checkbox"/> No	Year established:	
Description of origin and development			
Population size	Year:	No. of animals:	
N° of reproductive animals	Males in natural service		
	Males used for AI		
	Breeding females		
	Trend in breeding females	<input type="checkbox"/> Increase <input type="checkbox"/> Steady <input type="checkbox"/> Decrease	
Females mated pure (%)			
Adult size (male/female)	Withers height (cm)	M	F
	Live weight (kg)		
N° of farmer/breeders			
Main uses (e.g. meat, milk)			
Typical management conditions	Type		
	Housing		
	Feeding		
Conservation activities	<i>In situ</i> : Y / N	<i>Ex situ</i> : Y / N	Cryo: Y / N
Performance comparison	Relative to which breed:		
much higher in:	(e.g. milk yield)		
higher in:			
equal in:			
lower in:			
much lower in:			

Appendix 6

Software for genomic analysis

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This is a (*incomplete*) list of genetic analysis programs with a short specification, grouped according to purpose. Although the software are grouped, many of them can be applied to multiple purposes. Most programs can be downloaded freely from the internet, along with detailed instruction manuals. If not given in this list, details, literature references and URL of programs can be found by search engines. An internet search using the program name as the keyword may also be done if the links given in this appendix are no longer functional. Appearance on this list does not constitute an endorsement of the software by the Food and Agriculture Organization of the United Nations.

SOFTWARE FOR MANAGING AND/OR ANALYSING LARGE SNP DATASETS

AlphaPeel – software package for calling, phasing, and imputing genotype and sequence data in pedigree populations: <https://github.com/AlphaGenes/alphapeel>

AlphaFamImpute – genotype calling, phasing, and imputation algorithm for large full-sib families in diploid plants and animals which supports individuals genotyped with SNP array or GBS data: <https://github.com/AlphaGenes/alphafamimpute>

ANGSD – utility program for low to medium coverage WGS data: www.popgen.dk/angsd/index.php/ANGSD

diveRsity – R package (www.r-project.org) that calculates a range of genetic diversity statistics: <https://cran.r-project.org/web/packages/diveRsity/index.html>

KING v2.2.6 – toolset to explore family relationship inference and pedigree error checking, quality control and population substructure identification in genome-wide data: www.kingrelatedness.com

PLINK 1.8 & 2.0 – clearly documented high-density SNP handling and analysis program; requires special format; outputs to formats compatible with many other applications; performs a variety of data handling operations and calculations, such as allele-sharing between individuals and coordination analysis: www.cog-genomics.org/plink

SOFTWARE FOR HANDLING GENOMIC DATA

Samtools – suite of programs for working with high-throughput sequencing data, particularly data stored in SAM format files: www.htslib.org

VCftools – utility program for working with VCF files (Variant call format, a common system for storing of genetic variation information): <https://vcftools.github.io/index.html>

GENOTYPE BY SEQUENCING TOOLS

DECONVQC – repository that contains scripts used to process and manage output sequence data and metadata related to Illumina HiSeq and MiSeq machines, including running a number of GBS-specific Q/C steps for predominantly GBS-related sequencing output; this project is focused on immediate upstream Q/C and sequence delivery, rather than custom downstream analyses: <https://github.com/AgResearch/DECONVQC>

KGD – kinship (genetic relatedness) using GBS (genotyping-by-sequencing) with depth adjustment: <https://github.com/AgResearch/KGD>

TASSEL – software package used to evaluate traits associations, evolutionary patterns, and linkage disequilibrium; it can work with genotyping-by-sequencing data: www.maizegenetics.net/tassel

See also a range of software described in tutorials for analysis of low-coverage whole genome sequencing data: <https://github.com/nt246/lcwggs-guide-tutorial>

PHASING AND IMPUTATION

AlphaImpute – software that combines heuristics and HMM to perform both family-based and population-based imputation; requires phased data, but presents the AlphaPhase algorithm embedded into the software for phasing: <https://alphagenes.roslin.ed.ac.uk/wp/software-2/alphaimpute/>

AlphaPhase – software that performs genotype phasing and minor imputation through a heuristic algorithm that employs the concepts of surrogate parents and Erdős distance: <https://alphagenes.roslin.ed.ac.uk/wp/software-2/alphaphase/>

Beagle v4.1 – (current v5.1 does not handle genotype likelihood input from sequencing) – pedigree-free imputation of genotyping-by-sequencing data: https://faculty.washington.edu/browning/beagle/b4_1.html

Eagle – highly efficient and fast genotype phasing and minor imputation software that uses an HMM-based algorithm combined with a positional Burrows-Wheeler transform: <https://alkesgroup.broadinstitute.org/Eagle/>

fastPhase – performs genotype phasing and minor imputation using HMM fitted via an EM algorithm: <http://scheet.org/software.html>

Fimpute – implements a deterministic algorithm that performs both family-based and population-based imputation; does not require phased data: <https://animalbiosciences.uoguelph.ca/~msargol/fimpute/>

GLIMPSE – phasing and imputation method for large-scale low-coverage sequencing studies: <https://odelaneau.github.io/GLIMPSE>

IMPUTE – fast population-based imputation through HMM and positional Burrows-Wheeler transform; requires phased data: <https://jmarchini.org/software/#impute-5/>

Minimac – population-based imputation using HMM with state space reduction; requires phased data: <https://genome.sph.umich.edu/wiki/Minimac4>

SHAPEIT – fast genotype phasing and minor imputation via HMM and positional Burrows-Wheeler transform: <https://odelaneau.github.io/shapeit4/>

STITCH – R package for Sequencing To Imputation Through Constructing Haplotypes: <https://github.com/rwdavies/STITCH>

GENETIC DISTANCES, TREES AND PLOTS

Beast – performs Bayesian MCMC analysis of molecular sequences, inferring rooted, time-measured phylogenies using strict or relaxed molecular clock models; provides a framework for testing evolutionary hypotheses without conditioning on a single tree topology: http://beast.bio.ed.ac.uk/Main_Page

Mega – calculation of a wide variety of population genetics statistics and convenient tree reconstruction program by the most common algorithms except the Bayesian method: www.megasoftware.net/

MrBayes – command-line operated for handling nexus sequence files for Bayesian tree reconstructions: <https://nbisweden.github.io/MrBayes/>

neighborNet – R package for construction of phylogenetic networks based on the Neighbour-Joining algorithm: www.rdocumentation.org/packages/phangorn/versions/2.5.5/topics/neighborNet

Network – constructs Median-joining networks of haplotype data; generates evolutionary trees and networks from genetic and other data: www.fluxus-engineering.com/sharenet.htm

Netview – R package for network visualization of individuals of breeds using maximally k nearest neighbours: <https://github.com/esteinig/netview>

Phylip – classical command-line comprehensive package requiring its own file format for tree reconstruction according to the most common algorithms, but offering fewer options than PAUP: <http://phylip.com>

QPTools – package within **AdmixTools** <https://github.com/DReichLab/AdmixTools> (or <https://github.com/uqrmaie1/admixtools>) and **ADMIXTUREGRAPH** an R package https://github.com/mailund/admixture_graph; optimize the branch lengths and admixture proportions from Admixture graphs.

Relate – estimates genome-wide genealogies in the form of trees, based on inferred haplotypes: <https://myersgroup.github.io/relate/>

SNAPP – uses a likelihood-based approach to estimate the “true” phylogenetic tree of populations based on the genetic variation of markers across the genome; built upon the Beast software: www.beast2.org/snapp

SplitsTree – constructs neighbour-joining tree, SplitsTree graphs and NeighborNet graphs; accepts Nexus files; many graphical output options: www-ab.informatik.uni-tuebingen.de/software/splitstree4/welcome.html

Treemix – constructs maximum-likelihood trees based on the covariance of allelic frequencies; attempt to infer the number of admixture events among the populations: <https://speciationgenomics.github.io/Treemix>

Tsinfer – develops a tree sequence based on inferred haplotypes: <https://github.com/tskit-dev/tsinfer>

PRINCIPAL COMPONENT ANALYSIS

Eigensoft – analyses population structure by combining statistical genetics with principal components analysis (**Eigenstrat**) to explicitly model ancestry differences between cases and controls along continuous axes of variation: <https://reich.hms.harvard.edu/software>

POPULATION ASSIGNMENT AND CLUSTER ANALYSIS

Admixture – produces results for unsupervised clustering (i.e. without prior population information) comparable or identical to **Structure**, but faster by means of a more efficient algorithm: <https://dalexander.github.io/admixture/>

Baps – comparable to **Structure** but with increased flexibility in the definitions of levels at which genetic structure may exist: <https://github.com/ocbe-uio/rBAPS> or <https://swmath.org/software/35649>

Clumpp – accepts the output of **Structure** or other clustering programs in order to align the output of different runs: <https://rosenberglab.stanford.edu/clumpp.html>

Distruct – reads in tables of genomic components from the **Structure** output and files of options set by the user in order to provide graphical output of the Structure clustering: <https://rosenberglab.stanford.edu/distruct.html>

fineStructure – clustering program on the basis of ChromoPaint-based haplotype sharing: https://people.maths.bris.ac.uk/~madjl/finestructure/finestructure_info.html

Frappe – estimates individual ancestry proportions – comparable to Admixture: <https://med.stanford.edu/tanglab/software/frappe.html>

Geneland – clustering program that can make use of both geographic and genetic information to estimate the number of populations in a dataset and delineate their spatial organization: <https://rdr.io/cran/Geneland/> or <https://i-pri.org/special/Biostatistics/Software/Geneland>

Instruct – joint inference of population structure and inbreeding rates, eliminating the assumption of Hardy-Weinberg equilibrium and especially applicable in cases of self-fertilization or inbreeding: <http://cbsuapps.tc.cornell.edu/InStruct.aspx>

LEA – R package, estimates admixture proportions similar to Structure or Admixture software, but with reduced computation time; less affected by the inbreeding of the analysed populations: <http://membres-timc.imag.fr/Olivier.Francois/LEA/software.htm>

MixMapper – refines an existing hierarchical tree of breeds with additional, admixed populations. Optimizes topology, sources of gene flow and branch lengths: <http://cb.csail.mit.edu/cb/mixmapper/>

Spaida and Spaign – assigns individual animals to genetic clusters based on spatial autocorrelations: <http://notendur.hi.is/~snaebj/programs.html>

Structure – popular and user-friendly program for an informative visualization of patterns of diversity. Reconstructs model-based subdivision of individual genotypes into a user-specified number of clusters (k) by optimizing of Hardy-Weinberg equilibrium and minimizing linkage disequilibrium within clusters; estimates proportion of individual genomes derived from the inferred clusters. Clusters may correspond to ancestral components, but also to a relatively homogeneous breed or group of breeds. Optionally allows for admixture, linkage between markers, codominant markers, incorporation of prior population information and incorporation of spatial information. It is substantially slower than Admixture but has more options: <https://web.stanford.edu/group/pritchardlab/structure.html>

Whichrun – assigns individuals to populations based upon maximum likelihood theory: <https://whichrun.software.informer.com/4.1/> or <https://mybiosoftware.com/tag/whichrun>

IDENTIFICATION OF SELECTION SIGNATURES

cgaTOH – detection of intra-population selection signatures based on runs of homozygosity: <https://github.com/hernanmd/cgaTOH.2018>

HapFLK – implements the Lewontin and Krankakauer tests to identify inter-population signatures based on individual loci (FLK) or haplotypes (hapFLK): <https://pypi.org/project/hapflk/>

HierFstat – R package for detection of inter-population selection signatures based on fixation indexes (F_{ST}): <https://cran.r-project.org/web/packages/hierfstat/index.html>

PCAdmix – estimation of local ancestry by applying PCA based upon phased haplotypes: <https://bio.tools/pcadmix>

rehh – performs tests based on extended haplotype homozygosity to detect genomic regions subject to selection within and across populations: <http://cran.r-project.org/web/packages/rehh/index.html>

Sweed – identification of intra-population selection signatures based on the site frequency spectrum: <https://cme.h-its.org/exelixis/web/software/sweed/>

Sweep – large-scale intra-population analysis of haplotype structure in genomes for the primary purpose of detecting evidence of natural selection: <https://software.broadinstitute.org/mpg/sweep/>

Selscan – detection of intra-population selection based on extended haplotype homozygosity (EHH) and integrated haplotype score (iHS) methods; detection of inter-population selection based on cross population extended haplotype homozygosity (XP-EHH): <https://github.com/szpiech/selscan>

LANDSCAPE GENOMICS

LFMM2 – applies latent factor mixed models to test for association between genotypes and explanatory variables that can include environmental or geographical measures: <https://bcm-uga.github.io/lfmm/>

BAYENV – applies Bayesian method to estimate the pattern of covariance in allele frequencies between populations and then uses this as a null model for a test of environmental variables at individual SNPs: https://bitbucket.org/tguenther/bayenv2_public/

BAYPASS – identifies genetic markers subjected to selection or associated with population-specific covariates, including geographical and environmental variables: www1.montpellier.inra.fr/CBGP/software/baypass/index.html

BAYESCENV – performs F_{ST} -based genome scans to detect local adaptation: <https://github.com/devillemereuil/bayescenv>

R.SamBada – R-package providing a pipeline for landscape genomic analysis, spanning from the retrieval of environmental variables at sampling locations to gene annotation using the Ensembl genome browser. This application standardizes the landscape genomics pipeline, eases the search for candidate genes involved in adaptation processes, and enhances reproducibility of the studies: <https://github.com/SolangeD/R.SamBada>

GENOME-WIDE ASSOCIATION STUDIES

bayesR – Bayesian hierarchical model for complex trait analysis, including GWAS: <https://github.com/syntheke/bayesR>

BOLT – applies linear mixed-models for genetic association testing; includes an application that applies an algorithm for restricted maximum-likelihood estimation of variance components: <https://alkesgroup.broadinstitute.org/BOLT-LMM/>

EMMAX – performs large-scale GWAS while correcting for population sampling structure: <https://genome.sph.umich.edu/wiki/EMMAX>

FaST-LMM – genome-wide association testing, genomic prediction, heritability analysis: <https://github.com/fastlmm/FaST-LMM/>

GCTA – estimates genomic relationships, performs association testing, heritability analysis and variance partitioning: <https://cnsgenomics.com/software/gcta>

GCTB – a collection of Bayesian linear mixed models for complex trait analyses using genome-wide SNP data; simultaneously estimates joint effects of the SNP and the background genetic architecture: <https://cnsgenomics.com/software/gctb>

GEMMA – applies linear mixed models for association testing, multi-trait analyses and heritability analysis: <https://github.com/genetics-statistics/GEMMA>

JWAS – performs routine single-trait and multi-trait genomic prediction and GWAS using Bayesian mixed effects models and either complete or incomplete genomic data: <https://reworkhow.github.io/JWAS.jl/latest/>

LFMM2 – fits latent factor mixed models, estimating factors based on an exact least squares approach: <https://rdrr.io/bioc/LEA/man/lfmm2.html> (also suitable for landscape genomics)

SPECIAL PURPOSE PROGRAMS

AlphaAssign – parentage assignment algorithm that works with SNP array and GBS data: <https://github.com/AlphaGenes/alphaassign>

Rannala software – multiple packages that perform various specific functions, including LD mapping, data simulation and detecting migration by using multilocus genotypes: www.rannala.org

SNeP – performs estimations of effective population size trajectories over time by using genome-wide SNP data: <https://sourceforge.net/projects/snepnetrends>

SPAGeDi – characterizes the spatial genetic structure of individuals or populations based on genetic marker data; estimates genetic distance and other basic statistics: <http://ebe.ulb.ac.be/ebe/Software.html>

SIMULATION, MODELLING AND PARAMETER ESTIMATION

ABC and **ABCRF** – R packages that perform approximate Bayesian computation (ABC) for model selection and parameter inference, the latter by utilizing random forests: <https://cran.r-project.org/package=abc> and <https://rdrr.io/cran/abcrf>

ABCtoolbox – a series of open-source programs that performs all of the steps of a standard ABC analysis; offers the user the opportunity to choose among several algorithms: <https://bitbucket.org/wegmannlab/abctoolbox/wiki/Home>

dadi – simulates the joint frequency spectrum of genetic variation among several populations: <https://dadi.readthedocs.io/en/latest/>

FastSimcoal2 – flexible modelling and simulation software that allows comparison of alternative tree construction models: <http://cmpg.unibe.ch/software/fastsimcoal2/>

PSMC and **MSMC** – R packages for implementing the pairwise sequentially Markov coalescent and multiple sequential Markov coalescent methods for modelling population histories: <https://github.com/lh3/psmc> and <https://github.com/stschiff/msmc>

MULTIPURPOSE PROGRAMS ORIGINALLY DEVELOPED FOR MICROSATELLITE DATA

GenAlEx – estimates of variability based on allele and genotypic frequencies, genetic distances, Principal Component Analysis, formatting of data for other software; runs as a Microsoft Excel add-in. <https://biology-assets.anu.edu.au/GenAlEx/Welcome.html>

Genepop – undertakes simple population genetic analyses including tests for Hardy-Weinberg equilibrium, estimation of LD, and population differentiation: <https://genepop.curtin.edu.au/>

Annex 7

Genotyping arrays available by species across different genotyping platforms²

Kathiravan Periasamy, International Atomic Energy Agency, Vienna, Austria

Species	Platform	Array name	Marker density	SNPs (N)	SNP ascertainment and/or validation breeds/populations	Special features/potential applications
Buffalo	Affymetrix Axiom	Buffalo Genotyping Array	Medium	89 988	4 breeds (Mediterranean buffalo, Murrah, Jaffarabadi and Nili-Ravi)	Useful for biodiversity research, GWAS
Camelid – Bactrian	Affymetrix Axiom	Camelid-Multispecies Array	Medium	59 938	Bactrian populations from Mongolia and China	Useful for biodiversity research, GWAS, mapping studies
Camelid – Dromedary				59 958	Dromedary populations from Algeria, Ethiopia, Mauritania, Morocco, Sudan, United Arab Emirates	Useful for biodiversity research, GWAS, mapping studies
Camelid – New World				60 000	Alpaca (Huacaya and Suri), Llama and Vicugna populations from Peru, Chile, Bolivia (Plurinational State of), Ecuador	Useful for biodiversity research, GWAS, mapping studies
Cattle	Illumina Infinium	Bovine LD v2.0-Genotyping Beadchip	Low	7 931	26 breeds (Angus, Ayrshire, Beefmaster, Blonde d' Aquitaine, Brahman, Brown Swiss, Charolais, Fleckvieh, Friesian, Gelbvieh, Guernsey, Hereford, Holstein, Jersey (United States of America & Denmark), Limousin, Montbeliard, N'dama, Normande, Norwegian Red, Red Angus, Red Dairy, Red Danish (Denmark, Finland, Sweden), Santa Gertrudis)	Useful for genomic selection, genotype imputation, parentage verification, biodiversity research; includes 121 parentage markers
Cattle	Illumina Infinium	Bovine SNP50v3-Beadchip	Medium	53 218	17 breeds (Angus, Beefmaster, Gir, Nelore, Brahman, Charolais, Guernsey, Hereford, Holstein, Jersey, Limousin, N'dama, Piedontese, Red Angus, Romagnola, Santa Getrudis, Sheko)	Useful for genomic selection, GWAS, parentage verification, biodiversity research; includes 116 parentage markers
	Affymetrix Axiom	Bovine	Medium	54 560	10 breeds (Holstein, Angus, Jersey, Fleckvieh, Hereford, Limousin, Romagnola, Brahman, Nelore, Gir)	Useful for biodiversity research, GWAS, parentage verification; consists of 191 ISAG core markers for bovine parentage

(Cont.)

² Appearance on this list does not constitute an endorsement of the platform or array by the Food and Agriculture Organization of the United Nations.

Species	Platform	Array name	Marker density	SNPs (M)	SNP ascertainment and/or validation breeds/populations	Special features/potential applications
Cattle	Affymetrix Axiom	BovMDv3 Array	Medium	63 648	20 breeds; <i>Bos taurus</i> (Afrikander, Angus, Ayrshire, Boran, Blonde d'Aquitaine, Brown Swiss, Simmental, Hanwoo, Hereford, Holstein, Japanese Black, Jersey, Limousine, Norwegian Red, Rouge des Pres, Romagnola, Tuli); <i>Bos indicus</i> (Brahman, Nelore, Gir).	Useful for dairy evaluation (genomic selection), biodiversity research, copy number variations, parentage verification, markers for deleterious recessive traits, fertility traits and traceability; consists of 13K <i>Bos indicus</i> SNPs; includes several hundred SNPs that are optimized for short tandem repeat (STR) imputation
Cattle	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Angus-GS-Beadchip	Medium	75 000	Angus	Breed specific SNP array for breeding and improvement of Angus cattle; includes 22000 new SNP markers with 7800 from Angus sequences that are novel to all available arrays; also includes markers associated with fertility, feed efficiency, marbling, calving ease and vaccine response; has markers for detection of genetic conditions like arthrogyrosis multiplex, neuropathic hydrocephalus contractural arachnodactyly, osteoporosis, oculocutaneous hypopigmentation, developmental duplication, BVDV, M1, D2
	Illumina Infinium	GGP Bovine 100K	Medium	100 000	Derived from the original Illumina BovineHD-Genotyping and Bovine SNP50 Beadchip	Useful for GWAS, genetic evaluations, biodiversity research and parentage verification; has 85 percent overlap with the Council of Dairy Cattle Breeding (CDCB) virtual evaluation and 35 000 SNPs overlap with the Canadian Dairy Network (CDN) Evaluation; average imputation accuracy to the Illumina Bovine HD is > 99.5 percent in Angus and Holstein populations
Cattle	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Bovine 150K	Medium	150 000	NA	Useful for GWAS, genetic evaluations, biodiversity research, parentage, detection of markers for recessive disorders including fetal death and abnormalities that interfere with growth rate in cattle
	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Bovine-F250	High	235 000	NA	Useful for functional genomic studies in cattle, genomic selection, genotype imputation; includes SNPs enriched for functional variants such as non-synonymous, frameshift and premature stop codons

(Cont.)

Species	Platform	Array name	Marker density	SNPs (M)	SNP ascertainment and/or validation breeds/populations	Special features/potential applications
Cattle	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Bovine 150K	Medium	150 000	NA	Useful for GWAS, genetic evaluations, biodiversity research, parentage, detection of markers for recessive disorders including fetal death and abnormalities that interfere with growth rate in cattle
Cattle	Illumina Infinium	BovineHD-Genotyping-Beadchip	High	777 962	20 breeds; <i>Bos taurus</i> (Angus, Bonde d'Aquitaine, Brown Swiss, Charolais, Guernsey, Hereford, Holstein, Jersey, Lagunaire, Limousin, Montbeliard, N'dama, Norwegian Red, Piedontese, Red Angus, Romagnola, Senepol, Simental, Wagyu); <i>Bos indicus</i> (Brahma, Gir, Nelore); Crossbreeds (Beefmaster, Brangus, Santa Gertrudis, Sheko)	Useful for genomic selection, GWAS, QTL identification, biodiversity research, mapping
Cattle – Zebu	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Indicus	Medium	35 090	Brahman, Guzera, Gyr, Nelore, Droughtmaster, Santa Gertrudis, Tropical Composite, etc.	Useful for biodiversity research, GWAS, QTL identification
Chicken	Affymetrix Axiom	ChickenHD Genotyping Array	High	580 961	Commercial broiler (Aviagen), commercial layers (Hyline, Synbreed), Experimental inbred (IAH line), Non-selected (J. Line Roslin)	Useful for genetic evaluation of layers and broilers, GWAS, mapping and biodiversity research; includes markers associated with wild outbred lines
Goat	Illumina Infinium	Goat SNP50-Genotyping-Beadchip	Medium	50 000	Saanen, Alpine, Creole, Boer, Kacang, and Savanna	Useful for biodiversity research, GWAS, genome mapping
	Affymetrix Axiom	Ovicap Mutispecies Array		60 034	Milk and mixed breeds (Alpine, Saanen and Creole); Meat breeds (Boer, Katjang and Savanna); Norwegian dairy goat	Useful for biodiversity research, GWAS, parentage verification, mapping studies; includes 583 markers for parentage testing and traceability
Horse	Illumina Infinium	Equine SNP50	Medium	54 602	15 breeds (Andalusian, Arabian, Belgian, Franches Montagnes, French Trotter, Hanoverians, Icelandic, Mongolian, Norwegian Fjord, Quarter Horse, Saddlebred, Standardbred, Swiss Warmblood, Thoroughbred, Hokkaido)	Useful for biodiversity research, GWAS, genomic selection, identification of QTL, mapping.
	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Equine		71 947	Derived from Illumina's Equine SNP50 Beadchip	Useful for biodiversity research, GWAS, genomic selection, identification of QTL, general mapping
	EquineHD Genotyping Array	Affymetrix Axiom	High	670 796	32 breeds (including Arabian, Belgian, Black Forest, Duelmener, Edlblu-Haflinger, French Trotter, Haflinger, Hanoverian, Icelandic, Lusitano, Marremanno, Mongolian, Morgan, Old-Oldenburger, Quarter Horse, Sorraia, Standardbred, Süddeutsches Kaltblut, Thoroughbred and Welsh)	Useful for biodiversity research, GWAS, association mapping, genomic prediction of disease risk

(Cont.)

Species	Platform	Array name	Marker density	SNPs (M)	SNP ascertainment and/or validation breeds/populations	Special features/potential applications
Multiple cattle, pig, chicken, horse, goat and sheep	Affymetrix Axiom	IMAGE001 Multispecies Array	Low	60 000 total 10 000 per species	Many breeds/per species (from publicly available arrays and project data)	Designed for assessment of gene bank collections, but also useful for biodiversity research, causal mutation known traits, parentage study, ancestral information
Multiple buffalo, rabbit, duck, quail, pigeon and honey bee	Affymetrix Axiom	IMAGE002 Multispecies Array	Low	60 000 total 10 000 per species	Many breeds/per species (from publicly available arrays and project data)	Designed for assessment of gene bank collections, but also useful for biodiversity research, causal mutation known traits, parentage study, ancestral information
Pig	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Porcine50K	Medium	51 000	NA	Useful for biodiversity research, GWAS, parentage verification, evaluation of pure line, identification of multi-line reference populations, genetic evaluation; includes markers for detection of Porcine Stress syndrome and Rendement Napole (RN).
	Affymetrix Axiom	Porcine-Breeder Array		55 150	Same as Axiom-PorcineHD Genotyping Array, but SNP selection was targeted towards commercial pig breeds	Useful for genomic and trait selection, parentage testing; includes 64 ISAG core parentage markers, 42 trait-specific markers selected by USDA ³ and targeted for commercial breeds including Large White, Landrace, Duroc and Piétrain
	Illumina Infinium	Porcine SNP60 v2 Genotyping Beadchip		64 232	Berkshire, Duroc, Hampshire, Landrace, Large White, Meishan, Pietrain, Synthetic (Large White and Pietrain), Wild boar	Useful for biodiversity research, GWAS, genomic selection, identification of QTL, mapping, genetic evaluation, linkage disequilibrium studies
	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Porcine80K		~80 000	NA	Useful for biodiversity research, GWAS, parentage verification, evaluation of pure line, identification of multi-line reference populations, genetic evaluation
Pig	Affymetrix Axiom	PorcineHD Genotyping Array	High	658 692	27 domestic and 16 wild boar populations; Commercial domestic European (Large White, Landrace, Piétrain, Duroc, Hampshire); Traditional/Rare domestic European (Angler Sattelschwein, British Saddleback, Bunte Bentheimer, Casertana, Cinta Senese, Gloucester Old Spot, Large Black, Linderodssvin, Mangalica, Middle White, Negro Iberico, Retinto, Tamworth); European wild boar; Asian domestic (Jiangquhai, Jinhua, Leping Spotted, Meihan, Thai Native, Wannan Spotted, Wuzishan, Xiang, Zang); Asian wild boar (China, Japan, Thailand)	Useful for biodiversity research, GWAS, high resolution genetic mapping, QTL analysis, genomic prediction

(Cont.)

³ U.S. Department of Agriculture.

Species	Platform	Array name	Marker density	SNPs (N)	SNP ascertainment and/or validation breeds/populations	Special features/potential applications
Sheep	Affymetrix Axiom	OvineLD-Genotyping Array	Low	11 196	Belclare, Charollais, Suffolk, Texel, Vendeen	Useful for biodiversity research, GWAS, parentage verification, breed assignment, aneuploidy detection, mating designs, traceability.
	Affymetrix Axiom	Ovine50K-Genotyping Array	Medium	50 000	74 breeds sampled from Asian, African, South-West Asian, Caribbean, North American, South American, European and Australasian sheep	Useful for biodiversity research, GWAS, parentage verification, breed assignment, aneuploidy detection, mating designs, traceability.
	GeneSeek Genomic Profiler (Illumina-Infinium)	GGP Ovine50K		50 000	NA	Useful for biodiversity research, GWAS, genomic selection, identification of QTL, genetic evaluation, linkage disequilibrium studies
Sheep	Affymetrix Axiom	Ovicap Mutispecies Array	Medium	54 236	74 breeds sampled from Asian, African, South-West Asian, Caribbean, North American, South American, European and Australasian sheep	Useful for biodiversity research, GWAS, mapping studies
	Illumina Infinium	Ovine SNP50-Genotyping-Beadchip		54 241	> 70 breeds sampled from Asian, African, South-West Asian, Caribbean, North American, South American, European and Australasian sheep	Useful for biodiversity research, GWAS, genomic selection, genetic evaluation, linkage disequilibrium studies

Annex 8

Bioinformatics pipeline for quality control of genomic data

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Quality control (QC) of large datasets of single nucleotide polymorphisms is an important step to ensure reliable results. Fortunately, a large collection of software has already been developed to aid in this task. This appendix describes the step-by-step application of a “QC pipeline” that combines the use of command line and R commands to manipulate and perform quality checks on binary PLINK formatted data. This pipeline outlines a standard approach to quality check genotype data. However, parameters and QC steps and their order might change when applied to different data or depending on downstream analyses. Finally, although some of the visual checks and simplest calculations might be performed using other statistical tools (e.g. Excel spreadsheet) the use of R is highly recommended due to its scalability.

REQUIREMENTS

The dataset used here is a collection of 60k SNP chip genotype data of Italian goat breeds (Cortellari *et al.*, 2021). The dataset is available in binary PLINK format: Cortellari2021.{bed, bim, fam} at <https://data.mendeley.com/datasets/hnd59x-6gmg/1>

The software used are:

PLINK v1.9 www.cog-genomics.org/plink

PLINK v2.0 www.cog-genomics.org/plink/2.0

R v4.1.0 www.r-project.org

All PLINK commands are executed in the system command line terminal (identified here by a preceding “\$” symbol). R commands require the R console (identified here by a preceding “>” symbol).

The R package `data.table` is required to efficiently load file content in R.

```
> install.packages("data.table")
> library(data.table)
```

QUALITY CONTROL

Missingness

The first step is to determine the rates of missing data both at the individuals and loci level. The `--missing` flag is applied to compute both using one single command. The chromosomal setup (29 autosomes in goats) can be specified using the `--cow` flag (cattle also have 29 autosomes).

```
$ plink --cow --bfile Cortellari2021 --missing --out Cortellari2021
```

FIGURE A8.1
Screenshot following the test for missingness

```
PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021.log.
Options in effect:
  --bfile Cortellari2021
  --cow
  --missing
  --out Cortellari2021

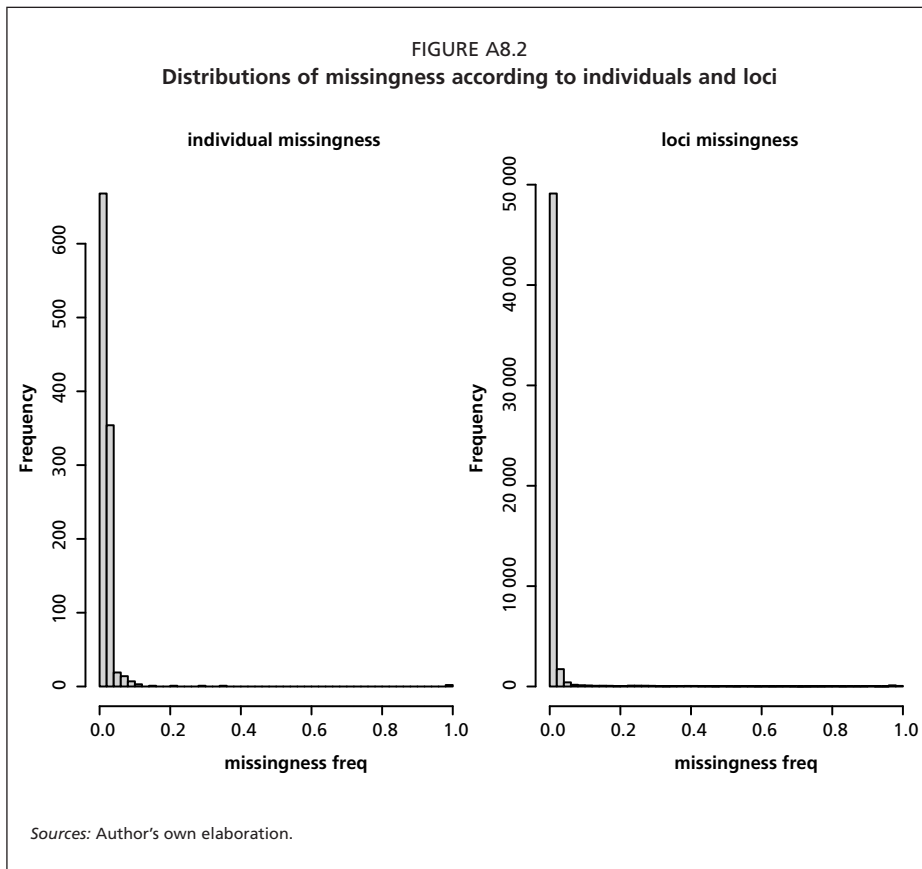
7993 MB RAM detected; reserving 3996 MB for main workspace.
53347 variants loaded from .bim file.
1071 cattle (16 males, 51 females, 1004 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1068 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.977196.
--missing: Sample missing data report written to Cortellari2021.imiss, and
variant-based missing data report written to Cortellari2021.lmiss.
```

Sources: Author's own elaboration.

The command produces two files with extensions, `.lmiss` and `.imiss`, containing loci and individual missingness information for loci and individual animals, respectively.

The missingness distribution is then assessed further by using R:

```
> imiss <- fread("Cortellari2021.imiss", select = 6)
> lmiss <- fread("Cortellari2021.lmiss", select = 5)
> par(mfrow = c(1, 2))
> hist(imiss$F_MISS, xlab = "missingness freq", main = "individual
missingness", breaks = 50)
> hist(lmiss$F_MISS, xlab = "missingness freq", main = "loci
missingness", breaks = 50)
> par(mfrow = c(1, 1))
```



To maximize the number of individuals, first prune for loci missingness, followed by individual missingness. Pruning for missingness can be performed in PLINK using the `--geno` and `--mind` for loci and individuals, respectively.

```
$ plink --cow --bfile Cortellari2021 --geno 0.05 --make-bed --out  
Cortellari2021_g05
```

FIGURE A8.3
Screenshot following pruning for loci missingness

```

PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05.log.
Options in effect:
  --bfile Cortellari2021
  --cow
  --geno 0.05
  --make-bed
  --out Cortellari2021_g05

7993 MB RAM detected; reserving 3996 MB for main workspace.
53347 variants loaded from .bim file.
1071 cattle (16 males, 51 females, 1004 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1068 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.977196.
2226 variants removed due to missing genotype data (--geno).
51121 variants and 1071 cattle pass filters and QC.
Note: No phenotypes present.
--make-bed to Cortellari2021_g05.bed + Cortellari2021_g05.bim +
Cortellari2021_g05.fam ... done.

```

Sources: Author's own elaboration.

```

$ plink --cow --bfile Cortellari2021_g05 --mind 0.05 --make-bed
--out Cortellari2021_g05m05

```

FIGURE A8.4
Screenshot following pruning for individual missingness

```

PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05.log.
Options in effect:
  --bfile Cortellari2021_g05
  --cow
  --make-bed
  --mind 0.05
  --out Cortellari2021_g05m05

7993 MB RAM detected; reserving 3996 MB for main workspace.
51121 variants loaded from .bim file.
1071 cattle (16 males, 51 females, 1004 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05.nosex .
23 cattle removed due to missing genotype data (--mind).
IDs written to cortellari2021_g05m05.irem .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate in remaining samples is 0.997105.
51121 variants and 1048 cattle pass filters and QC.
Note: No phenotypes present.
--make-bed to Cortellari2021_g05m05.bed + Cortellari2021_g05m05.bim +
Cortellari2021_g05m05.fam ... done.

```

Sources: Author's own elaboration.

The missingness statistics are then checked:

```
$ plink --cow --bfile Cortellari2021_g05m05 --missing --out
Cortellari2021_g05m05
```

FIGURE A8.5
Screenshot when checking missingness statistics

```
PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05.log.
Options in effect:
  --bfile Cortellari2021_g05m05
  --cow
  --missing
  --out Cortellari2021_g05m05

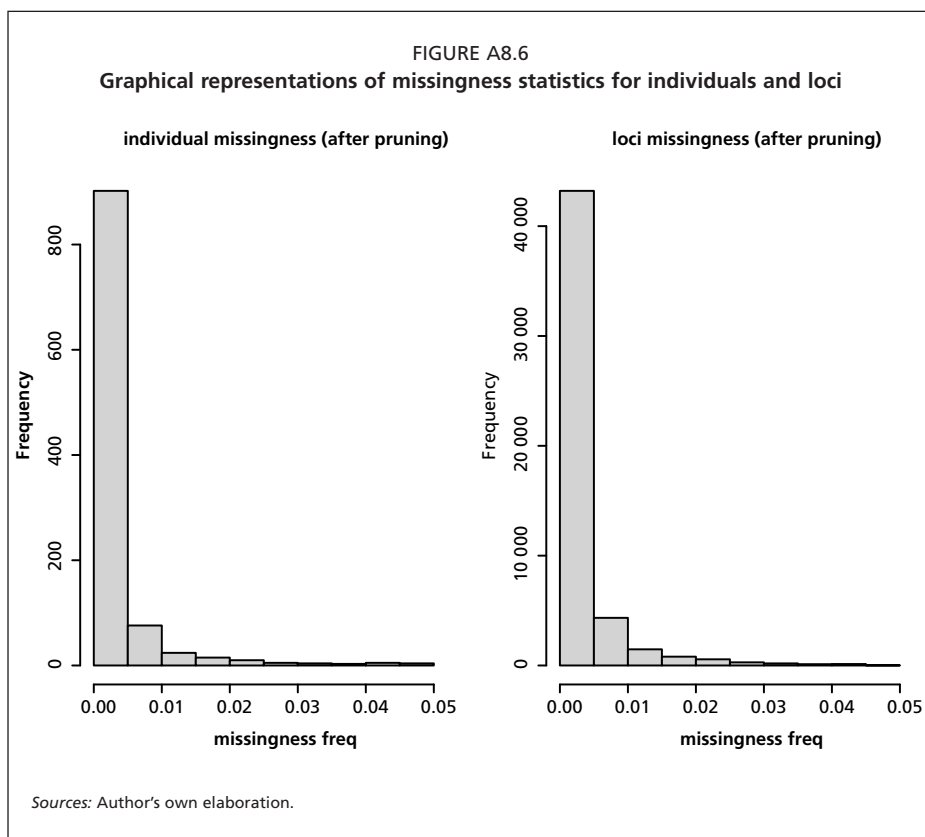
7993 MB RAM detected; reserving 3996 MB for main workspace.
51121 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.997105.
--missing: Sample missing data report written to Cortellari2021_g05m05.imiss,
and variant-based missing data report written to Cortellari2021_g05m05.lmiss.
```

Sources: Author's own elaboration.

Missingness ratios are now negligible; a call rate of ~0.998 was obtained at the cost of only 2 228 loci (from 53 347 to 51 119) and 23 individuals (1 071 to 1 048), comparing Figure A8.4 and Figure A8.5.

Results are then visualized in R:

```
> imiss.pruned <- fread("Cortellari2021_g05m05.imiss", select = 6)
> lmiss.pruned <- fread("Cortellari2021_g05m05.lmiss", select = 5)
> par(mfrow=c(1, 2))
> hist(imiss.pruned$F_MISS, xlab = "missingness freq", main =
"individual missingness (after pruning)")
> hist(lmiss.pruned$F_MISS, xlab = "missingness freq", main =
"loci missingness (after pruning)")
> par(mfrow = c(1, 1))
```



Minor allele frequency

The allele frequency spectrum is then compared using the `--freq` flag in PLINK.

```
$ plink --cow --bfile Cortellari2021_g05m05 --freq --out  
Cortellari2021_g05m05
```


FIGURE A8.7
Screenshot when assessing allelic frequencies

```

PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05.log.
Options in effect:
  --bfile Cortellari2021_g05m05
  --cow
  --freq
  --out Cortellari2021_g05m05

7993 MB RAM detected; reserving 3996 MB for main workspace.
51121 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.997105.
--freq: Allele frequencies (founders only) written to cortellari2021_g05m05.frq.

```

Sources: Author's own elaboration.

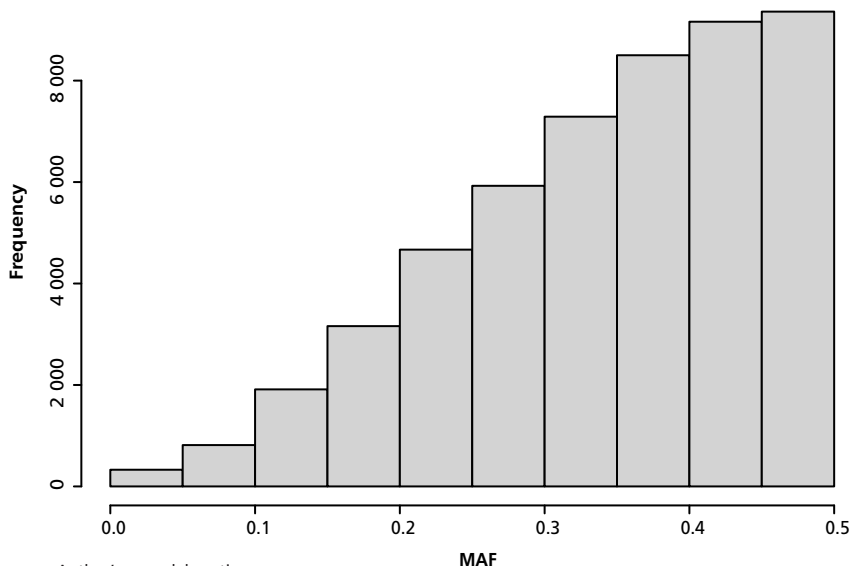
Results are then plotted with R (Figure A8.8).

```

> maf <- fread("Cortellari2021_g05m05.frq")
> hist(maf$MAF, xlab = "MAF", main = "Allele Frequency Spectrum")

```

FIGURE A8.8
Distribution of minor allele frequencies (MAF)
Allele Frequency Spectrum



Sources: Author's own elaboration.

Low frequency variants appear heavily under-represented due to ascertainment bias. We set the MAF threshold at 0.02 following the rule of thumb $10/N$ where N = number of individuals.

```
> round(10/nrow(fread("Cortellari2021_g05m05.fam")), 2)
0.01 (Output)
```

MAF pruning can be performed with the `--maf` flag in PLINK.

```
$ plink --cow --bfile Cortellari2021_g05m05 --maf 0.01 --make-bed
--out Cortellari2021_g05m05f01
```

FIGURE A8.9
Screenshot when pruning based on allelic frequencies

```
PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang  GNU General Public License v3
Logging to Cortellari2021_g05m05f01.log.
Options in effect:
  --bfile Cortellari2021_g05m05
  --cow
  --maf 0.01
  --make-bed
  --out Cortellari2021_g05m05f01

7993 MB RAM detected; reserving 3996 MB for main workspace.
51121 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05f01.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.997105.
151 variants removed due to minor allele threshold(s)
(--maf/--max-maf/--mac/--max-mac).
50970 variants and 1048 cattle pass filters and QC.
Note: No phenotypes present.
--make-bed to Cortellari2021_g05m05f01.bed + Cortellari2021_g05m05f01.bim +
Cortellari2021_g05m05f01.fam ... done.
```

Sources: Author's own elaboration.

Linkage disequilibrium reduction

A dataset with reduced linkage disequilibrium (LD) can be used to feed all those analyses requiring approximate linkage equilibrium. The LD is scanned in sliding windows of 50 SNPs, sliding forward 5 SNPs at each step. The LD for each window is reduced to $r^2 = 0.2$.

```
$ plink -cow -bfile Cortellari2021_g05m05f01 -indep-pairwise 50 5
0.2 -out Cortellari2021_g05m05f01
```

FIGURE A10
Screenshot when pruning based on linkage disequilibrium among loci

```

PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05f01.log.
Options in effect:
--bfile Cortellari2021_g05m05f01
--cow
--indep-pairwise 50 5 0.2
--out Cortellari2021_g05m05f01

7993 MB RAM detected; reserving 3996 MB for main workspace.
50970 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05f01.nosex .
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.997108.
50970 variants and 1048 cattle pass filters and QC.
Note: No phenotypes present.
--indep-pairwise: Ignoring 597 chromosome 0 variants.
Pruned 367 variants from chromosome 1, leaving 2816.
Pruned 266 variants from chromosome 2, leaving 2473.
Pruned 250 variants from chromosome 3, leaving 2051.
Pruned 281 variants from chromosome 4, leaving 2095.
Pruned 217 variants from chromosome 5, leaving 1983.
Pruned 280 variants from chromosome 6, leaving 2078.
Pruned 253 variants from chromosome 7, leaving 1885.
Pruned 243 variants from chromosome 8, leaving 2027.
Pruned 127 variants from chromosome 9, leaving 1711.
Pruned 212 variants from chromosome 10, leaving 1825.
Pruned 248 variants from chromosome 11, leaving 1849.
Pruned 235 variants from chromosome 12, leaving 1475.
Pruned 155 variants from chromosome 13, leaving 1456.
Pruned 230 variants from chromosome 14, leaving 1652.
Pruned 156 variants from chromosome 15, leaving 1447.
Pruned 158 variants from chromosome 16, leaving 1403.
Pruned 114 variants from chromosome 17, leaving 1286.
Pruned 156 variants from chromosome 18, leaving 1119.
Pruned 93 variants from chromosome 19, leaving 1096.
Pruned 118 variants from chromosome 20, leaving 1337.
Pruned 145 variants from chromosome 21, leaving 1259.
Pruned 126 variants from chromosome 22, leaving 1017.
Pruned 94 variants from chromosome 23, leaving 898.
Pruned 135 variants from chromosome 24, leaving 1158.
Pruned 71 variants from chromosome 25, leaving 784.
Pruned 88 variants from chromosome 26, leaving 931.
Pruned 89 variants from chromosome 27, leaving 812.
Pruned 68 variants from chromosome 28, leaving 841.
Pruned 76 variants from chromosome 29, leaving 881.
Pruned 376 variants from chromosome 30, leaving 1301.
Pruning complete. 5427 of 50373 variants removed.
Marker lists written to Cortellari2021_g05m05f01.prune.in and
Cortellari2021_a05m05f01.prune.out .

```

Sources: Author's own elaboration.

The SNPs to retain are listed in the output file with extension `.prune.in`. This latter file can be used in combination with the PLINK flag `-extract` to subsets the input file accordingly.

Extreme heterozygosity

Genotypic counts can be obtained by using the PLINK `--het` flag. This analysis is influenced by LD, hence, the `--extract` flag is applied in conjunction with the `.prune.in` file to limit the SNP list to loci in approximate linkage equilibrium.

```
$ plink --cow --bfile Cortellari2021_g05m05f01 --extract
Cortellari2021_g05m05f01.prune.in --het --out Cortellari2021_
g05m05f011d02
```

FIGURE A8.11
Screenshot when obtaining genotypic counts

```
PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05f011d02.log.
Options in effect:
  --bfile Cortellari2021_g05m05f01
  --cow
  --extract Cortellari2021_g05m05f01.prune.in
  --het
  --out Cortellari2021_g05m05f011d02

7993 MB RAM detected; reserving 3996 MB for main workspace.
50970 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05f011d02.nosex .
--extract: 44946 variants remaining.
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1045 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate is 0.997124.
44946 variants and 1048 cattle pass filters and QC.
Note: No phenotypes present.
--het: 43645 variants scanned, report written to
Cortellari2021_g05m05f011d02.het ..
```

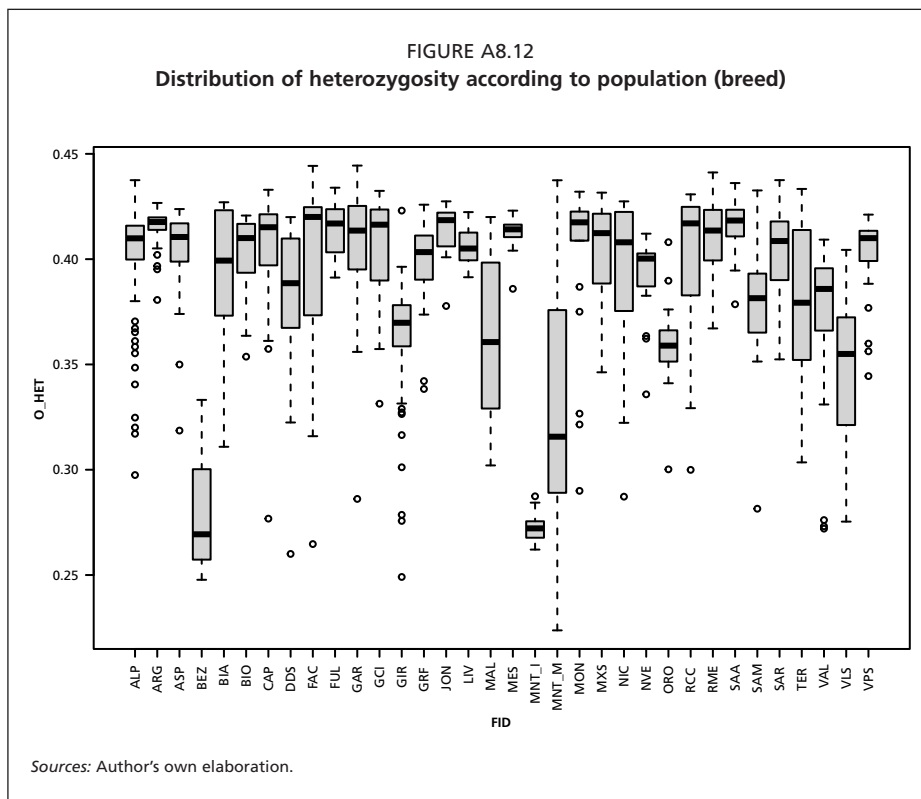
Sources: Author's own elaboration.

The output with extension `.het` contains the observed Homozygotes counts for each individual. These data are then used to compute in R the observed heterozygosity.

```
> het <- fread("Cortellari2021_g05m05f011d02.het")
> het$O_HET = (het$`N(NM)` - het$`O(HOM)`)/het$`N(NM)`
```

The heterozygosity distribution per population can then be plotted (Figure A8.12).

```
> boxplot(O_HET~FID, data = het, las = 2)
```



R can then be used to identify those individuals having within-population extreme (> 3 SD) heterozygosity.

```
> ExtHet <- NULL
> for(p in unique(het$FID)){
> subS = het[het$FID == p, ]
> ExtHet = rbind(ExtHet, subS[as.vector(abs(scale(subS$O_HET)) >
3), c("FID", "IID")])
> }
> table(ExtHet$FID)
```

Output:

ALP	ARG	ASP	CAP	DDS	FAC	GAR	GCI	GIR	MES	NIC	SAA
4	1	1	1	1	1	1	1	1	1	1	1

The output above shows that the process identified 15 Individuals (i.e. 4 from the ALP breed and 1 from each of the others) showing within population extreme Heterozygosity. The list is then saved in a tab delimited file.

```
> fwrite(ExtHet, "IIDtoremove.txt", sep = "\t")
```

The data can then be filtered to remove individuals with extreme observed heterozygosity (H_o) while keeping SNP in approximate linkage equilibrium by combining the PLINK flags `--remove` (to exclude individuals) and `--extract` (to retains SNPs).

```
$ plink --cow --bfile Cortellari2021_g05m05f01 --extract Cortel-
lari2021_g05m05f01.prune.in --remove IIDtoremove.txt --make-bed
--out Cortellari2021_g05m05f01ld02h3
```

FIGURE A8.13
Screenshot when filtering animals with extreme heterozygosity

```
PLINK v1.90b6.24 64-bit (6 Jun 2021)          www.cog-genomics.org/plink/1.9/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05f01ld02h3.log.
Options in effect:
  --bfile Cortellari2021_g05m05f01
  --cow
  --extract Cortellari2021_g05m05f01.prune.in
  --make-bed
  --out Cortellari2021_g05m05f01ld02h3
  --remove IIDtoremove.txt

7993 MB RAM detected; reserving 3996 MB for main workspace.
50970 variants loaded from .bim file.
1048 cattle (16 males, 50 females, 982 ambiguous) loaded from .fam.
Ambiguous sex IDs written to Cortellari2021_g05m05f01ld02h3.nosex .
--extract: 44946 variants remaining.
--remove: 1033 cattle remaining.
Using 1 thread (no multithreaded calculations invoked).
Before main variant filters, 1030 founders and 3 nonfounders present.
Calculating allele frequencies... done.
Total genotyping rate in remaining samples is 0.997109.
44946 variants and 1033 cattle pass filters and QC.
Note: No phenotypes present.
```

Sources: Author's own elaboration.

Relatedness

Plink v2.0 implements the KING robust kinship estimation (Manichaikul *et al.*, 2010). We can generate a table of kinship to check pairwise kinship across the dataset using the `--make-king-table` flag:

```
$ plink2 --cow --bfile Cortellari2021_g05m05f01ld02h3 --make-king-
table --out Cortellari2021_g05m05f01ld02h3
```

FIGURE A8.14
Screenshot when generating a kinship table

```

PLINK v2.00a3 64-bit (1 Jul 2021)          www.cog-genomics.org/plink/2.0/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05f011d02h3.log.
Options in effect:
  --bfile Cortellari2021_g05m05f011d02h3
  --cow
  --make-king-table
  --out Cortellari2021_g05m05f011d02h3

Start time: Tue Jun 14 11:21:01 2022
7993 MiB RAM detected; reserving 3996 MiB for main workspace.
Using up to 8 compute threads.
1033 samples (49 females, 15 males, 969 ambiguous; 1030 founders) loaded from
Cortellari2021_g05m05f011d02h3.fam.
44946 variants loaded from Cortellari2021_g05m05f011d02h3.bim.
Note: No phenotype data present.
Excluding 1301 variants on non-autosomes from KING-robust calculation.
--make-king-table pass 1/1: Scanning for rare variants... done.
6 variants handled by initial scan (43639 remaining).
--make-king-table pass 1/1: writing... done.
--make-king-table: 43645 variants processed.
Results written to Cortellari2021_g05m05f011d02h3.kin0 .
End time: Tue Jun 14 11:21:02 2022

```

Sources: Author's own elaboration.

The output is then loaded into R:

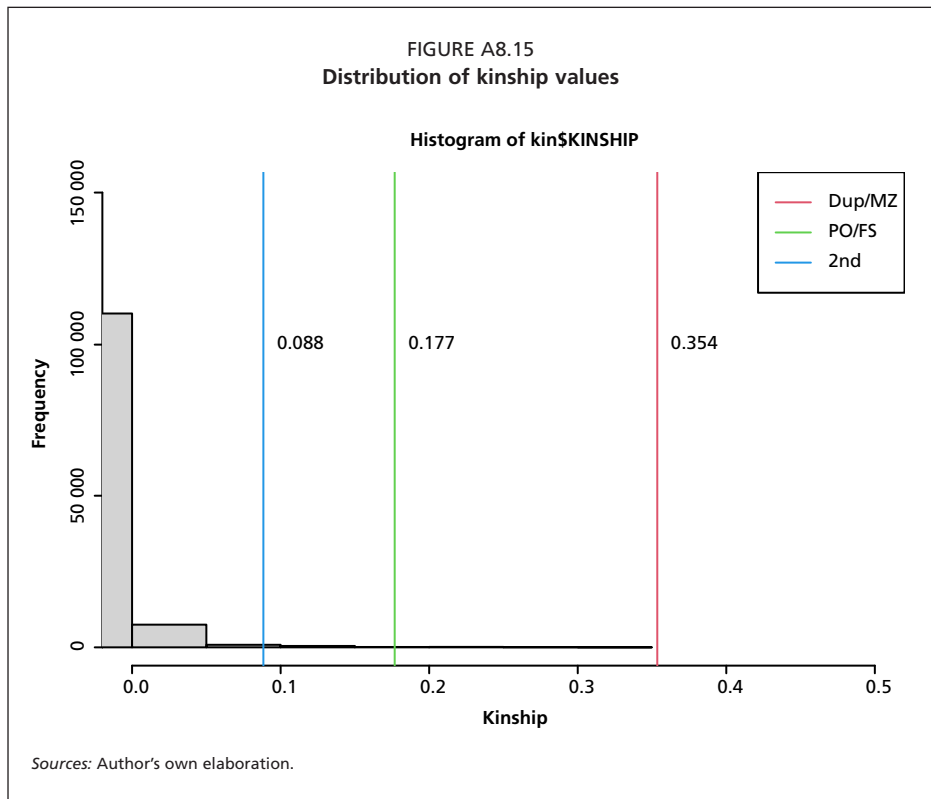
```
> kin <- fread("Cortellari2021_g05m05f011d02h3.kin0")
```

A cutoff threshold of 0.354 is used to identify duplicate samples and monozygotic twins, with 0.354 being the geometric mean of kinship of 0.5 (for duplicates and monozygotic twins) and kinship of 0.25 (for parent-child, full siblings). The inferred kinship values can then be plotted (Figure A8.15) and thresholds set to identify relatedness proportions in the data:

```

> hist(kin$KINSHIP, xlim = c(0, 0.5))
> rel <- c(sqrt(0.5*0.25), sqrt(0.25*0.125), sqrt(0.125*0.0625))
> abline(v = rel, col = 2:4)
> text(rel+.02, 100000, round(rel, 3))
> legend("topright", legend = c("Dup/MZ", "PO/FS", "2nd"), col =
2:4, lty=1)

```



The `--king-cutoff` flag is then used in PLINK v2.0 to exclude one member of each pair of samples with relatedness \geq PO (0.177 in the following example).

```
$ plink2 --cow --bfile Cortellari2021_g05m05f011d02h3 --king-cutoff 0.177 --make-bed --out Cortellari2021_g05m05f011d02h3rel
```


FIGURE A8.16
Screenshot when removing closely related (and possible duplicate) animals

```

PLINK v2.00a3 64-bit (1 Jul 2021)          www.cog-genomics.org/plink/2.0/
(C) 2005-2021 Shaun Purcell, Christopher Chang GNU General Public License v3
Logging to Cortellari2021_g05m05f011d02h3re1.log.
Options in effect:
--bfile Cortellari2021_g05m05f011d02h3
--cow
--king-cutoff 0.177
--make-bed
--out Cortellari2021_g05m05f011d02h3re1

Start time: Tue Jun 14 11:22:08 2022
7993 MiB RAM detected; reserving 3996 MiB for main workspace.
Using up to 8 compute threads.
1033 samples (49 females, 15 males, 969 ambiguous; 1030 founders) loaded from
Cortellari2021_g05m05f011d02h3.fam.
44946 variants loaded from Cortellari2021_g05m05f011d02h3.bim.
Note: No phenotype data present.
Excluding 1301 variants on non-autosomes from KING-robust calculation.
--king-cutoff pass 1/1: Scanning for rare variants... done.
6 variants handled by initial scan (43639 remaining).
--king-cutoff pass 1/1: Condensing... done.
--king-cutoff: 43645 variants processed.
--king-cutoff: Excluded sample IDs written to
Cortellari2021_g05m05f011d02h3re1.king.cutoff.out.id , and 897 remaining sample
IDs written to Cortellari2021_g05m05f011d02h3re1.king.cutoff.in.id .
writing Cortellari2021_g05m05f011d02h3re1.fam ... done.
writing Cortellari2021_g05m05f011d02h3re1.bim ... done.
writing Cortellari2021_g05m05f011d02h3re1.bed ... done.
End time: Tue Jun 14 11:22:08 2022

```

Sources: Author's own elaboration.

For these data, 136 individuals were removed due to excessive relatedness, leaving 897 individuals for further analysis (Figure A8.16).

Importantly, although it is often preferred to remove excess heterozygosity or relatedness from a working dataset, such features can be diagnostic of underlying population dynamics of interest. Hence, it is always advisable to perform these pruning steps keeping in mind the demographic history of the populations.

REFERENCES

- Cortellari, M., Barbato, M., Talenti, A., Bionda, A., Carta, A., Ciampolini, R., Ciani, E., et al.** 2021. The climatic and genetic heritage of Italian goat breeds with genomic SNP data. *Scientific Reports*, 11: 10986. <https://doi.org/10.1038/s41598-021-89900-2>
- Manichaikul, A., Mychaleckyj, J.C., Rich, S.S., Daly, K., Sale, M. & Chen, W-M.** 2010. Robust relationship inference in genome-wide association studies. *Bioinformatics*, 26(22): 2867–2873. <https://doi.org/10.1093/bioinformatics/btq559>

Appendix 9

Steps to be taken in a typical genomic characterization study

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ALL DATASETS

- Genotyping
- Formatting of dataset (PLINK)³
- Quality control (PLINK, KING, and R; see Appendix 8)
- Optional: merging with literature data (PLINK)
- Calculation and display of interindividual distances for detection of duplicates and outliers (mislabelling, crossbreeding) and for checking breed-level differentiation (PLINK)
- Calculation of summary statistics per breed: nucleotide diversity (genomes), expected and observed heterozygosity, heterozygote deficiency (PLINK)

MOST DATASETS

- Inferring of relationships of breeds and genetic clines
 - PCA (Eigensoft, PLINK and specialized R functions)
 - model-based clustering (Admixture, Frappe, Structure)
 - NeighborNet graphs (SplitsTree)

DEPENDING ON THE DATASET AND OBJECTIVES OF THE STUDY

- Phasing and detection of haplotype-based clusters by fine-structure (AphaPhase, EAGLE, fastPhase)
- ROH content (KING, PLINK)
- Inferring of gene flows by the f_3 , f_4 and of the D statistics (ADMIXTUREGRAPH, MIXMAPPER, TreeMix,)
- Examine the genetic basis of traits
 - GWAS – when individual phenotypes are available (BayesR, CGTA, EMMAX, PLINK,)
 - Selection signatures for population-wide characteristics
 - Within breeds (cgaTOH, rehh, Selscan, SweeD, Sweep)
 - Across breeds (HierFstat, PLINK, rehh)
- Coalescence analysis (MSMC and PSMC R packages)
- Reconstructing the history of a population (ABCtoolbox, Fastsimcoal2, SliM)³

⁴ Common software options are indicated in parenthesis.

FAO ANIMAL PRODUCTION AND HEALTH GUIDELINES

1. Collection of entomological baseline data for tsetse area-wide integrated pest management programmes, 2009 (En)
2. Preparation of national strategies and action plans for animal genetic resources, 2009 (En, Fr, Es, Ru, Zh)
3. Breeding strategies for sustainable management of animal genetic resources, 2010 (En, Fr, Es, Ru, Ar, Zh)
4. A value chain approach to animal diseases risk management – Technical foundations and practical framework for field application, 2011 (En, Zh, Fr**)
5. Guidelines for the preparation of livestock sector reviews, 2011 (En)
6. Developing the institutional framework for the management of animal genetic resources, 2011 (En, Fr, Es, Ru)
7. Surveying and monitoring of animal genetic resources, 2011 (En, Fr, Es)
8. Guide to good dairy farming practice, 2011 (En, Fr, Es, Ru, Ar, Zh, Pt^e, Az)
9. Molecular genetic characterization of animal genetic resources, 2011 (En, Zh**)
10. Designing and implementing livestock value chain studies – A practical aid for Highly Pathogenic and Emerging Disease (HPED) control, 2012 (En)
11. Phenotypic characterization of animal genetic resources, 2012 (En, Fr^e, Zh^e)
12. Cryoconservation of animal genetic resources, 2012 (En)
13. Handbook on regulatory frameworks for the control and eradication of HPAI and other transboundary animal diseases – A guide to reviewing and developing the necessary policy, institutional and legal frameworks, 2013 (En)
14. *In vivo* conservation of animal genetic resources, 2013 (En, Zh**)
15. The feed analysis laboratory: establishment and quality control – Setting up a feed analysis laboratory, and implementing a quality assurance system compliant with ISO/IEC 17025:2005, 2013 (En)
16. Decision tools for family poultry development, 2014 (En)
17. Biosecurity guide for live poultry markets, 2015 (En, Fr^e, Zh^e, Vi)
18. Economic analysis of animal diseases, 2016 (En, Zh)
19. Development of integrated multipurpose animal recording systems, 2016 (En, Zh)
20. Farmer field schools for small-scale livestock producers – A guide for decision-makers on improving livelihoods, 2018 (En, Fr^e)
21. Developing sustainable value chains for small-scale livestock producers, 2019 (En, Zh**)
22. Estimation des bilans fourragers dans la région du Sahel d’Afrique de l’Ouest et Centrale, 2020 (Fr)
23. Carcass management guidelines – Effective disposal of animal carcasses and contaminated materials on small to medium-sized farms, 2020 (En, Fr, Es, Ru, Zh, Ar, Sq, Sr, Mk)
24. Technical guidelines on rapid risk assessment for animal health threats, 2021 (En, Fr)
25. Good beekeeping practices for sustainable apiculture, 2021 (En)
26. Responsible use of antimicrobials in beekeeping, 2021 (En, Es)
27. Developing field epidemiology training for veterinarians – Technical guidelines and core competencies, 2021 (En)
28. Making way: developing national legal and policy frameworks for pastoral mobility, 2022 (En)
29. Rift Valley fever action framework, 2022 (En)
29. Rift Valley fever action framework, 2022 (En)
30. Developing an emergency vaccination plan for foot-and-mouth disease in free countries, 2022 (En)
31. Guidelines for livestock vaccination campaigns, 2022 (En)

Availability: January 2023

Ar – Arabic
Az – Azerbaijani
En – English
Es – Spanish
Fr – French
Mk – Macedonian
Pt – Portuguese
Ru – Russian
Sq – Albanian
Sr – Serbian
Vi – Vietnamese
Zh – Chinese

Multil – Multilingual

* Out of print

** In preparation

^e E-publication

The *FAO Animal Production and Health Guidelines* are available through authorized FAO Sales Agents or directly from Sales and Marketing Group, FAO, Viale delle Terme di Caracalla, 00153 Rome, Italy.

Whole genome sequencing and related tools now offer opportunities to evaluate the genetic diversity of livestock populations and individuals with a much higher precision than ever before, at a fraction of previous costs. Applying genomics is technically demanding, however. This publication constitutes a practical tool to guide scientists and other stakeholders in their activities of genomic characterization of animal genetic resources.

This document is part of a series of guidelines published by FAO to support implementation of the *Global Plan of Action for Animal Genetic Resources*, which was adopted in 2007 and remains the internationally agreed framework for the management of biodiversity in the livestock sector.

These guidelines on *Genomic characterization of animal genetic resources* update the previous edition of guidelines on *Molecular genetic characterization of animal genetic resources*, published in 2011. The guidelines describe the key processes of undertaking a study to characterize one or more livestock populations on the basis of their DNA sequences. The publication opens by reviewing the rationale for characterization of animal genetic resources and summarizing the history and prospects. The basics of carrying out a genomic diversity study are then addressed, followed by explanations of the main approaches for assessing genomes (i.e. whole genome sequencing and genotyping of single nucleotide polymorphism markers). Finally, the most important applications of genomics for assessing the genetic variation within and across populations are described. These guidelines are intended to provide countries with another tool for sustainable management and conservation of their animal genetic resources.

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