Topic 4: Genetic test for identification of parents (parentage testing)
Laboratory examples

In this lecture we will be looking at genetic parentage testing in animals, specifically the laboratory procedures for this analysis. The lecture is part of Module number 4, Precision Livestock Breeding. The development of this presentation was supported by an ERASMUS+ KA2 grant within the ISAGREED project, Innovating the content and structure of study programmes in the field of animal genetic and food resources management using digitisation.
What are the steps of the analysis itself? First, DNA must be isolated from the biological material and its quality verified. Next, amplify the fragments using multiplex PCR and separate the amplicons obtained by fragmentation analysis. A special computer program then automatically compares the results of the fragmentation analysis with the calibration curve of the standard and determines the alleles of the individual microsatellites. After obtaining the genetic profile of the individual, the parentage verification is then performed. Let us now describe the steps in more detail:
The first and necessary step in most molecular genetic methods is DNA isolation. DNA can be isolated from almost any biological material, e.g. blood, muscle, buccal swabs, milk, feathers, hair or hair bulbs, semen, etc. The quality and quantity of DNA isolated greatly influences the course of subsequent reactions. Several isolation methods exist. The method is chosen according to the material from which the DNA is isolated and the quantity and quality of the biological material. At present, one of the most commonly used methods of DNA isolation is the spin-column method, which belongs to the group of methods using solid particles for DNA isolation.
The advantage of the spin-column method is primarily speed, simplicity, high reliability and good quality and purity of the obtained DNA. The essence of this method is the adsorption of DNA onto a silicate membrane, its washing and subsequent release into a tube. The first step is the preparation of the biological material from which the DNA will be isolated and its possible homogenisation. The images show the most common materials used for DNA isolation in animals, i.e. blood, tissue (e.g. muscle) and hair bulbs, which have the advantage of easy non-invasive collection and easy storage and sending of material from breeders to the laboratory for analysis.
Lysing buffer and proteinase K are added to the biological material, after incubation at an elevated temperature, a lysate is formed in which DNA is released from the cell nucleus and proteins are degraded. The resulting lysate is pipetted onto a silicate marticulate column. During subsequent cetrifugation, the DNA is captured and bound to the silicate.
In the next step, the DNA is bound to the silicate matrix inside the column by centrifugation. In the next two steps, the bound DNA is washed with wash buffer to remove all impurities. In the last step, the purified DNA is released into the elution buffer solution. We will use the DNA isolated in this way for further analyses.
After DNA isolation, it is always necessary to verify its amount, i.e. if the isolation was successful. The most common way to verify the amount of isolated DNA is agarose gel electrophoresis. In this case, we will use a 2% agarose gel. First, we weigh 2g of agarose, which is a purified polysaccharide from seaweed. We boil this white powder in 100 ml of electrophoretic buffer, in our case it is TBE buffer (TRIS-BORATE + EDTA). This gives us a 2% concentration of the gel. Boiling takes place in a microwave oven until the solution is completely homogeneous and clear.
Once thoroughly cooked, add the visualization paint, in this case GoodView, pour everything into the prepared tub and insert the ridges. Allow the gel to set.
After solidification, the gel is placed in an electrophoretic bath filled with electrophoretic TBE buffer, the combs are removed and DNA samples are applied to the resulting wells. The DNA

must be mixed with a loading buffer that contains an indicator dye (usually bromophenol blue) and a thickener such as sucrose or glycerol. This will allow the DNA to be properly applied to the bottom of the well.
Each gel must include at least one column with a size standard. This is a mixture of DNA of different and precisely defined sizes, which is used to infer or verify the size of the samples.
After all the DNA samples have been applied, we connect the electrophoretic bath to a power source using electrodes, set the necessary parameters - in our case 130V and 30 minutes, and start the electrophoresis. Once the separation is complete, we visualize the result using UV light. In the resulting electrophoretogram we can see in the first well the so-called size standard pipetted for rough quantification of the results, then in the next wells the isolated DNA of different intensity, i.e. different quantity. After this electrophoretic verification of the isolation, the DNA is used for the next steps of the molecular genetic analysis.
The last figure on electrophoresis illustrates the basic steps of agarose gel electrophoresis.
The next step is multiplex PCR. Multiplex PCR is one of many modifications of the PCR reaction. The idea is that multiple DNA fragments are amplified in a single reaction, depending on the number of primers added to the reaction. All this takes place in a thermal cycler. Here, the temperature profile of each reaction step and the number of repetitions is set.
The result is a microtube containing amplicons of different lengths. The number of amplicons corresponds to the number of MS determined in a given individual. But how to evaluate this reaction, how to subtract the sizes of the individual PCR products? Using conventional agarose gel electrophoresis, the result is not clearly readable. Because fluorescently labelled primers have been added to the reaction, capillary electrophoresis run in a genetic analyser can be used to read the results. Multiplex PCR is therefore followed by fragmentation analysis.
The fragmentation analysis itself takes place in a special genetic analyser. The analysis is based on capillary electrophoresis followed by fluorescence detection of individual amplified fragments. The aim is to detect the length of the DNA fragment, which is defined by two primers (one primer is fluorescently labelled, the other is not). The advantage of using this method is that multiple fragments can be analysed simultaneously. The fragments must differ in length or be fluorescently labelled. A size standard must be added to each sample. The operation of the genetic analyzer is similar to sequencing, and you can see a demonstration of this instrument in Module 1: Sequencing Demonstration.
After the fragmentation analysis is completed, the results are evaluated in a special program, e.g. GeneMapper. First, the quality of the raw data needs to be checked. They look like this.
On the picture you can see the resulting electrophoretogram of the set of 17 MS in horses. The individual MS are divided into 4 colours, in order to avoid overlapping alleles and to ensure that the resulting genotypes are correctly read and determined. Each MS consists of one or two peaks that indicate the alleles of that MS. If one allele is present in the genotype of the selected MS, the individual under study is homozygous for that allele. If the MS has two alleles detected, it is heterozygous. The result of the fragmentation analysis is therefore the determined MS set of the individual. When verifying parentage in animals, selected sets of MS of the parent and offspring are compared.
Based on this, it can then be determined whether the individual is a descendant of those parents. We will illustrate all this with a concrete example. On this slide you can see the resulting genotypes of a set of 17 MS horses for the offspring and its possible parents. The challenge is to determine if the listed parents are indeed the parents of the offspring. If they are, the offspring will have one allele from the sire and one from the dam in its MS genotypes. As we can see in the offspring, the genotypes of each locus really consist of one allele inherited from the father (marked in blue) and one allele inherited from the mother (marked in red). In this case, genetic testing confirmed the parentage. There may be other cases where genetic analysis rules out one parent or both parents.