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KINSHIP DETERMINATION IN COMMON ELAND (*Taurotragus oryx*) AND GIANT ELAND (*Taurotragus derbianus*) BRED IN CAPTIVITY

Diploma thesis

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ANNOTATION

Subject: Kinship determination in Common eland (*Taurotragus oryx*) and Giant eland (*Taurotragus derbianus*)

Abstract:

The antelopes of the genus *Taurotragus* are the largest antelopes in the world. Their number in the wild decreases especially these of Giant eland's (*Taurotragus derbianus derbianus*) in Senegal. With the conservation of the Giant eland in Senegal deal the project of Czech University of Life Sciences Prague "Support to Natural reserves and National parks of Senegal".

Some individuals of both species are bred in captivity – the Giant elands are bred only in semi-captivity in Senegal in frame of this conservation project, the Common elands are bred (besides) in the farm breeding of Czech University of Life Sciences Prague in Lány. In populations held in captivity is necessary to know the kinship relations especially because of inbreeding prevention. These relationships are usually based on direct observations of animals´ maternal behaviour and for confirmation of these we looked for the possibility to use genetic markers. We used the method of cross-amplification with markers originally developed for other relative species.

Forty-four polymorphic microsatellite markers from bovids (cattle, sheep and goats) and Grant's gazelle were tested on 22 Common elands and 33 Giant elands for the polymorphism detection and applicability in kinship determination. Ten markers were polymorphic in the Common eland and six in the Giant eland. These markers could be used for determination of kinship relations.

Keywords: microsatellites, kinship, cross-amplification, *Bovidae*, *Taurotragus*

ANOTACE

Téma: Určování příbuznosti u antilopy losí (*Taurotragus oryx*) a antilopy Derbyho (*Taurotragus derbianus*)

Souhrn:

Antilopy rodu *Taurotragus* patří mezi největší antilopy na světě. Jejich počet ve volné přírodě klesá, zejména u antilopy Derbyho (*Taurotragus derbianus derbianus*) v Senegalu. Ochranou antilopy Derbyho se zabývá projekt České zemědělské univerzity v Praze „Podpora přírodních rezervací a národních parků Senegalu".

Několik jedinců obou druhů je chováno v zajetí – antilopy Derbyho jsou chovány pouze v polozajetí v Senegalu, antilopy losí jsou chovány na farmovém chovu České zemědělské univerzity v Praze. U populací chovaných v zajetí je nutné znát příbuzenské vztahy, zejména kvůli prevenci příbuzenské plemenitby (inbreedingu). Tyto vztahy jsou založeny na přímém pozorování mateřského chování zvířat a pro jejich ověření jsme testovali možnost použití genetických markerů. Byla použita metoda cross-amplifikace s markery původně vyvinutými pro jiné příbuzné druhy.

Čtyřicet čtyři polymorfních mikrosatelitních markerů od turovitých (skotu, ovcí a koz) a gazely Grantovy bylo testováno na 22 antilopách losích a 33 antilopách Derbyho pro zjištění polymorfismu a možné použitelnosti pro určování příbuznosti. Deset markerů bylo polymorfních u antilopy losí a šest u antilopy Derbyho. Tyto markery mohou být použity pro určování příbuzenských vztahů.

Klíčová slova: mikrosatelity, příbuznost, cross-amplifikace, *Bovidae*, *Taurotragus*

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1. INTRODUCTION

The Common and the Giant eland belong to the largest antelopes in the world. The Common eland (*Taurotragus oryx*) is more numerous and there is more information about it, it has the status "Lower risk, conservation dependent" on the IUCN Red list of endangered species. The population of the Giant eland (*Taurotragus derbianus*) is smaller in the wild. The subspecies of the Giant eland are placed into different categories on the Red list – the Western giant eland (*Taurotragus derbianus derbianus*) has the status "Endangered" and the Eastern giant eland (*Taurotragus derbianus gigas*) "Lower risk, near threatened".

The samples collected in this study were obtained from individuals of the Common elands bred in captivity in experimental farm Lány, which belongs to the University of Life Sciences in Prague, and from the Giant elands bred in semi-captivity in two natural reserves in Senegal – Bandia and Fathala reserves. The Giant eland individuals belong to the Western subspecies (*Taurotragus derbianus derbianus*) and they strand to the Czech Development Aid project "Support to Natural reserves and National parks in Senegal" oriented to Conservation of the Western Giant eland in Senegal.

The both populations in captivity are relatively small (approximately 30 Common elands and 46 Giant elands + calves under 1 year), so the risk of inbreeding is very high. The herd of Common elands originates from the breeding of ZOO Dvůr Králové and there are new coming individuals from others zoos. The herd of the Giant elands has been established with only 6 founders – 5 females and only 1 male captured in 2000 in the wild – Niokolo Koba National Park in Senegal and it was not possible till now to obtain a new unrelated male from the wild. To minimize inbreeding the observations of the kinship relations (maternity and paternity) in herds are registered and serve as the base for studbook. The maternity is determinated by observations of the maternal care – mainly suckling. The dominant male in the herd is assumed to be the father of the calves. The animals are divided into the breeding herds according to their ancestors.

The observation can be inaccurate, especially the paternity is uncertain in Common eland herds, where several males are present. In Giant elands only one adult male is present in all breeding herds, but contrary the observation in the field during calving time are more difficult. The genetic tests can provide a clear confirmation of results obtained by observations and so help in proper genetic management.

The method of cross-amplification of microsatellite markers from relative species was chosen for the genetic tests. The main advantage of microsatellites is their variability. The problem is that the microsatellites for the elands have not been evolved yet. Searching of suitable microsatellites sets is very time-consuming and expensive, so the microsatellites developed originally for cattle, goats, sheep and Grant's gazelle were tested for their ability to amplify in elands. At least 6 verified polymorphic microsatellite markers are necessary to the accurate kinship determination so it could be very problematic to find the appropriate set for kinship determination in elands.

In this study the 44 markers were tested by means of the polymerase chain reaction (PCR) with various PCR conditions.

2. AIM OF THE THESIS

The aim of the thesis is to verify the applicability of selected polymorphic bovine, ovine, caprine and Grant's gazelle's microsatellite markers in antelopes of the genus *Taurotragus*.

Another object is to choose the set of identified polymorphic markers, which could be used in kinship relations studies, specifically in parentage testing.

3. LITERATURE OVERVIEW

3.1 <u>The natural history of antelopes</u>

The antelopes of the genus *Taurotragus* – the Common eland (*Taurotragus oryx*) and Giant eland (*Taurotragus derbianus*) belong to the tribe *Tragelaphini*, family *Bovidae* (bovids), suborder *Ruminantia* (ruminants), order *Artiodactyla* (even-toed ungulates), class *Mammalia* (mammals), phylum *Chordata* (chordates), kingdom *Animalia* (animals) (Anděra et Červený 2000, Burnie et al. 2002, Estes 1991).

The order *Artiodactyla* has 10 families and the family *Bovidae* has around 138 species, from the smallest antelopes - duikers (*Cephalopus spp.*) to the biggest – oxen (*Bos spp.*) (Anděra et Červený 2000, Burnie et al. 2002).

The elands belong to the antelopes of the tribe *Tragelaphini*, they are also known as spiral-horned antelopes. Except the elands belong to this tribe also the bushbuck (*Tragelaphus scriptus*), nyala and mountain nyala (*Tragelaphus angasii* and *T. buxtoni*), greater and lesser kudu (*Tragelaphus strepsiceros* and *T. imberbis*), bongo (*Tragelaphus euryceros*) and sitatunga (*Tragelaphus spekii*) (Estes 1991, Grzimek 1990).

These antelopes have typical spiral horns, which are present in males, only by elands and bongos also in females. Further typical trait are white vertical strips and the scent glands, located ahead of the teats (by bushbuck, sitatunga, lesser kudu and mountain nyala) and glands around false hooves in hindfeet (these glands are absent in bushbuck, sitatunga and bongo). The females have 4 mammae (Estes 1991, Grzimek 1972, Grzimek 1990, Kingdon 1982).

The sexual dimorphism is distinctive for the *Tragelaphini*. Except the horns by males, as was described above, to the sexual dimorphism belong the different coloration of males and females (the males are darker than the females), common is also the size dimorphism (the females are usually smaller than the males) (Estes 1991, Kingdon 1982).

The antelopes of this tribe are found in all kinds of wooded habitats in the sub-Saharan Africa – elands in savannas and subdeserts. The exception is the Common eland, which ventures to live on the open habitats, not under the protection of the forest. The elands and kudus are adapted to the arid conditions; elands are able to reduce the loss of water by concentrating the urine, excreting dry faeces, lowering the metabolic rate, by slower and deeper breathing and by seeking shade in the heat of the day. The other *Tragelaphini* are water-dependent (Estes 1991, Kingdon 1982).

Social organization of the spiral-horned antelopes is very various, from the solitary and sedentary bushbuck to the gregarious and nomadic eland. The most usual is sedentary way of the life in small unisexual groups. Adult males join the herds of females to find some female in estrus, the young males create the bachelor herds. The bonds among the individuals in the herd are very weak, except the bonds between the mother and calf. The hierarchy obeys by the dominance of the elderly over youngers and of the larger males over smaller females. The society of *Tragelaphini* is open and loose. Notable is the low level of aggression, all of the spiral-horned antelopes are nonterritorial (Estes 1991, Kingdon 1982).

The Tragelaphines are hidders – after birth the calf spends about two weeks concealed in the grass and during this time it is totally dependent on its mother. The mother visits the calf for suckling 2 – 4 times a day. After these two weeks the calves join the herd and form the nursery group. Young calves suckle for 5 – 10 minutes. Weaning is relatively early in the spiral-horned antelopes, between 4 and 6 months (Estes 1991, Grzimek 1990, Kingdon 1982).

3.1.1 The Common eland (*Taurotragus oryx*)

The Common eland (Figure 1) has 3 subspecies – Cape eland (*Taurotragus oryx oryx*), Livingstone's eland (T*aurotragus oryx livingstonii*) and East African eland (*Taurotragus oryx pattersoni*) (Kingdon 1997), but Estes (1991) mentions only 2 subspecies (*T. o. oryx* and *T. o. pattersoni*).

The males weigh up to 1000kg, they have spiral horns, which could be up to 1,2m long. The females are smaller than the males, they weigh up to 600kg (Anděra et Červený 2000, Burnie et al. 2002, Grzimek 1972, Grzimek 1990). The Livingstone's eland and East African eland have 10 - 16 white or creamy stripes on their flanks, the adult Cape elands are tawny without stripes (Estes 1991, Kingdon 1997, Nowak 1991).

The Common eland lives in East and South Africa. It occupies grassy and shrubby biotopes and the edges of the forest (Anděra et Červený 2000, Burnie et al. 2002, Kořínek 2008 [online]).

The digestive system is by intermediate type, adapted to a low fibre, high protein diet (Kingdon 1982). The Common eland eats grass, leaves and branches, fruits, tubers and roots (Kořínek 2008 [online]). The both eland species use their horns to break off the branches of trees, which they want to nibble. This behaviour is probably learnt (Dorst et Dandelot 1970, Estes 1991, Grzimek 1972, Grzimek 1990, Kingdon 1982, Kingdon 1997).

The Common elands live in herds up to 200 individuals, which are created mainly by females and calves. The older males live solitary. (Anděra et Červený 2000, Burnie et al. 2002, Dorst et Dandelot 1970, Grzimek 1972).

The gestation lasts 8 – 9 months. The female has usually 1 calf, which suckles up to 6 months (Estes 1991, Grzimek 1972, Grzimek 1990, Kořínek 2008 [online]).

The life expectancy is 23 - 25 years in captivity (Anděra et Červený 2000, Nowak 1991).

The Common elands are not aggressive and they are readily tamed (Dorst et Dandelot 1970). They are bred in captivity. In Africa they have been utilized for meat, milk and skin production (Burnie et al. 2002), breeding with the longest history exists in the southern Ukraine in Askania Nova. The females of the Common elands are milked regularly there (Dorst et Dandelot 1970, Grzimek 1972, Grzimek 1990, Nowak 1991). The Common elands were also bred in Asia or USA on farms (Beazley 1973, Burnie et al. 2002).

In recent time the Common eland numbers more than 115 000 individuals in the natural environment (East 1998). According to IUCN, the Common eland is placed into the category "Lower Risk, conservation dependent" (*2007 IUCN Red list of threatened species* 2007 [online], East 1998).

The Czech University of Life Sciences (CULS) started with breeding of Common elands in 1998, now they are placed in the farm Lány. They adapted to the central European environment and prosper very well; they also reproduce (Kotrba 2004). In recent time there is approximately 30 individuals (Annexe 1).

Figure 1: The Common eland (*Morris* 2005 [online])

3.1.2 The Giant eland (*Taurotragus derbianus*)

We distinguish two subspecies of the Giant eland (called also the Derby's eland), the Western giant eland (*Taurotragus derbianus derbianus*) (Figure 2) and the central African eland, called also the Eastern giant eland (*Taurotragus derbianus gigas*) (Dorst et Dandelot 1970, East 1998).

The Giant eland is considered to be the largest antelope in the world, but it is very disputable, because its weight and size is comparable with the Common eland's measurements. The male could weigh up to 1000kg, the female weigh up to 440kg. Both sexes have spiral horns up to 1 – 1,2m long (Dorst et Dandelot 1970, Estes 1991). The Giant eland has about 15 white stripes on his flanks (Anděra et Červený 2000, Dorst et Dandelot 1970, Nežerková et al. 2004).

The Giant eland lives in the savanna from Senegal to southern Sudan. The Eastern giant eland ranges in Cameroon, Central African Republic and Sudan, but the Western subspecies lives in the recent time probably only in one place – the Niokolo Koba National Park in Senegal (*2007 IUCN Red list of threatened species* 2007 [online], Antonínová et al. 2006, East 1998, Nežerková et al. 2004, Nowak 1991). Its natural environment is woodland and forested savannas (Dorst et Dandelot 1970).

The Giant eland is mainly the browser (Kingdon 1982). It feeds mostly on leaves and shoots, sometimes on grasses (Dorst et Dandelot 1970).

The herds of the Giant elands number around 60 individuals, usually 15 – 25 individuals. The males are solitary or assemble in small groups. The animals are very shy (Dorst et Dandelot 1970, Kingdon 1982).

The information about the reproduction and life expectancy in captivity are similar as in the Common eland (Grzimek 1990).

The Giant elands bred in captivity (in about 10 institutions in USA and South African Republic) belong to the eastern subspecies (*Taurotragus derbianus gigas*). The only Western giant elands bred in semi-captivity are in Senegal in Bandia reserve (Nežerková et al. 2004) and in Fathala reserve (Antonínová 2007, personal communication).

According to IUCN the Western giant eland is placed into the category "Endangered". In the Niokolo Koba remain only 100 – 150 individuals (Nežerková et al. 2004). The Eastern giant eland has the status "Lower risk, near threatened". It numbers in the recent time about 15 – 20 000 individuals (*2007 IUCN Red list of threatened species* 2007 [online], East 1998).

With the conservation of the Western giant eland deal since the 2000 the project of the Institute of Tropics and Subtropics of the Czech University of Life Sciences in Prague, in cooperation with the Directorate of National Parks in Senegal (DPNS) and the Society for the Protection of the Environment and Wildlife in Senegal (SPEFS). (Antonínová et al. 2006, Nežerková et al. 2004).

Breeding individuals originate from Niokolo Koba National Park, where they were captured in 2000. They adapted well and create the basic breeding herd (Nežerková et al. 2004). The Giant elands reproduce very well, in present time there are two herds in Bandia reserve and one in the Fathala reserve. The main breeding herd in Bandia reserve consist of 30 adult or subadult animals and calves. The second herd is created only by 3 animals. In February 2008 a new breeding herd in Fathala reserve has been created and in this group there is 1 male and 5 females (Annexe 2).

The whole herd originates from 6 animals. The maternal relations were found out on the basis of the suckling observations and there is only one dominant male, which is assumed to be the sire of all youngs. The new herds are created on the basis of knowledge of the kinship relations, registered in studbook. The breeding herd is created by the males and females, which are least related after the maternal line.

Figure 2: The Giant eland – female (left) and male (right) (Photo by author).

3.2 **Genetics and small animal populations**

3.2.1 Small populations and the risk of extinction

The populations of species bred in captivity are often very small and they also often originate from small number of founders, because of the costs for establishment of the captive population, lack of space. Capture of the animals (especially big animals as the antelopes) in the wild is very expensive too and, from view of epizootology, a very difficult process. And also there is not possibility to obtain many animals (particularly unrelated animals) from the wild because of limited size of their natural population (Frankham et al. 2003).

The population of the Giant eland in Senegal is very small. And in small populations there increases the probability of inbreeding, it means mating between related individuals (father – daughter, brother sister etc.). Consequence of inbreeding is the decrease of the heterozygosity, the risk of increased incidence of genetically conditional diseases and decrease of genetic diversity that is connected with decrease of reproduction fitness and survival. The decrease of the reproductive fitness is called inbreeding depression (Frankham et al. 2003, Kočárek 2004). More detailed correlation of heterozygosity and inbreeding are the subject of discussions and further research (Balloux et al. 2004).

It follows that small population (as is the Giant eland population) are more endangered by extinction. Restoration of the fitness of the inbred population is possible by means of the outcross to another unrelated population if it is possible. If not there is the necessity of studbooks creation and on their basis to propose the breeding management (Frankham et al. 2003).

In small populations proves also the genetic drift. Some alleles will be transmitted into the next generation in smaller amount or they will not be transmitted at all. So the consequences of the genetic drift are decrease of genetic diversity of the population and again increase of the risk of extinction (Frankham et al. 2003, Kočárek 2004, Nečásek 1993).

3.2.2 <u>Management of small populations</u>

3.2.2.1 <u>Wild populations</u>

Management of wild population consists in increasing of the population size, recovery of small inbred populations and genetically manage fragmented population. The increasing of the population size is possible by several ways, for example by stopping hunting of the species, protection and translocation or captive breeding and release programs (Frankham et al. 2003).

The first step to the genetic management is the determination of the genetic status of the population. The recovery of inbred population is performed by introduction of individuals from other population. The result is improvement of the reproductive fitness and restoration of the genetic diversity (Frankham et al. 2003).

For the single population the management deal with increasing of the population size by means of establishment of the populations in several locations (to minimize the risk of catastrophes), by improvement of the environment to maximize the reproduction rate and by protection against some environmental change (Frankham et al. 2003).

The genetic diversity in fragmented populations could be recovered by increasing of the habitat area, increasing suitability of the habitat, increasing migration rate by translocation, creating habitat corridors and re-establishing of extinct populations in the suitable habitats (Frankham et al. 2003).

3.2.2.2 <u>Captive populations</u>

The animal species are bred in captivity in zoos and in various wildlife parks or reserves. The objectives of captive breeding programs are preservation of the species and increase of number. Animals bred in captivity can serve also as the "insurance" against extinction of endangered species in the wild (Frankham et al. 2003).

The main strategy in the management of captive populations is minimization of kinship. It is very difficult because the institution, which wants to breed some species, often has only several individuals, so the risk of inbreeding is very high. The solution is in the regional and international cooperation of breeding and conservation programs. These programs keep and share the information about each individual and recommend breeding of suitable (not related) individuals. The example of such program or database is the International Species Information System (ISIS) (Frankham et al. 2003).

Inbreeding can be prevented when the individuals come from different sources or their origin is unknown and also by exchanging individuals between the populations (Frankham et al. 2003).

The captive population should be established before the wild population approaches extinction. According to World Conservation Union (IUCN) there is recommended to establish the captive population before the number of wild population drops below 1000 individuals. Simultaneously the new captive population should be established with minimally 20 animals to insure the adequate genetic base (Frankham et al. 2003).

Many species, for example addax (*Addax nasomaculatus*), Arabian oryx (*Oryx leucoryx*) or Przewalski's horse (*Equus przewalskii*), which became extinct in the wild, have been rescued due to their breeding programmes in captivity (Frankham et al. 2003).

The captive populations can provide animals for reintroduction programs. Reintroduction means releasing of captive-born animals back into the wild (Frankham et al. 2003).

One of the negative consequences (except inbreeding and loss of genetic diversity) of breeding of captive population is genetic adaptation to captivity. It is more extensive in larger populations and it is disadvantageous for the populations, which are predetermined for reintroduction. The animals in captivity are not endangered by predators, diseases and pests are controlled and there is usually no competition. The genetic adaptation to captivity can be minimized by reducing number of the generations in captivity, by minimizing captive population size and selection in captivity and also by fragmentation of the population (Frankham et al. 2003).

3.2.3 Kinship

The related individuals have identical part of their genetic outfit. The offspring gets one half of genetic outfit from mother and one half from father. The relatedness between two individuals, r (the **coefficient of relatedness**) can be interpreted as the expected fraction of alleles that are shared of these two individuals (Blouin 2003). In unrelated individuals $r = 0$; in full siblings or parents and children $r = 0,5$; in half-siblings or grandparents and grandchildren $r = 0,25$ and so on (Gilbert et al. 1991) (Figure 3).

Figure 3: The coefficient of relatedness (Kenyon C. A. P. 2006 [online])
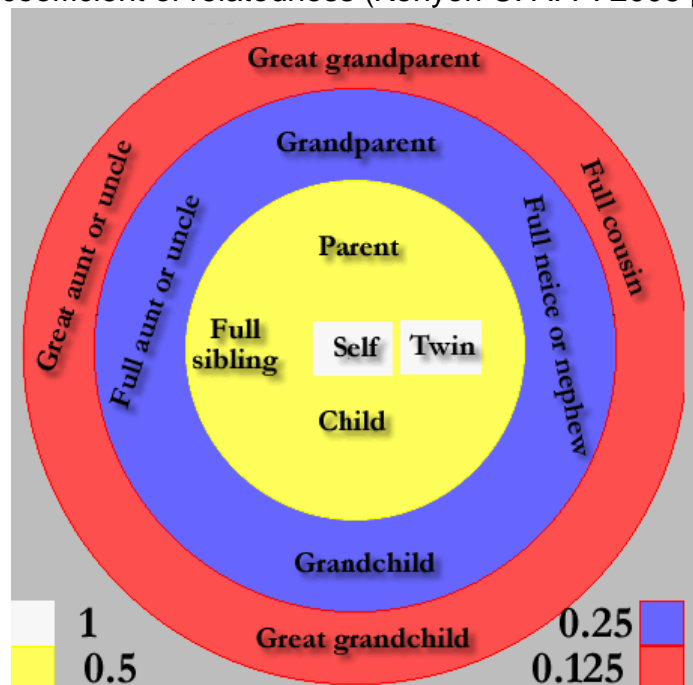
To prevent the decrease of the genetic diversity, inbreeding or even extinction of the small population there is necessary to know the kinship relations in the population. Due to the knowledge of the kinship relations there could be possible to breed only "suitable" individuals and in this way to rescue the population (Frankham et al. 2003).

There is possible to find out, if the two individuals are related or not, due to genetic testing. It is important for small endangered populations, such as the Giant elands.

3.3 <u>Genetic methods for kinship determination</u>

There exist several methods of kinship determination. The method called DNA fingerprints (or variable number tandem repeats (VNTR) or minisatellites) uses minisatellite sequences (repeat sequences with lengths of 10 to 100 bases). It is used in human forensics. In RAPD (randomly amplified polymorphic DNA) are used random primer sequences, usually 10 – 20 base pairs long. Closely related with RAPD is AFLP (amplified fragment length polymorphism). The both methods have been used more in plants than in animals. RFLP (restriction fragment length polymorphism) requires large amount of DNA and cannot be performed when the samples are obtained by non-invasive way, similarly to DNA fingerprints. The next method SSCP (single strand conformational polymorphisms) is using mainly mitochondrial DNA (Frankham et al. 2003).

These methods have uniform some basic steps, like DNA isolation, searching and selection of the primers, polymerase chain reaction or evaluation of successfulness of the method by means of electrophoresis.

3.3.1 <u>DNA isolation</u>

There is possible to isolate DNA from whatever animal tissue, which contains live cells. Isolation from the blood, whose cells have the nucleus (avian blood) is simpler, isolation from the mammalian blood is not so simple, because the erythrocytes do not have the nucleus – it is necessary to use higher volume of the blood (even a few millilitres) (Zima et al. 2004).

Except blood and tissue, there is possible to isolate the DNA from semen, skin, fur, nails or claws, feather, salivas, urine but also from the odorous marks, food, excrements and so on (Queller et al. 1993, Zima et al. 2004).

In the recent time there is possible to use ready kits for DNA isolation. The classical method is based on the dissolution of the proteins by means of the proteinase K, extraction by the mixture of phenol and chloroform and precipitation with ethanol. The isolated DNA can be diluted with distilled water. The samples are stored in the freezer (Zima et al. 2004).

3.3.2 Microsatellites

For kinship determination in the elands there was chosen the method of microsatellites. This method is popular and often used, because the microsatellites are very variable – highly polymorphic, it means that they often have many alleles per locus, which vary in the number of repeats and can be used also by the samples, which were obtained by non-invasive way (Frankham et al. 2003, Kim et al. 2004, Queller et al. 1993).

This technique is called also simple sequence repeats (SSR) or short tandem repeats (STR). Microsatellites are tandem repeats of short DNA segments, typically 1 – 6 bases (Frankham et al. 2003, Jarne and Lagoda 1996, Queller et al. 1993, Zima et al. 2004). They are possible to be detected by amplifying DNA using PCR (polymerase chain reaction) (Kim et al. 2004, Queller et al. 1993). The primers (DNA segments of known sequences) flanking microsatellites are used to amplify specific part of DNA. The primers are usually 17 – 25 bases long (rarely they can be longer, shorter primers are suitable for example for RAPD) (Zima et al. 2004). The products of PCR are separated using electrophoresis on agarose (or polyacrylamide) gels (Frankham et al. 2003).

The disadvantage is that the microsatellites have to be developed for each species; only some primers can work in closely related species (Frankham et al. 2003, Queller et al. 1993).

3.3.3 Polymerase Chain Reaction (PCR)

Due to polymerase chain reaction there is possible to amplify *in vitro* the specific DNA section, even though there is only very small amount of original sample of the DNA (theoretically one molecule of DNA suffices) (Zima et al. 2004).

For the performance of PCR there is needed isolated DNA, *Taq* polymerase (polymerase isolated from the gram-negative bacteria *Thermus aquaticus*, that is stable even by high temperatures, when the DNA denatures), primers (DNA sections with known sequence of the bases; each primer has two parts – "Forward" and "Reverse"), free deoxynucleotidtriphosphates (dNTPs = the mixture of 4 nucleotides), $MgCl_2$, PCR buffer (contains some additives like detergents, dimethylsulphoxid or glycerol, formamide or potassic chloride and another, which should increase the effectiveness of PCR) and redistilled water. The polymerase chain reaction proceeds in the machine called thermocycler, which is able to change the temperatures according to the set program (Rosypal et Doškař 1997, Šmarda et al. 2005, Zima et al. 2004).

The principle of this method is following. The initial step is denaturation of the double-stranded DNA by warming on the high temperature (over 90°C) (Rosypal et Doškař 1997, Zima et al. 2004).

The next step is "hybridization" or "annealing". Zima et al. (2004) adduces the temperature in this step 45 – 60°C, but Rosypal et Doškař (1997) and Šmarda et al. (2005) adduce the range of temperatures 30 – 65°C (the annealing temperature is specific for each primer). The primers bind on the complementary sections of the DNA (but each primer on the opposite DNA strand), they lie on borders of the section, that is required to amplify. The primers are very short, so they tie in the DNA strand faster than the complementary DNA strand (Zima et al. 2004).

The annealing temperature is one of key factors of PCR. Higher temperatures increase the specificity of the primers and the accuracy of PCR. Lower temperatures enable less specific primers to tie in the templates (Zima et al. 2004).

In the last step – extension – new chains of DNA are synthesized. The extension runs at 72°C (this temperature is optimal for the *Taq* polymerase). The *Taq* polymerase enables connection of dNTPs and elongation of the chain (Zima et al 2004) (Figure 4).

The whole procedure repeats in several cycles (usually 20 – 40 cycles). In each cycle the number of copies doubles, while the new chains serve at the same time as the matrices. The last extension is longer, it takes usually several minutes and it helps eliminate the occurrence of incomplete short fragments (Zima et al. 2004).

Figure 4: Scheme of the Polymerase chain reaction. P = Polymerase (Modified according to *Polymerase chain reaction – Wikipedia* 2007 [online], Rosypal et Doškař 1997, Šmarda et al. 2005, Zima et al. 2004)
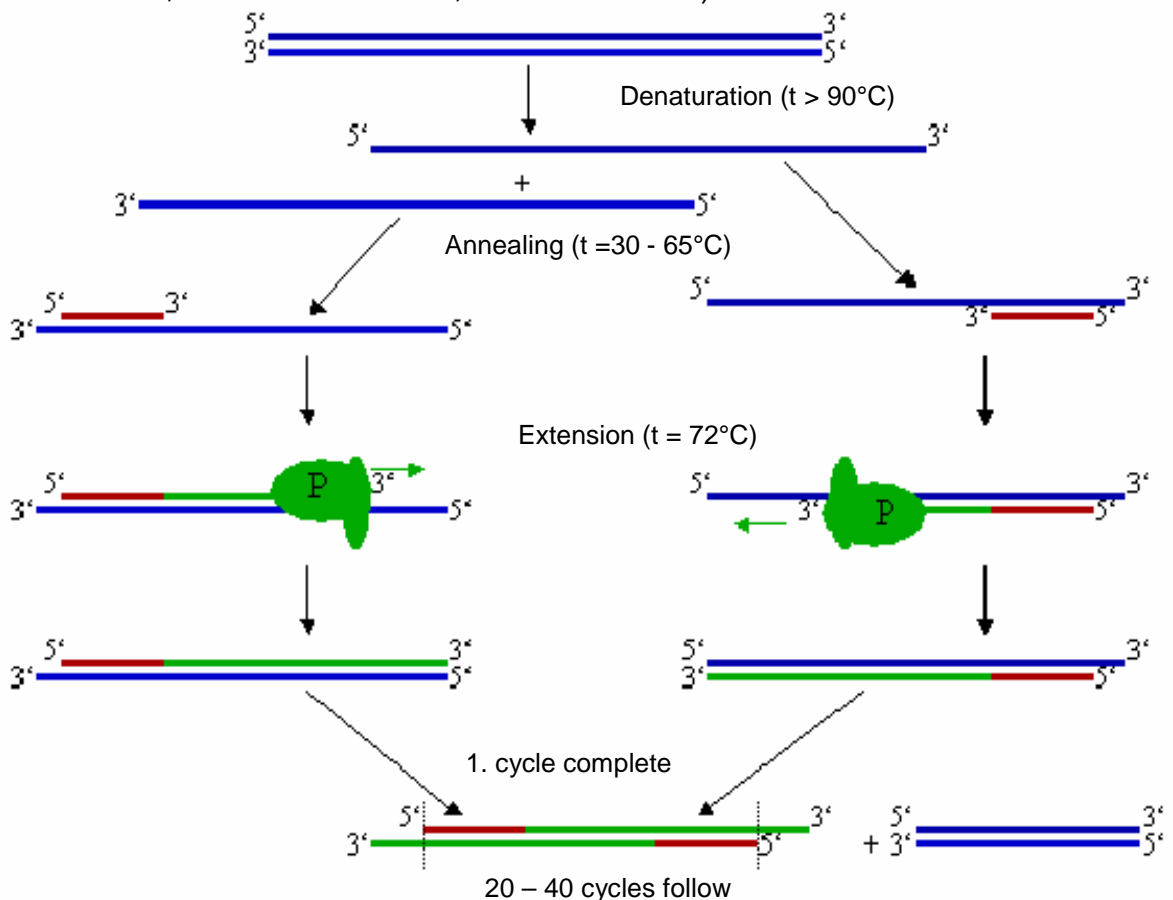
The principle of PCR appears very simple, but the results are influenced by many co-operating factors. One of the problems is for example different traits of the examined templates and primers. Another problem is risk of contamination, which is very high (Zima et al. 2004).

The PCR can be used for the detection of the deviations in the sequences of the nucleic acids, it is important for the correct diagnostics and therapy, for example by cancer or leukaemia, for the detection of bacillary and viral infections, for the prenatal diagnostics of hereditary diseases, in archaeology, judicature, criminalistics and so on (Šmarda et al. 2005).

3.3.3.1 PCR modifications

There exist many modification of the standard PCR, only some are described below.

One of the modifications is reverse PCR (RT-PCR), which allows the amplification of the RNA molecules. The RNA is transcribed to DNA and then the amplification continues by the standard way (Rosypal et Doškař 1997, Šmarda et al. 2005, Zima et al. 2004).

Inverse PCR (IPCR) serves to the amplification of the DNA sections with unknown sequences of the bases, which border the DNA sections with known sequences of their bases (Rosypal et Doškař 1997, Šmarda et al. 2005).

In asymmetric PCR is used only one primer. This modification of PCR is utilized in automatic sequencing (Rosypal et Doškař 1997).

In situ-PCR allows amplification of the specific sequences of nucleic acids straight in the cells or in the cytological preparations of tissues and chromosomes (Rosypal et Doškař 1997).

Real-time PCR allows finding out the amount of the product during the each single step. The special thermocycler is necessary (Šmarda et al. 2005, Zima et al. 2004).

By hot-start PCR some components of the reaction mixture (DNA polymerase or Mg^{2+} ions) are separated from others, till the temperature exceeds the optimal temperature for binding of the primer. In place of the separation of the components the DNA polymerase can be inactivate for the initial phase (Šmarda et al. 2005).

The nested PCR is performed in two steps. The first step contains 15 – 30 cycles with one pair of so-called external primers. The product of this first step is transferred into the new test-tube. In the second step is used the pair of the internal primers, which are specific for the internal part of the sequence, that has been amplified using the external pair of primers. The second step has 15 – 30 cycles and the product is detected by electrophoresis. The transfer of the product from the first step to the second carries the risk of contamination (Šmarda et al. 2005).

Another modification of the classical PCR, which has been used, is **"touchdown"**. Touchdown increases the specificity of the primers and so improves the results of PCR. The annealing temperature in the first cycles is higher than the specific temperature of the primer, and decreases with further cycles (Zima et al. 2004).

Another modification of the PCR is **multiplex PCR**. The PCR is performed with using of more than one pair of the primers, so several genes together are detected in one reaction mixture. There is necessary to optimise the reaction conditions for the simultaneous amplification of all products. Use of this modification reduces costs and time (Galan et al. 2003, Luikart et al. 1999, Šmarda et al. 2005).

Cross-amplification is the modification of PCR, which is performed, when the primers for investigated species are not known, so the primers for another species are used. It is better when these two species are closely related. For example the bovine microsatellites are used in cross-species amplification in goats (Galan et al. 2003, Kim et al. 2004, Zima et al. 2004).

This method is frequently applied too. It is used for testing applicability of microsatellite markers of one species on another species for various purposes – markers are tested for parentage testing, genetic diversity estimation and further population studies, and so on (Beja-Pereira et al. 2004, Kim et al. 2004, Mommens et al. 1998).

3.3.4 Electrophoresis of the PCR products

The electrophoresis of the PCR products serves for the control, if some PCR product has been amplified or not. The principle of this method is that the nucleic acids have negative charge (the negative charge is carried by the phosphate groups), so they will migrate in the electric field to the positive electrode. The shorter fragments migrate faster and they pass greater distance than the longer fragments (Rosypal et Doškař 1997, Zima et al. 2004).

It is performed on agarose or polyacrylamide gel. Agarose gels are usually horizontally situated and completely dipped in the buffer (Figure 5). The gel is prepared by dissolution of agarose in TBE buffer (Tris-borate-EDTA) and addition of the fluorescent dye – usually ethidiumbromide. Gel is filled into the form and the comb is given into the gel. The comb creates the holes in which the samples are given. The samples – the PCR products – are mixed with loading buffer, which contains a dye and glycerol or sucrose, which help to quicker falling of the sample to the bottom of the hole. To one hole there is given standard or "ladder" – the mixture of fragments with known length. It serves to comparison of the length of amplified fragments (Zima et al. 2004).

When the gel is solid, the comb can be removed. The gel is given into the electrophoresis bath with the buffer (TBE and ethidiumbromide) and the samples are applied into the holes. The source of the electrical voltage is connected and the fragments of amplified DNA migrate to the positive electrode. The fluorescent dye binds on the DNA, so there is possible to see the amplified fragments under the UV light by means of the transilluminator (Zima et al. 2004).

Figure 5: The horizontally electrophoresis (Bříza 2008 [online])
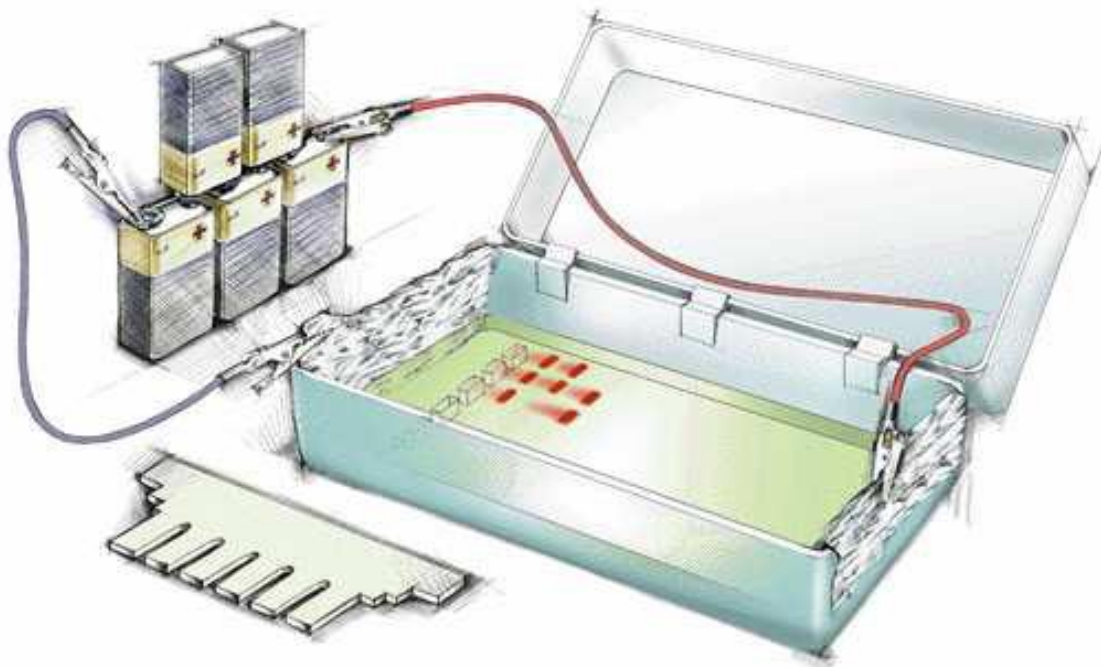
3.3.4.1 Modifications of electrophoresis

Another way of the electrophoresis is pulse gel electrophoresis. The charge do not move directly to the positive electrode, but the direction of the electric field changes periodically under the specific angle (90 – 180°) in time intervals. This technique is used for separation of the DNA molecules, several megabase large (Rosypal et Doškař 1997, Šmarda et al. 2005).

Special method of the electrophoresis of the PCR products is single-strand conformational polymorphism (SSCP). This method uses special polyacrylamide gels, which increase the sensitivity of the analyses, and is cheaper than sequencing. To the PCR the marked primers or dNTPs are used and the amplified products are denatured by adding of the formamide and sodium hydroxide and by warming. Then the products are chilled and given on the gel. On the similar principle works also denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (Zima et al. 2004).

Except the horizontally electrophoresis, which has been described above, there exist also the vertical gel electrophoresis, where the gel is situated vertically, and the capillary electrophoresis, where the gel is inside the capillary (Šmarda et al. 2005).

3.3.5 <u>DNA sequencing</u>

DNA sequencing is the way to identify the order of the bases in the DNA sequence. For this method the radioactively or fluorescently marked primers are used. There are two methods used in DNA sequencing. The first, chemical cleavage procedure or Maxam-Gilbert sequencing is based on the base-specific chemical modification and fission of the DNA fragments. The second, Sanger's enzymatic method, called also chain termination method or dideoxy method, is based on the termination of replication of the new chain according to the template of the examined sequence with dideoxynucleotidetriphosphate (ddNTP). These nucleotides have different structure than the classical dNTPs. This structure prevent binding of the next dNTP, so when the ddNTP is bound into the chain, the chain elongation terminates (Kočárek 2004). The enzymatic method is in the recent time the most usually used method. Modification of the second method is the thermal-cyclical sequencing, which uses the automatic sequencing machines. (Rosypal et Doškař 1997, Šmarda et al. 2005, Zima et al. 2004).

The sequencing in the automatic sequencing machine uses for the DNA synthesis the asymmetric PCR in the thermocycler (with *Taq* DNA-polymerase). For the detection of the PCR products the four different fluorescent marks are used, each for the detection of the PCR products, which are terminated by the specific base. For the sequencing the fluorescently marked primers are used. The primer must be synthesized four times. The specific fluorescently marked base is connected to the primer. Then the four reactions for the asymmetric PCR are prepared, each contains the DNA, four dNTPs, specific ddNTP, specific fluorescently marked primer and other reagents. The products of the four reactions are mixed and applied to the one track of the automatic analyser (Šmarda et al. 2005).

The detection of the products of sequencing proceeds automatically during the electrophoresis due to the laser detector, which is connected with the computer. In the recent time the sequencing machines are equipped with capillaries where the electrophoresis proceeds (Šmarda et al. 2005).

The fluorescently marked terminators (ddNTPs) can be used instead of the fluorescently marked primers. The PCR is performed in one test-tube, containing the DNA, primer, four dNTPs, four marked ddNTPs and other reagents. The products are separated by the electrophoresis in one track. The results of this method are better-quality, because there are visualized only the products ending with fluorescently marked ddNTP (Šmarda et al. 2005).

The prerequisites of the successful sequencing are the homogeneity of examined sequences, the high purity of template DNA and the accuracy of the amplification (Zima et al. 2004). DNA sequencing is still very expensive (Frankham et al. 2003, Zima et al. 2004).

3.3.6 Heterozygosity estimation and Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium describes the relation between genetic and genomic frequencies, which is valid for **ideal population** (model used in the population genetic). Ideal population is defined as interminably large population in which the frequencies of genes are constant. Every individual can mate with whatever individual in this population. This way of mating is called random mating or panmixia, and the population is called also panmictic population (Nečásek 1993, Zima et al. 2004).

Hardy-Weinberg equilibrium said that the allelic and genomic frequencies are constant in the ideal population. For Hardy-Weinberg equilibrium there are valid these two equations:

$$p^2 + 2pq + q^2 = 1 \qquad (1)$$

p^2 ... frequency of dominant homozygote (AA)

2pq ... frequency of heterozygote (Aa)

q^2 ... frequency of recessive homozygote

$$p + q = 1 \qquad (2)$$

p ... frequency of the dominant allele (A)

q ... frequency of the recessive allele (a)

The heterozygosity estimation is possible by means of Hardy-Weinberg equilibrium. At first the numbers of individual genomes is found out through genetic analysis. Than the genetic and genome frequencies are calculated. The observed frequencies are compared with expected frequencies (expected frequencies are deduced from the model of ideal population) (Nečásek 1993, Zima et al. 2004).

3.3.7 Use of genetic methods in praxis

The genetic methods are used to many purposes. Today is possible to create the genome map of the organism. The genetic linkage map has been created for cattle (Bishop et al. 1994).

3.3.7.1 Use of microsatellites in kinship determination

Another purpose is the parentage analysis. By this analysis there is possible to choose the most likely parents from more candidates for target offspring (Blouin 2003).

Using of microsatellite markers for kinship determination is based on the variability of the certain microsatellite locus at all tested individuals, specifically on detection of all different alleles of this locus at all tested individuals. Further it is assumed that the offspring can inherit from its parents only such alleles, which its parents have. So the kinship determination consists in the determination of all alleles of the certain microsatellite locus at the offspring and their potential parents, and finding, if the offspring really have such alleles, which they could obtain from their potential parents (*Katedra buněčné biologie a genetiky, PřF UP v Olomouci* 2008 [online]).

If the offspring have some allele, which does not occur by its potential parent, so the parent cannot be the biological parent of this offspring. For confirmation of this fact there is necessary to test several microsatellite loci, usually 3 to 6 (*Katedra buněčné biologie a genetiky, PřF UP v Olomouci* 2008 [online]).

The problems, which can cause the error, are the mutations or so-called null alleles. Null alleles occur in the genome, but there is not possible to detect them, so it seems that they are absent (*Katedra buněčné biologie a genetiky, PřF UP v Olomouci* 2008 [online], Munclinger 2007 [online]).

In praxis the microsatellite is amplified by using PCR. Resultant PCR products are evaluated by means of electrophoresis or sequencing. The same alleles have the same length, so they pass the same distance and in the electrophoresis they are side-by-side (Figure 6). Similarly in DNA sequencing the same alleles occupies the same locus (Figure 7) (Munclinger 2007 [online]).

Figure 6: Kinship determination on the basis of electrophoresis (Munclinger 2007 [online])
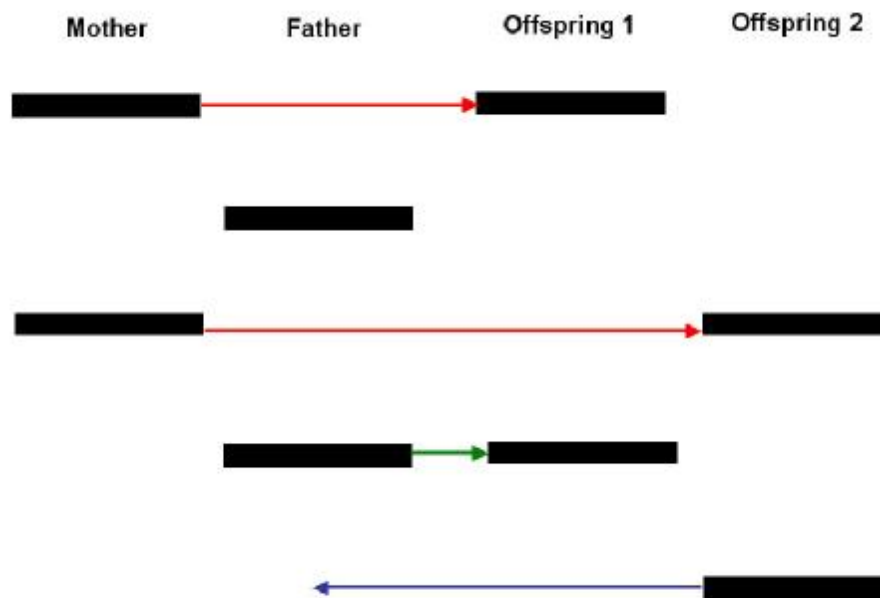
Figure 7: Kinship determination on the basis of DNA sequencing (Munclinger 2007 [online])
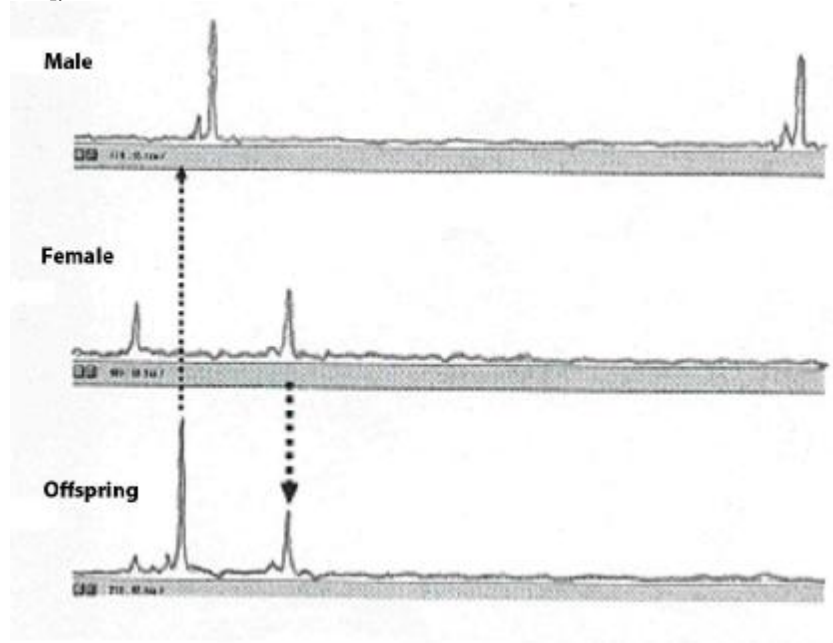
The method of DNA fingerprinting can be used, similarly as the microsatellites, for determination of the parentage, kinship and genetic diversity. It was performed for example in the populations of African and Asian lions (Gilbert et al. 1991).

Microsatellites are also used to determination of the population structure by means of the statistical methods (Balloux et Lugon-Moulin 2002).

The microsatellites were also used as supplementary method to confirm the relationships in the sheep population, which has been studied for paternal inheritance of mitochondrial DNA. The mitochondrial DNA was considered to be hereditable only from mother, male mitochondria are absent in the cells , or they are degraded after entering into the egg (Kočárek 2004). To this study were used 172 sheep samples, 6 markers for mitochondrial DNA and 12 microsatellite markers. The PCR was followed by RFLP (restriction fragment length polymorphism) analysis. The experiment confirmed the paternal inheritance of mitochondrial DNA in sheep. This phenomenon is explained by entering of the paternal mitochondrial DNA into the egg in sperm and maintaining in the egg (in small amount) or by recombination of the mitochondrial DNA into the nucleus (Zhao et al. 2004).

4. METHODOLOGY

The kinship relations in herds of the Common elands and Giant elands bred in captivity were determined according to the observation of suckling and maternal behaviour and according to the presence of the dominant male in the herd during the time of assumed mating. These direct observations, the base of studbook records, could be verify or disprove using following procedures.

4.1 <u>Sample collection</u>

The Common elands´ blood and tissue samples were collected from 22 individuals living in the farm Lány (the farm belongs to the Czech University of Life Sciences Prague) by experienced veterinarian. The blood samples were obtained during the translocation of the herd from Březová to Lány in spring 2006.

Blood and tissue samples from the Giant elands were collected from the 33 individuals living in Bandia and Fathala reserves in Senegal by experienced veterinarian. The blood samples were obtained during the translocation of animals from Bandia to Fathala reserve in March 2006 and the tissue samples were obtained by biopsy darts in September 2006 or by collecting from the dead animals.

The blood samples were heparinized and stored in the freezer by the temperature −18 or −20°C. The tissue samples were stored in the 96% ethanol in room temperature and after in 6°C.

After processing the rests of the blood and tissue samples are stored for possible further use.

4.1.1 <u>Biopsy darts</u>

For the collecting of the tissue samples there were used the Biopsy Darts by Pneu-Dart Inc. The biopsy darts have plastic body with 3 barbs and the ferrule cutter. After the shot the sharp edges of the cutter cut a piece of the skin and tissue of the animal, the barbs catch the tissue and than the whole dart falls on the ground. The ferrule is removed and the tissue is stored or further used (Figure 8) (*Pneu-Dart tranquilizer guns and remote drug delivery systems 2008* [online]).

Figure 8: The biopsy dart (*Pneu-Dart tranquilizer guns and remote drug delivery systems* 2008 [online])

4.2 DNA extraction

DNA was extracted from the blood and tissues using DNeasy Blood and Tissue kit by Qiagen (*Qiagen – Sample & Assay Technologies* 2008 [online]). The procedure was performed according to the manual enclosed by the kit.

The procedure for the blood and tissue samples is a little different. At the first step the small piece of the tissue (up to 25mg) or the small amount of anticoagulated blood (50 – 100µl) is put into the microcentrifuge tube and 20µl of the proteinase and 200µl of the buffer for tissue lysis is added. The microcentrifuge tube is mixed by the vortex and incubated by 56°C in the water bath for 10 minutes for blood samples or for a few hours for tissue samples (the incubation runs so long until the tissue is completely lysed).

After the incubation a few steps of washing follow. The one step of washing consists of transfer of the mixture into the DNeasy Mini spin column (which contains the filter) in a collection tube, addition of the washing buffer and centrifugation.

The last step serves to the elution of the DNA. The DNeasy Mini spin column is placed into the microcentrifuge tube, the elution buffer is added, the centrifugation is performed and the pure DNA (in the solution) is obtained (Figure 9).

The DNA samples are stored in the freezer by the temperature −18 or −20°C till processing. They must be marked very well.

Figure 9: Scheme of DNA extraction (DNeasy Blood and Tissue kit manual; *Qiagen – Sample & Assay Technologies* 2008 [online])
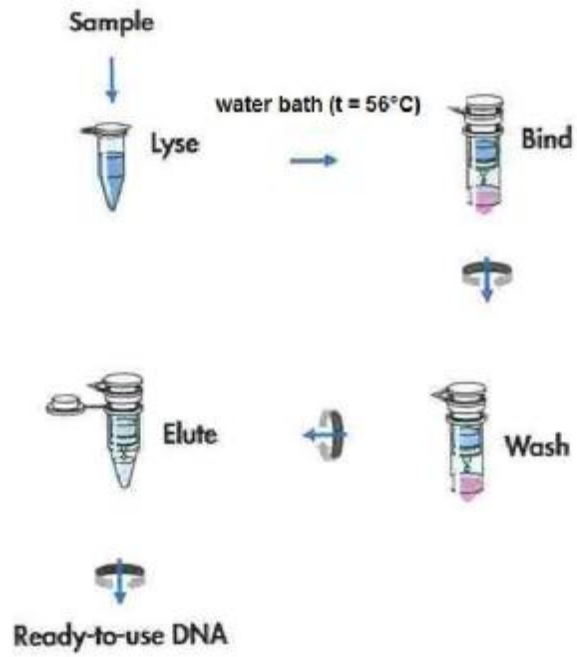
4.3 Polymerase chain reaction (PCR)

For the performation of PCR the suitable primers are necessary. The microsatellites for elands have not been developed yet. Finding of eland's microsatellites would be very laborious and expensive and so the primers for the near relatives – bovids – were used. These primers were searched in the scientific articles; also on the websites – National Centre for Biotechnology Information (*NCBI* 2008 [online]) and Goatmap (Samson et al. 2008 [online]). The primers originating from the Grant's gazelle were searched from their microsatellite sequences using the program GeneFischer (Antonínová 2007, personal communication, *BiBiServ – Bielefeld University Bioinformatics Service* 2008 [online]). These microsatellite sequences were searched on the NCBI websites before (Annexe 3).

The PCR was conducted in 20μl reaction mixture containing 1μl of the extracted DNA, 2μl of the primer (1μl of the forward and 1μl of the reverse primer), 0,4μl of the mixture of dNTPs, 2μl of $MgCl_2$, 2μl of PCR buffer (containing $(NH_4)_2SO_4$) and 0,16μl of *Taq* polymerase, replenished to the total volume with 12,44μl ddH_2O (Antonínová 2007, personal communication). The reaction mixture was prepared on the ice to prevent the activation of the *Taq* polymerase and the formation of nonspecific products.

The PCR conditions were set according to the literature, in which the primers were used before. The different conditions were tested to find the most suitable (Annexe 4, Annexe 5). Touchdown was used in some PCR – the annealing temperature was by 10°C higher than the specific annealing temperature of the primer, the temperature decreases with each primer (10 cycles were performed). When the annealing temperature reached the specific annealing temperature, the appropriate number of cycles was performed according to the given PCR conditions.

Peelman et al. (1998) devise for the PCR conditions to the websites of International Society for Animal Genetics (*ISAG:: Comparison Test Guidelines* 2008). The websites usher only actual information, so the PCR conditions were adapted (initial temperature and the times) according to the actual information on ISAG.

The PCR underwent in the DNA engine Peltier Thermal Cycler – 200 by MJ Research.

The final PCR products have been stored by the temperature of -18°C or directly used for electrophoresis.

4.4 <u>Electrophoresis</u>

The PCR products were separated on a 1% agarose gel for verification of the presence of some PCR product. 3µl of the PCR product were mixed with 0,5µl of loading dye (6x Loading Dye Solution by Fermentas). The electrophoresis ran 20 – 30 minutes by 120V. For the transillumination the Electronic UV Transilluminator Ultra-Lum was used. The photos of the gels were performed by Canon PowerShot G5 camera.

If the PCR product was present, another electrophoresis on 3% high-resolution agarose gel was performed for polymorphism determination. The electrophoresis ran 1,5 - 2 hours by 100V.

For comparison of the length of fragments the ladder GeneRuler^{TM} 100bp DNA Ladder Plus by Fermentas was used.

4.5 <u>DNA sequencing</u>

The 2 polymorphic loci, BM 1818 and L 37 208, were used for sequencing. The next PCR was performed with using of fluorescently marked primers (BM 1818 – FAM and L 37 208 – HEX), for each primer was used 15 samples of the Common eland and in 1 sample the DNA was replaced by the water. The PCR products were separated on 3% high-resolution agarose gel.

The samples with PCR product were purified with QiaQuick Purification kit by Qiagen (*Qiagen – Sample & Assay Technologies* 2008 [online]). The purification is necessary to remove the rests of components, which have been used in PCR. The procedure was performed according to the manual enclosed by the kit. At first the admixtures are washed away in the QiaQuick column with the filter by the washing buffer and centrifugation. In the second step the QiaQuick column is placed into the centrifuge tube, the elution buffer is added and the PCR product (amplified DNA) is eluted by centrifugation.

The concentration of the purified PCR product is measured by the spectrophotometer Jenway Genova. The mixture for DNA sequencing contains 1µl of the forward primer (not fluorescently marked), specific amount of the PCR product – according to its concentration and the mixture is replenished to the volume 14µl by the redistilled water. The samples are analysed in the Sequencing laboratory of the Faculty of Science (Charles University in Prague, *SEQLAB* 2008 [online]).

The Sequencing laboratory uses the four-capillary 3100 Avant Genetic Analyser, or four-capillary 3130 Genetic Analyser. For the sequencing the 50cm capillary is used, the sample is carried by the polymer POP-6 by 3100 Avant Genetic Analyse, or in the case of the 3130 Analyser by the polymer POP-7.

For the sequencing reaction the Sequencing laboratory uses the kit BigDye[®]Terminator v3.1 Cycle Sequencing Kit by Applied Biosystems.

5. RESULTS

In all the 44 markers were tested, 17 from cattle (*Bos taurus*), 20 from goats (*Capra hircus*), 4 from sheep (*Ovis aries*) and 3 from Grant's gazelle (*Gazella granti*) (Annexe 3).

Each primer was tested on 6 samples – 3 samples of the Common eland, 3 samples of the Giant eland and additional sample "negative control" – the DNA was replaced by water (so called blind sample). The ability of amplification was found out on the 1% agarose gel (Figure 10), the level of polymorphism was found out on the 3% high-resolution agarose gel (Figure 11).

Figure 10: Gel transilluminated with UV light.
L = ladder, numbers = samples, A = negative control (Photo by author)
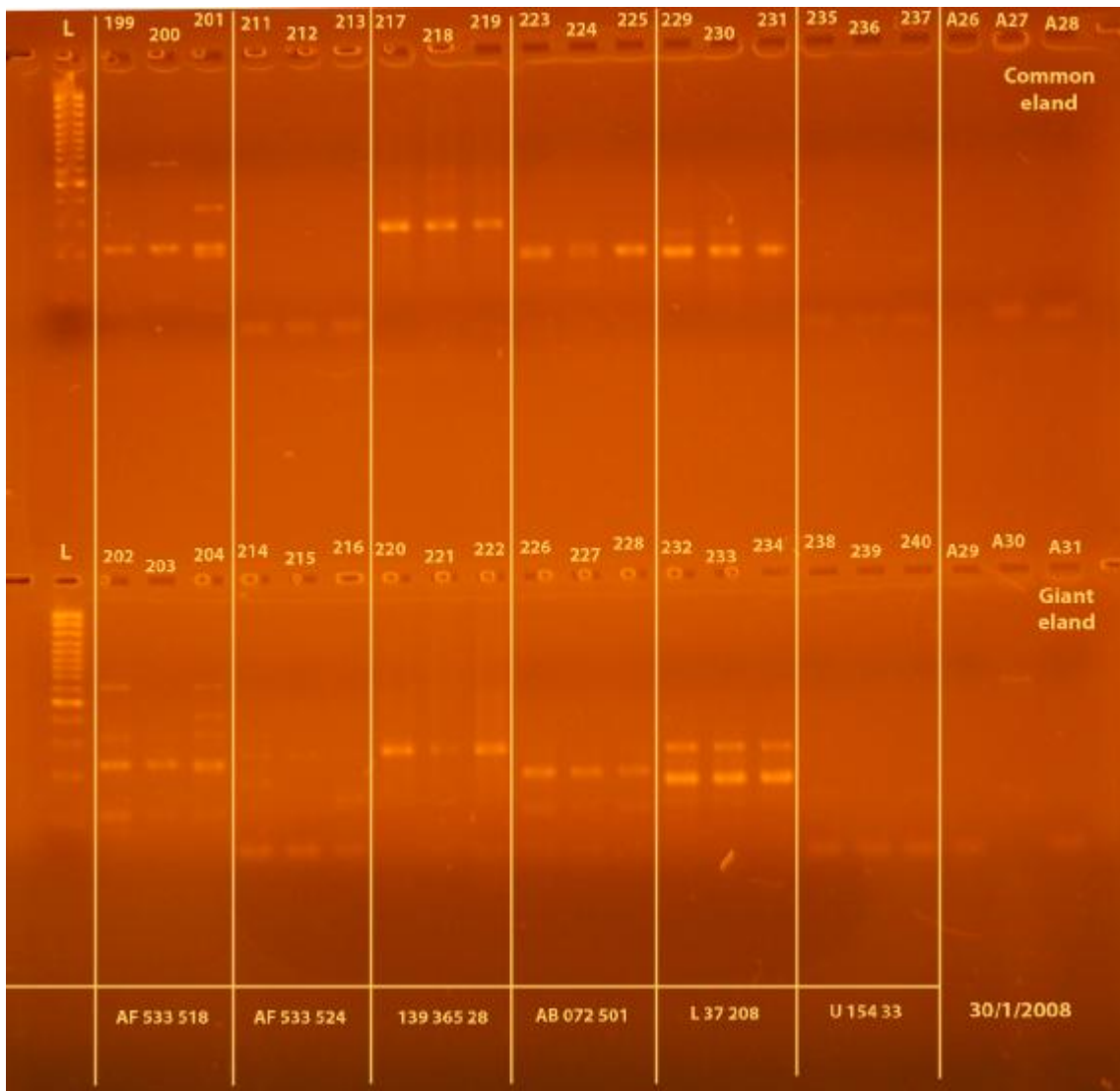
Figure 11: High-resolution gel.
L = ladder, numbers = samples, double stripes = polymorphic loci (Photo by author)
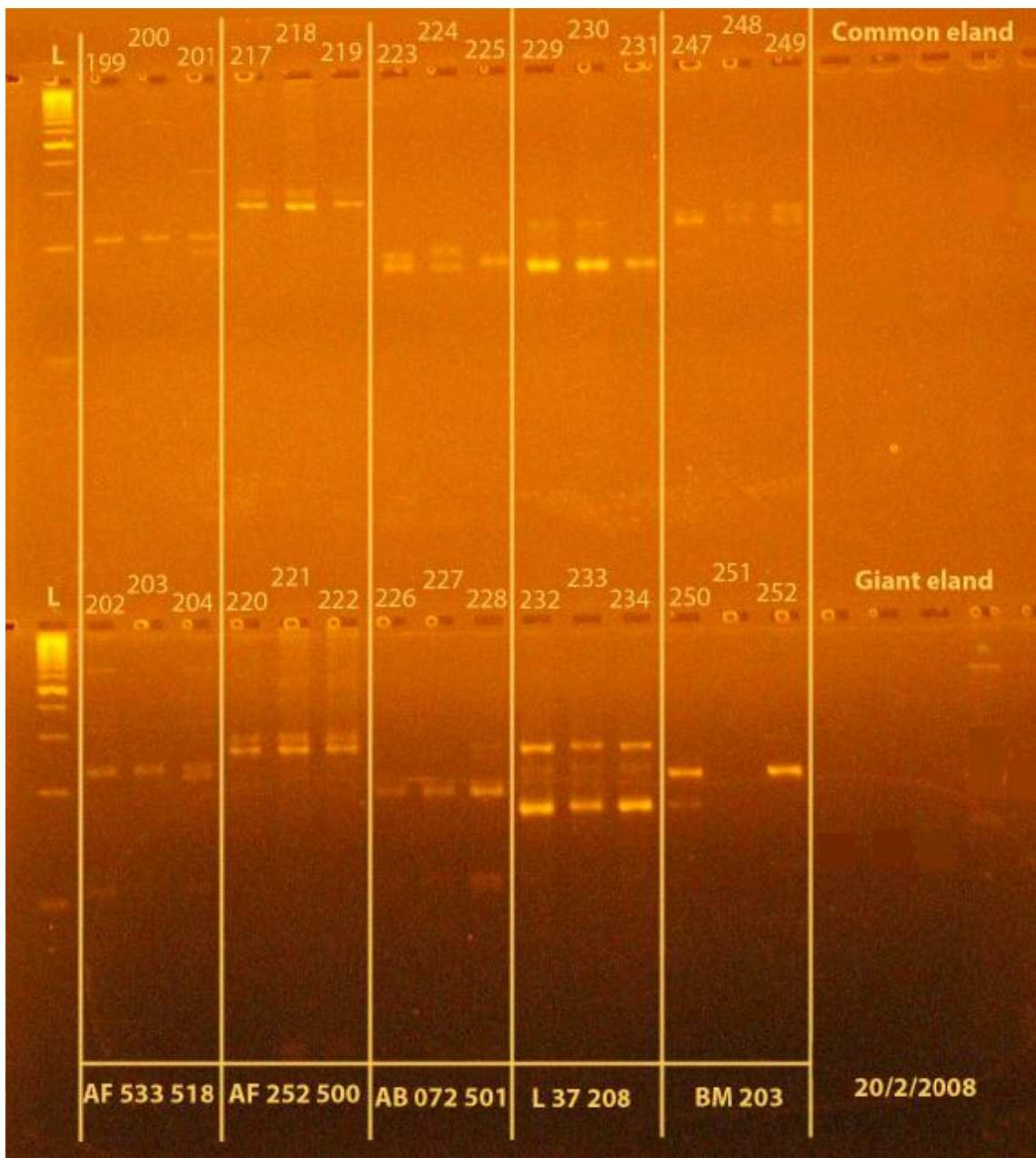
Various PCR conditions were tested to find the most suitable, it means the conditions, when the primer gave the best amplification (Annexe 5). All, except one primer, had to be tested more than once. The Figure 12 describes how many reactions were performed for how many primers.

Figure 12: Number of primers and number of reactions performed for them.
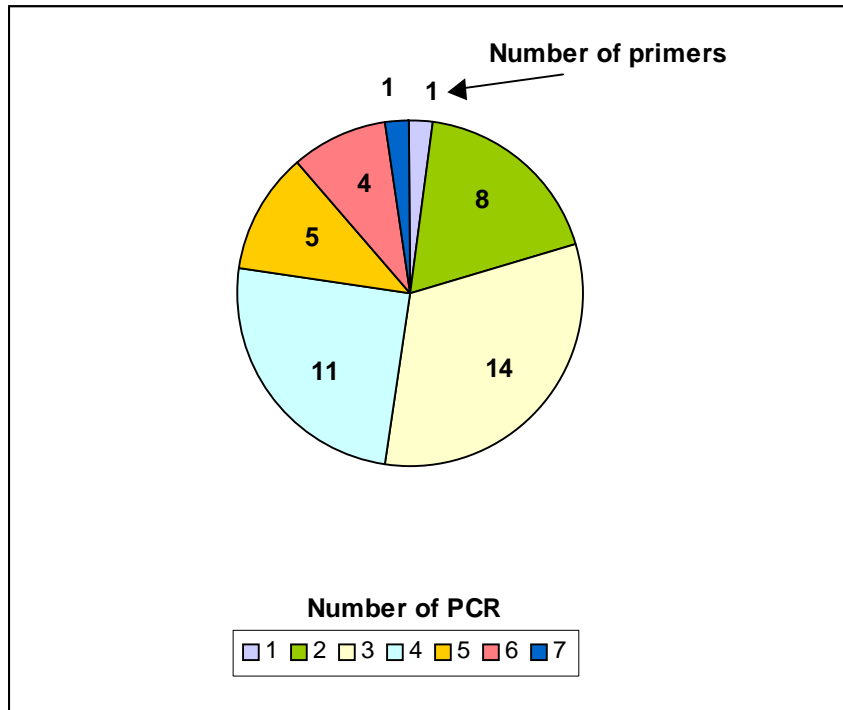
In the Common eland 10 primers were polymorphic, 9 monomorphic, 23 gave poor or no amplification and 2 gave nonspecific amplification products.

In the Giant eland 6 primers were polymorphic, 15 monomorphic, 21 gave poor or no amplification and 2 gave nonspecific amplification products (Figure 13).

Figure 13: The ability of amplification of the 44 primers in the Common eland and Giant eland.
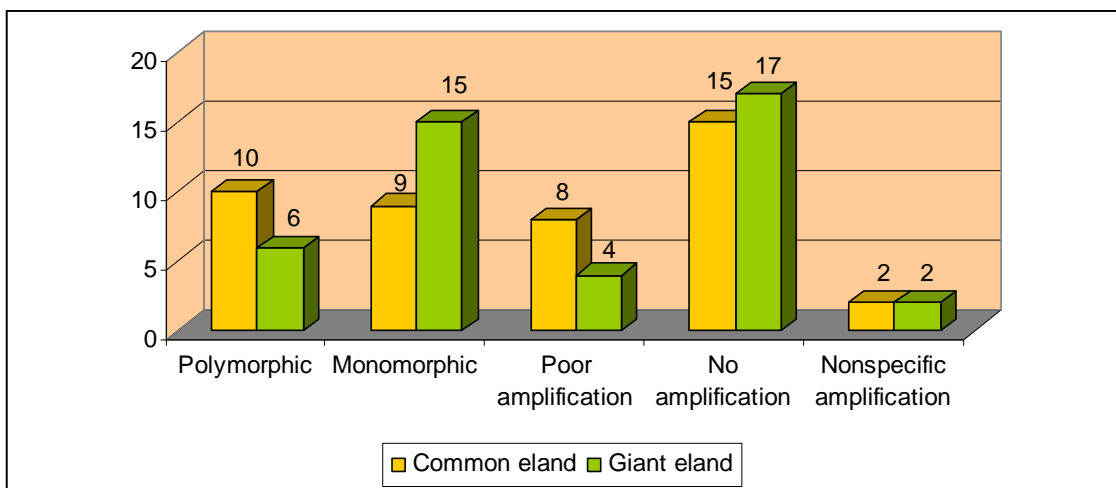
There was assumed that the primers polymorphic in the Common eland could be polymorphic also in the Giant eland, but 3 primers (AF 533 518, BM 4505 and IDVGA 8) were polymorphic in Giant eland and they were monomorphic in Common eland, and 1 primer (SRCSRP 24) was polymorphic in the Giant eland but in the Common eland showed only poor amplification. Only 2 primers (AF 252 500 and L 37 208) were polymorphic both in the Common eland and in the Giant eland (Table 1).

Table 1: Polymorphic primers in the Common and Giant eland

The Common eland			
Locus	Annealing temperature (°C)	Origin	PCR conditions
AB 072 501	55	*Bos taurus*	Adapted conditions
AF 252 500	50	*Bos taurus*	Adapted conditions
AF 533 521	50	*Gazella granti*	Adapted conditions
BM 203	50	*Capra hircus*	Beja-Pereira et al. (2004)
BM 1443	50	*Ovis aries*	Beja-Pereira et al. (2004)
BM 1818	50	*Capra hircus*	Beja-Pereira et al. (2004)
CSSM 39	50	*Bos taurus*	Galan et al. (2003)
ETH 10	50	*Capra hircus*	Beja-Pereira et al. (2004)
INRABERN 192	55	*Capra hircus*	Antonínová (2007, personal communication)
L 37 208	55	*Bos taurus*	Adapted conditions
The Giant eland			
Locus	Annealing temperature (°C)	Origin	PCR conditions
AF 252 500	50	*Bos taurus*	Adapted conditions
AF 533 518	50	*Gazella granti*	Adapted conditions
BM 4505	50	*Ovis aries*	Beja-Pereira et al. (2004)
IDVGA 8	55	*Bos taurus*	Galan et al. (2003)
L 37 208	55	*Bos taurus*	Adapted conditions
SRCRSP 24	50	*Capra hircus*	Beja-Pereira et al. (2004)

From the tested PCR conditions the best worked the conditions used in the reference articles of Galan et al. (2003) and Beja-Pereira et al. (2004). These conditions worked also for the primers, which were not used in these articles. Conversely as less functional proved the PCR conditions used in the articles of Luikart et al. (1999), Kim et al. (2004) and Peelman et al. (1998), which did not worked neither for the primers used by the authors (Annexe 5). This fact can have many causes because the PCR can be influenced by many factors.

Adapted conditions were used for some primers, which were not mentioned in the articles and I have insufficient information about them or for the primers, which did not amplify by the conditions originally used by the authors. Similarly the conditions according to Antonínová (2007, personal communication) were tested for these primers.

Adaptation of the PCR condition consisted of the adaptation of the initial temperature and individual times for each step of the reaction (Annexe 4). Also the amount of the components (MgCl$_2$, DNA...) in the PCR reaction mixture was adapted to increase the possibility of obtaining of the best results.

On the basis of electrophoresis results 2 primers for DNA sequencing examination were chosen - BM 1818 and L 37 208. These primers were fluorescently marked: BM1818 – FAM, L 37 208 – HEX and used in PCR for 15 pilot samples with Common eland DNA. The first results of DNA sequencing gave poor sequencing products insufficient for heterozygosity estimation.

6. DISCUSSION

Twenty-five from 44 tested primers did not amplified, gave poor or none amplification in the Common eland and 23 in the Giant eland. The inability to amplify or give applicable product can be caused by many reasons, because the results of PCR can be influenced by many factors.

Very important is the annealing temperature, which is specific for each primer, but different authors used different annealing temperature – for example Beja-Pereira et al. (2004) uses for the primer INRA 005 the annealing temperature 45°C, but Kim et al. (2004) uses 55°C.

When I tested primer with different annealing temperatures in different references, at first I chose the annealing temperature randomly (but usually the lower temperature was tested at first, because the lower temperature decreases the specificity of the primer (Zima et al. 2004)). When the primer did not amplify, the PCR reaction was performed again with different annealing temperature, but the results were in all cases the same – no amplification. This is the case of CSSM 014, CSSM 022, ETH 3, INRA 005 and INRA 063 (Annexe 5).

With the annealing temperature relates the PCR conditions, which differ also in different tasks. The temperature in the last step – extension – is uniform in all authors, but the temperatures in the first step differ – from 94°C (Galan et al. 2003, Kim et al. 2004) to 96°C (Antonínová 2007 personal communication, Beja-Pereira et al. 2004). Also the times for which the temperatures are held on are very different. Some authors use the same times for the temperatures in the second step (Beja-Pereira et al. 2004, Galan et al. 2003, Kim et al. 2004) but some authors also use the different times in this step (Antonínová 2007 personal communication, Luikart et al. 1999). The times vary from 15 seconds (Luikart et al. 1999) to 1 minute (Antonínová 2007 personal communication, Galan et al. 2003, Kim et al. 2004). Also the times in the first and last step differ. All authors, except Antonínová (2007, personal communication) use the time 10 minutes in the last step. The times in the first, initial step vary from 2 minutes (Antonínová 2007, personal communication) to 12 minutes (Galan et al. 2003).

In adapted conditions were used the 95°C in the first step and the same time in the second step – 30 seconds.

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The number of cycles is the next thing diverging by different authors. The number of cycles is 30 (Kim et al. 2004), 35 (Galan et al. 2003, Luikart et al. 1999) or 40 (Antonínová 2007 personal communication, Beja-Pereira et al. 2004). Beja-Pereira et al. (2004) uses touchdown in addition. The adapted conditions had 35 cycles and touchdown was also used.

Further factor influencing the successfulness of the PCR is the rate of the components in the mixture for PCR. Again the authors use different volumes of the PCR mixture and different rates of the components. For example Galan et al. (2003) and Luikart et al. (1999) used the total volume of the PCR mixture 25μl, Beja-Pereira et al. (2004) 15μl, Kim et al. (2004) 12,5μl and so on. I used 20μl of the PCR mixture.

The inability of the primers to amplify in antelopes can be also caused by the primer alone – there is possible that the sequence of the primer is not suitable for the antelopes and the primer is not able to amplify in the elands.

Some primers were tested in the Common elands before, but the results are different than the results of this thesis. For example the primer MCHII – DR was classified as polymorphic (Antonínová 2007, personal communication), but in this thesis it gave only poor amplification in the Common eland by using identical conditions and also identical composition of the PCR mixture. Zima et al. 2004 explains such cases so, that the results of PCR reactions performed with identical conditions can differ when the reactions are performed in different laboratories.

The ability of primers´ amplification diminishes also the cross-amplification, especially when the primers are used for distantly related species. The bovine, caprine and ovine microsatellites are usually used for cross-amplification studies, but also the microsatellites from reindeer (Galan et al. 2003). The animals, which were tested for applicability of these microsatellites in cross-amplification studies before are African buffalo (Van Hooft et al. 1999, Van Hooft et al. 2000), roe deer (Galan et al. 2003), dorcas gazelle (*Gazella dorcas*) and Barbary sheep (*Ammotragus lervia*) (Beja-Pereira et al. 2004), American bison (Mommens et al. 1998), or Korean goral (Kim et al. 2004) – closed related of bovids. The numbers of tested individuals move again between 6 and 60, and the number of tested markers between 14 and 168 (Beja-Pereira et al. 2004, Galan et al. 2003, Galan et al. 2004, Kim et al. 2004, Mommens et al. 1998, Van Hooft et al. 1999, Van Hooft et al. 2000).

In this thesis 3 - 15 individuals from each species were tested for each of 44 primers.

Cross–amplification is not used only for bovids, but also for example for the rodents of the genus *Mastomys* (Galan et al. 2004).

In parentage analysis is very popular also the multiplex method. The bovine markers, divided into several multiplexes are tested on cattle or goats, often on different breeds. The numbers of tested animals are very various - from 128 (Luikart et al. 1999) or 200 (Peelman et al. 1998) to 1333 individuals (Heyen et al. 1997). Also the numbers of tested microsatellite markers are very various – from about 20 (Galan et al. 2004, Heyen et al. 1997, Peelman et al. 1998) to 41 (Luikart et al. 1999). The multiplexes have usually 3 or 4 markers (Galan et al. 2003, Heyen et al. 1997, Peelman et al. 1998), but the multiplex of 2 (Galan et al. 2003) or 11 markers (Galan et al. 2004, Luikart et al. 1999) are also possible.

In this thesis were used only separate primers, not multiplexes. In multiplexes there is possible to combine the primers with the same annealing temperatures. The same PCR conditions are advantageous, but not necessary – they can be modified. But there is essential to create the multiplexes with primers, which give good amplification.

Our study is in the first phase – we are searching for several reliable polymorphic markers, which could be suitable for multiplex reaction. These primers must be properly tested.

The primary sequencing was performed with two chosen primers (BM1818 and L37 208) in Common eland. The results are not satisfactory, it can be caused by incorrectly chosen amount of the PCR product and the further experimental work is necessary to optimise the sequencing conditions.

The populations of the Common and Giant eland in captivity are very small. In the Giant eland population in captivity occurs inbreeding, because of the population has been established with 6 founders (1 male and 5 females), there is no chance in present time to reinforce this population with individuals from the wild. The kinship relations are managed with regard on these relations registered in studbook.

The situation of the population of Common elands is a little bit better. The Common elands are also bred in zoos, these populations are small too, but there is possible to change the animals among the institutions and facilitate mating of different individuals. The selection of breeding individuals conducts the coordinator (for the certain species) on the basis of studbooks. The problem could be that the individuals bred in captivity for longer time have often the same ancestors in the past.

Leading of the studbook is necessary for management of captive populations. On the basis of the studbooks there is possible to decide which individuals are suitable for further reproduction and choose the most suitable mating partner to them. There also exist computer programs (Breeders manager, GenoPro, Genepop...), which are able to create the pedigree, count the heterozygosity and also count the probability of extinction of the population.

Obtaining information by means of observing is also difficult – especially in wildlife populations, but in captive populations can also come the problem, for example when in the herd is present except the adult male also the young, almost adult male. So in the cases, when the parents are not certain and observation is not successful, genetics can help.

By genetic analyses there can be another problem (except high costs and time-consumption) – samples obtaining. There is possible to obtain the tissue samples by means of the biopsy darts, but the darts are quite expensive and in the field sometimes difficult to find. Blood samples are more difficult to be obtained, it is necessary to immobilize the animals by professional veterinarian. There is not possible to narcotize the animals bred in captivity only because of sample taking and taking of samples without narcotization is rather exceptional – like by freshly born hidden calf, where is taken again the tissue sample rather than blood.

Another possibility of DNA obtaining are non-invasive methods like DNA isolation from faeces. The DNA isolation from faeces is more complicated, expensive and the amount of DNA is smaller and quality lower than in invasive methods. Big advantage are the commercial kits for obtaining DNA by non-invasive way (like QIAamp DNA Stool Mini Kit, *Qiagen – Sample & Assay Technologies* 2008 [online]). This method was also successfully tested and applied in various species (Creel et al. 2003, Flagstad et al. 1999, Morin et al. 2001)

7. CONCLUSION

We tested bovine, caprine, ovine and gazelles' microsatellite markers on two small populations of antelopes, which belong to the same genus *Taurotragus*. These markers were tested for the purpose of verifying of their applicability in parentage testing.

There exist many genetic methods for kinship determination, but the microsatellites are most suitable for the parentage testing, because of their variability. Also the cross-amplification is very popular and successful method, but it requires range of tests for finding of the most suitable markers and reaction conditions.

Forty-four markers were tested on selected individuals of Common and Giant elands. The 10 markers in the Common eland and 6 in the Giant eland were classified as polymorphic. These markers can be utilized for determination of the kinship.

The meaning of parentage testing in the populations is to find out the kinship relations among the individuals. It has the big value especially in small population, where inbreeding occurs. The best solution how to prevent inbreeding would be to outcross an unrelated individual (preferably the male from the wild). But it is very complicated and in many cases not performable. Most acceptable solution under these conditions is choice of as few as possible related individuals for the next reproduction. To this can help the studbook, which gather the information about the individuals of some species. According to the data in studbook the unrelated individuals for breeding can be chosen.

Inbreeding prevention is important for conservation of the genetically healthy population, especially when this population is predetermined for reintroduction.

The genetic tests are very useful, when the suitable and working markers and methods are used. The genetic tests can help with population management. The disadvantages of the genetic analyses are higher costs in comparison with other methods like observation. That is why the observation is in present time on the first position between breeders and then, if it is necessary, the results are verified genetically.

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9. ANNEXES

LIST OF ANNEXES

Annexe 1: The family tree of the Common elands bred in captivity in farm breeding of Czech University of Life Sciences Prague in Lány.

¨ = male, ¡ = female, ? = gender unknown, × = dead individual, colours = related individuals (families), smaller symbols = animals bred in zoos, not in Lány (Created in the programme GenoPro 2007)
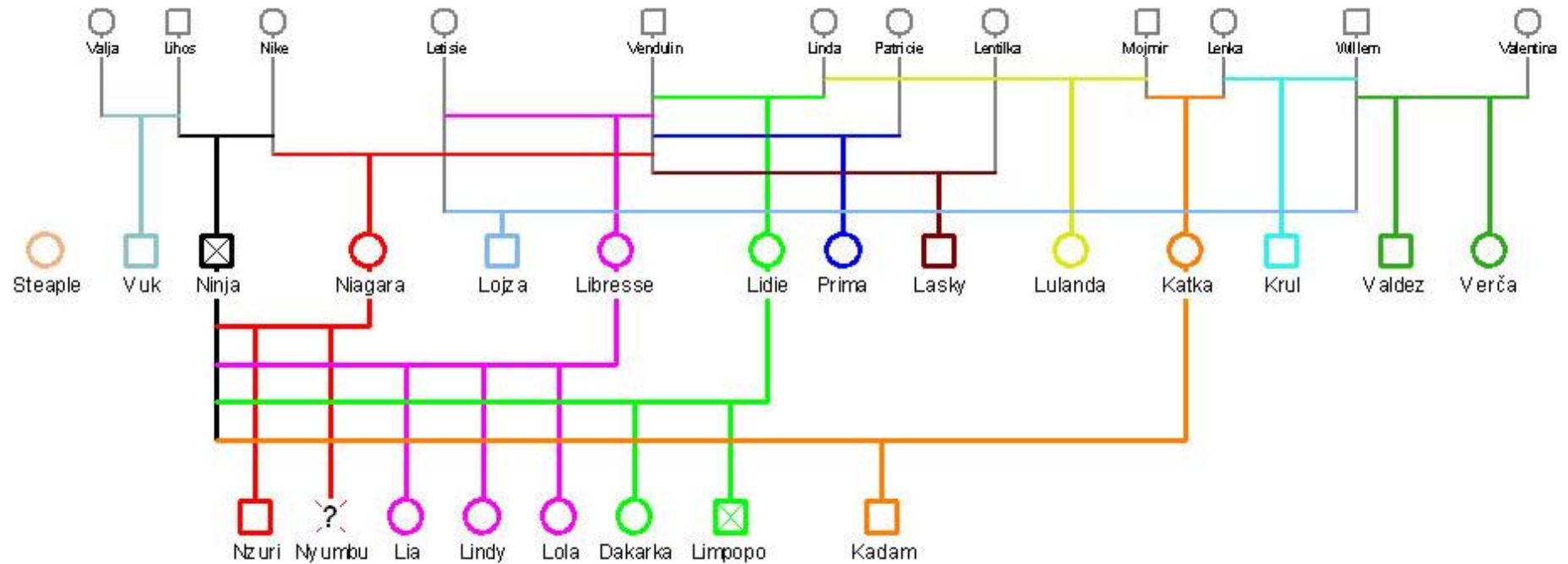
Annexe 2: The family tree of the Giant elands bred in semi-captivity in Bandia and Fathala reserves.
¨ = male, ¡ = female, ? = gender unknown, × = dead individual, colours = related individuals (families) (Created in the programme GenoPro 2007)
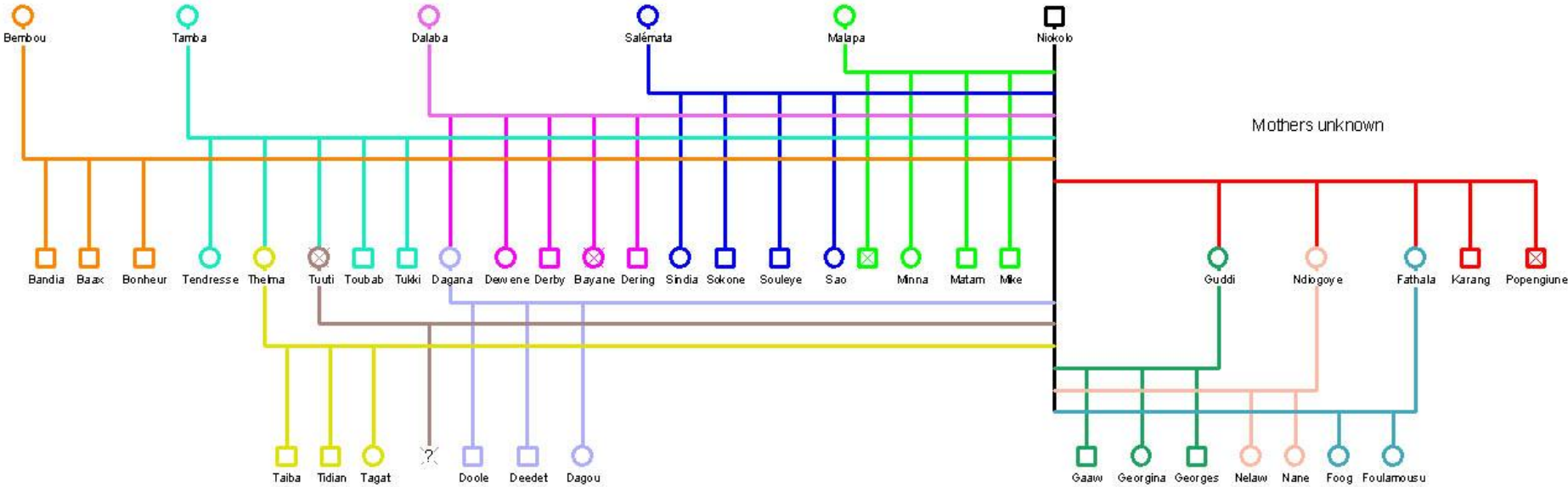
Annexe 3: Results of cross-amplification tests and level of polymorphism

Locus	Primer sequence	Annealing temperature (°C)	Amplification		Origin	References
			in Common eland	in Giant eland		
AB 072 501	GCA GTC CAA GGG ACT CTC AA TAC CCT CCA ATC CCA TGT TG	55	Polymorphic	Monomorphic	*Bos taurus*	Antonínová (2007, personal communication)
AF 252 500	AGG AGT TGC TGA TGG ACA TCT GTT CAG CTT GGG TGA	50	Polymorphic	Polymorphic	*Bos taurus*	Antonínová (2007, personal communication)
AF 533 518	CAG GAA GAC CTG TAT GGA AAT CTA TGC CTG GGA GGA	50	Monomorphic	Polymorphic	*Gazella granti*	Antonínová (2007, personal communication)
AF 533 521	TCC AGA TGG TAT TTT CCT CA CCA GTG TTT TAC CGA GCA	50	Polymorphic	Monomorphic	*Gazella granti*	Antonínová (2007, personal communication)
AF 533 524	CAT GCT TCT CTA TCT GGG TA CCT TTC TTA GGG ACT GGA A	50	No amplification	No amplification	*Gazella granti*	Antonínová (2007, personal communication)
BM 203	GGG TGT GAC ATT TTG TTC CC CTG CTC GCC ACT AGT CCT CT	50	Polymorphic	Monomorphic	*Capra hircus*	Beja-Pereira et al. (2004)
BM 848	TGG TTG GAA GGA AAA CTT GG CCC TCT GCT CCT CAA GAC AC	55	Monomorphic	Monomorphic	*Bos taurus*	Galan et al. (2003)
BM 1443	AAT AAA GAG ACA TGG TCA CCG G TCG AGG TGT GGG AGG AAG	50	Polymorphic	Monomorphic	*Ovis aries*	Beja-Pereira et al. (2004)
BM 1706	ACA GGA CGG TTT CTC CTT ATG CTT GCA GTT TCC CAT ACA AGG	50	Poor amplification	Monomorphic	*Bos taurus*	Galan et al. (2003)
BM 1818	AGC TGG GAA TAT AAC CAA AGG AGT GCT TTC AAG GTC CAT GC	50	Polymorphic	Monomorphic	*Capra hircus*	Beja-Pereira et al. (2004), Kim et al. (2004), Peelman et al. (1998), Antonínová (2007, personal communication)
BM 1824	GAG CAA GGT GTT TTT CCA ATC CAT TCT CCA ACT GCT TCC TTG	55	Poor amplification	No amplification	*Bos taurus*	Kim et al. (2004), Peelman et al. (1998)

Locus	Primer sequence	Annealing temperature (°C)	Amplification in Common eland	in Giant eland	Origin	References
BM 2113	GCT GCC TTC TAC CAA ATA CCC CTT CCT GAG AGA AGC AAC ACC	55	Poor amplification	Poor amplification	*Bos taurus*	Kim et al. (2004), Peelman et al. (1998)
BM 4505	TTA TCT TGG CTT CTG GGT GC ATC TTC ACT TGG GAT GCA GG	50	Monomorphic	Polymorphic	*Ovis aries*	Beja-Pereira et al. (2004), Antonínová (2007, personal communication)
BMC 1009	GCA CCA GCA GAG AGG ACA TT ACC GGC TAT TGT CCA TCT TG	50	Monomorphic	Monomorphic	*Bos taurus*	Galan et al. (2003), Antonínová (2007, personal comunication)
CSSM 014	AAA TGA CCT CTC AAT GGA AGC TTG AAT TCT GGC ACT TAA TAG GAT TCA	60	No amplification	No amplification	*Bos taurus*	Peelman et al. (1998)
CSSM 022	TCT CTC TAA TGG AGT TGG TTT TTG ATA TCC CAC TGA GGA TAA GAA TTC	60	No amplification	No amplification	*Bos taurus*	Peelman et al. (1998)
CSSM 39	AAT CGG AAC CTA GAA TAT TTT GAG AGA TAA AAT GTG AGT GTG GTC TCC	50	Polymorphic	Monomorphic	*Bos taurus*	Galan et al. (2003), Antonínová (2007, personal comunication)
ETH 3	GAA CCT GCC TCT CCT GCA TTG G ACT CTG CCT GTG GCC AAG TAG G	65	No amplification	No amplification	*Bos taurus*	Peelman et al. (1998), Kim et al. (2004)
ETH 10	GTT CAG GAC TGG CCC TGC TAA CA CCT CCA GCC CAC TTT CTC TTC TC	50	Polymorphic	Monomorphic	*Capra hircus*	Luikart et al. (1999), Peelman et al. (1998), Antonínová (2007, personal communication)
ETH 225	GAT CAC CTT GCC ACT ATT TCC T ACA TGA CAG CCA GCT GCT ACT	45	Poor amplification	Poor amplification	*Bos taurus*	Peelman et al. (1998), Beja-Pereira et al. (2004), Kim et al. (2004)
HUJ 1177	TCC ATC AAG TAT TTG AGT GCA A ATA GCC CTA CCC ACT GTT TCT G	55	Monomorphic	Monomorphic	*Bos taurus*	Galan et al. (2003), Antonínová (2007, personal comunication)
IDVGA 8	CTC TTG GGG GCG TGT TGT CT TAG CAG AAA GCA CAG GAG TC	55	Monomorphic	Polymorphic	*Bos taurus*	Galan et al. (2003), Antonínová (2007, personal comunication)

Annexe 3 – continued:

Locus	Primer sequence	Annealing temperature (°C)	Amplification in Common eland	in Giant eland	Origin	References
IDVGA 29	CCC ACA AGG TTA TCT ATC TCC AG CCA AGA AGG TCC AAA GCA TCC AC	50	Monomorphic	Monomorphic	*Bos taurus*	Galan et al. (2003), Antonínová (2007, personal comunication)
INRA 005	CAA TCT GCA TGA AGT ATA AAT AT CTT CAG GCA TAC CCT ACA CC	45	No amplification	No amplification	*Capra hircus*	Beja-Pereira et al. (2004), Kim et al. (2004), Antonínová (2007, personal communiation)
INRA 040	TCA GTC TCC AGG AGA GAA AAC CTC TGC CCT GGG GAT GAT TG	45	Poor amplification	Poor amplification	*Capra hircus*	Beja-Pereira et al. (2004), Galan et al. (2003), Antonínová (2007, personal communication)
INRA 063	ATT TGC ACA AGC TAA ATC TAA CC AAA CCA CAG AAA TGC TTG AAA G	50	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999), Kim et al. (2004), Antonínová (2007, personal communication)
INRABERN 192	AGA CCT TTA CAG CCA CCT CTT C GTC CCA GAA ACT GAC CAT TTT A	55	Polymorphic	Monomorphic	*Capra hircus*	Antonínová (2007, personal communication)
L 37 208	AGT CTG AAG GCC TGA GAA CC CTT ACA GTC CTT GGG GTT GC	55	Polymorphic	Polymorphic	*Bos taurus*	Antonínová (2007, personal communication)
MAF 065	AAA GGC CAG AGT ATG CAA TTA GGA G CCA CTC CTC CTG AGA ATA TAA CAT G	55	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
MAF 70	GCA GGA CTC TAC GGG GCC TTT GC CAC GGA GTC ACA AAG AGT CAG ACC	50	Nonspecific amplification	Nonspecific amplification	*Capra hircus*	Beja-Pereira et al. (2004)
MCHII - DR	GGA CAC GTT CTT GCA GAT ACA ACT AC GAA CTC TCC TTA AGC ATA CTT GCT C	45	Poor amplification	No amplification	*Capra hircus*	Luikart et al. (1999), Antonínová (2007, personal communication)
MM 12	CAA GAC AGG TGT TTC AAT CT ATC GAC TCT GGG GAT GAT GT	45	Poor amplification	Poor amplification	*Ovis aries*	Beja-Pereira et al. (2004), Kim et al. (2004), Antonínová (2007, personal communiation)
Oar FCB 048	GAC TCT AGA GGA TCG CAA AGA ACC AG GAG TTA GTA CAA GGA TGA CAA GAG GCA	55	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)

Annexe 3 – continued:

Locus	Primer sequence	Annealing temperature (°C)	Amplification in Common eland	in Giant eland	Origin	References
Oar FCB 304	CCC TAG GAG CTT TCA ATA AAG AAT CGG CGC TGC TGT CAA CTG GGT CAG GG	50	Monomorphic	Monomorphic	*Ovis aries*	Galan et al. (2003), Antonínová (2007, personal comunication)
SRCRSP 5	GGA CTC TAC CAA CTG AGC TAC AAG TGA AAT GAA GCT AAA GCA ATG C	55	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
SRCRSP 7	TCT CAG CAC CTT AAT TGC TCT GGT CAA CAC TCC AAT GGT GAG	50	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
SRCRSP 9	AGA GGA TCT GGA AAT GGA ATC GCA CTC TTT TCA GCC CTA ATG	55	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
SRCRSP 12	TGA CCA GGT GAC TAA CAC AAT CTG ATT TCA TTT CAT G	50	Nonspecific amplification	Nonspecific amplification	*Capra hircus*	Beja-Pereira et al. (2004), Antonínová (2007, personal communication)
SRCRSP 13	GAT GGG ACA GGG AAG CCT GG TAA CGT ACT GGT ACT TGA AAT TG	50	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
SRCRSP 23	TGA ACG GGT AAA GAT GTG TGT TTT AAT GGC TGA GTA G	50	No amplification	No amplification	*Capra hircus*	Luikart et al. (1999)
SRCRSP 24	AGC AAG AAG TGT CCA CTG ACA G TCT AGG TCC ATC TGT GTT ATT GC	50	Poor amplification	Polymorphic	*Capra hircus*	Beja-Pereira et al. (2004)
U 154 33	GTG AGT ACA GGC GCT TTC TG AGA ACA AAT GTG ACA CTC ACA	55	No amplification	No amplification	*Bos taurus*	Antonínová (2007, personal communication)
X 800214	CGA GTT TCT TTC CTC GTG GTA GGC GCT CGG CAC ATC TTC CTT AGC AAC T	50	Monomorphic	Monomorphic	*Capra hircus*	Antonínová (2007, personal communication)
X 80217	CTC ATT CTC CAG GAG AGA AAA CGT TCT GCC CTG AGG ATG TGT ATT GTG T	45	No amplification	No amplification	*Capra hircus*	Antonínová (2007, personal communication)

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Annexe 4: PCR conditions

Adapted conditions - touchdown:

1)	95°C	10 min
2)	95°C	30 s
	Ann. t	30 s } 35 cycles
	72°C	30 s
3)	72°C	10 min

Galan et al. (2003):

1)	94°C	12 min
2)	94°C	1 min
	58°C or ann. t	1 min } 35 cycles
	72°C	1 min
3)	72°C	10 min

Luikart et al. (1999):

1)	95°C	10 min
2)	95°C	15 s
	Ann. t	15 s } 35 cycles
	72°C	30 s
3)	72°C	10 min

Beja-Pereira et al. (2004) - touchdown:

1)	96°C	10 min
2)	96°C	30 s
	Ann. t	30 s } 40 cycles
	72°C	30 s
3)	72°C	10 min

Kim et al. (2004):

1)	94°C	3 min
2)	94°C	1 min
	55°C	1 min } 30 cycles
	72°C	1 min
3)	72°C	10 min

Antonínová (2007, personal communication):

1)	96°C	2 min
2)	96°C	45 s
	Ann. t	30 s } 40 cycles
	72°C	1 min
3)	72°C	2 min

Peelman et al. (1998) + ISAG (*ISAG:: Comparison test Guidelines* 2008 [online])

1)	94°C	5 min
2)	94°C	1 min
	Ann. t	1 min } 30 cycles
	72°C	1 min
3)	72°C	10 min

Annexe 5: Testing of various PCR conditions

Primer	Original conditions	Further tested conditions	Best working conditions	Number of PCR
AB 072 501	Adapted conditions	None	Adapted conditions	2
AF 252 500	Adapted conditions	None	Adapted conditions	2
AF 533 518	Adapted conditions	None	Adapted conditions	2
AF 533 521	Adapted conditions	Galan et al. (2003)	Adapted conditions	2
AF 533 524	Adapted conditions	Galan et al. (2003), Beja-Pereira et al. (2004)	None	3
BM 848	Galan et al. (2003)	Beja-Pereira et al. (2004)	Galan et al. (2003), Beja-Pereira et al. (2004)	4
BM 203	Beja-Pereira et al. (2004)	Galan et al. (2003)	Beja-Pereira et al. (2004)	3
BM 1443	Beja-Pereira et al. (2004)	None	Beja-Pereira et al. (2004)	3
BM 1706	Galan et al. (2003)	None	Galan et al. (2003)	3
BM 1818	Beja-Pereira et al. (2004)	None	Beja-Pereira et al. (2004)	2
BM 1824	Kim et al. (2004)	Galan et al. (2003), Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	4
BM 2113	Kim et al. (2004)	Galan et al. (2003)	Galan et al. (2003)	2
BM 4505	Beja-Pereira et al. (2004)	Galan et al. (2003)	Beja-Pereira et al. (2004)	4
BMC 1009	Galan et al. (2003)	Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	3
CSSM 014	Peelman et al. (1998)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	4
CSSM 022	Peelman et al. (1998)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	4
CSSM 39	Galan et al. (2003)	Galan et al. (2003), Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	3
ETH 3	Kim et al. (2004)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	4
ETH 10	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	5
ETH 225	Beja-Pereira et al. (2004)	Galan et al. (2003)	Beja-Pereira et al. (2004)	3
HUJ 1177	Galan et al. (2003)	Beja-Pereira et al. (2004)	Galan et al. (2003), Beja-Pereira et al. (2004)	3
IDVGA 8	Galan et al. (2003)	None	Galan et al. (2003)	2

Primer	Original conditions	Further tested conditions	Best working conditions	Number of PCR
IDVGA 29	Galan et al. (2003)	Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	4
INRA 005	Beja-Pereira et al. (2004)	Galan et al. (2003)	None	4
INRA 040	Galan et al. (2003)	Beja-Pereira et al. (2004)	Galan et al. (2003)	3
INRA 063	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	6
INRABERN 192	Antonínová (2007, personal communication)	None	Antonínová (2007, personal communication)	1
L 37 208	Adapted conditions	None	Adapted conditions	2
MAF 065	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	5
MAF 70	Beja-Pereira et al. (2004)	Galan et al. (2003)	None	3
MCHII - DR	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004), Antonínová (2007, personal communication)	Beja-Pereira et al. (2004)	7
MM 12	Beja-Pereira et al. (2004)	Galan et al. (2003)	Galan et al. (2003), Beja-Pereira et al. (2004)	4
Oar FCB 048	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	5
Oar FCB 304	Galan et al. (2003)	Beja-Pereira et al. (2004)	Beja-Pereira et al. (2004)	4
SRCRSP 5	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	5
SRCRSP 7	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	6
SRCRSP 9	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	5
SRCRSP 12	Beja-Pereira et al. (2004)	None	None	3
SRCRSP 13	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	6
SRCRSP 23	Luikart et al. (1999)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	6
SRCRSP 24	Beja-Pereira et al. (2004)	Galan et al. (2003)	Beja-Pereira et al. (2004)	3
U 154 33	Adapted conditions	Galan et al. (2003), Beja-Pereira et al. (2004)	None	3
X 800214	Adapted conditions	Galan et al. (2003), Beja-Pereira et al. (2004)	Adapted conditions, Beja-Pereira et al. (2004)	3
X 80217	Peelman et al. (1998)	Galan et al. (2003), Beja-Pereira et al. (2004)	None	4