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Czech University of Life Sciences

**Institute of Tropics
and Subtropics**

Genetic variability of Czechoslovakian wolfdogs

DIPLOMA THESIS

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Declaration

I declare I wrote this study, Genetic variability of Czechoslovakian wolfdogs, myself just using quoted literature.

In Rohatce, 17th of April 2012

.....

Signature

Acknowledgement:

I would like to thank to my advisor, Mgr. Barbora Bolfíková for her enormous help and patience. She was the person who got me into problematic of molecular genetics and guided me through all my work.

I acknowledge to the Club of breeders of Czechoslovakian wolfdog that let me study the breed and of course to all owners of sampled dogs. Also to Helena Hubáčková, who is one of the Czech most successful breeders of CSW, for bringing me the idea to write my thesis about CSW and for providing me her experiences with breeding of dogs. Also I must thank to all people from kennel club in Roudnice nad Labem for supporting me in my work.

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Abstrakt:

V průběhu 50. let bylo v Libějovicích u Vodňan započato pokusné křížení vlka (*canis lupus lupus*) a německého ovčáka. Cílem křížení bylo získat jedince s vysokým temperamentem, dobrými fyziologickými vlastnostmi, odolností a ovladatelností. Tento pokus nakonec vedl až ke vzniku nového národního plemene pod názvem československý vlčák. Plemeno bylo Mezinárodní kynologickou organizací (FCI) oficiálně uznáno 13.6.1989.

Pro diplomovou práci byly odebrány vzorky bukálních stěrů 75 jedincům plemene československý vlčák, 10 německým ovčákům a 6 vlkům. Pro analýzy bylo použito 19 mikrosatelitových lokusů. Koeficient inbreedingu byl u československých vlčáků velmi nízký (0,0222) a nebyly zjištěny signifikantní odchylky mezi pozorovanou a očekávanou heterozygotitou. Pomocí použitých markerů nebyla zjištěna žádná vnitřní struktura u československého vlčáka a nebyly nalezeny stopy vlčí introgrese. Analýzy přesto dokázaly odlišit jednotlivá plemena a čistou populaci vlků. Tato metodika je velmi vhodná pro detekci vlků, či vlčích hybridů ve volné přírodě.

Klíčová slova: československý vlčák, vlk, genetická variabilita, mikrosatelity, domestikace

Abstract:

In fifties experimental crossbreeding between wolves (*canis lupus lupus*) and German shepherd dogs has begun in Libějovice near to Vodňany. Aim of this crossbreeding was to obtain individuals with high temperament, good physiological attributes and good controllability. This experiment finally led to creation of new national breed under the name Czechoslovakian wolfdog. The breed was officially accepted by the Fédération Cynologique Internationale (FCI) 13.6.1989.

Samples of buccal swabs were collected from 75 individuals of Czechoslovakian wolfdog breed, 10 individuals of German shepherd and 6 wolf individuals for the study. 19 microsatellite loci were used for analyses. Inbreeding coefficient was very low (0,0222) in Czechoslovakian wolfdogs and we did not detected significant difference between observed and expected heterozygosity. No internal structure was detected among Czechoslovakian wolfdogs. Also any evidences of wolf genome introgression were not found by used markers. Single breeds and pure wolf population were recognized and distinguished by analyses. These methods are suitable for recognition of pure wolf or wolf hybrids in the wild.

Key words: Czechoslovakian wolfdog, wolf, genetic variability, microsatellites, domestication

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1 Introduction and aims of the study:

Czechoslovakian wolfdog (CSW) breed is one of couple of breeds that has been bred in our republic. Nowadays Czechoslovakian wolfdogs are becoming more and more popular in general public even though the breed was originally developed for the needs of border army. CSW breed has been developed from four Carpathian wolves crossbred with German shepherds during 25 years. Because the breed is still in his birth it is very important for breeders to control genetic variability to may pretend genetic losses to other continuing development and viability of the breed.

Aims of my study were to analyze genetic variability of CSW breed and to confirm its origin. Also the methodology for genetic determination between dogs and wolves was tested, which can be later used for recognizing of samples taken from the wild. Tested genetic markers will be applied to confirm occurrence of wolves in the nature of Beskydy Mountains.

This study developed on financial support of Internal Grant Agency under the number 51120/1312/3125.

Theoretical Background

1.1 Domestication of wolves

The dog (*Canis lupus familiaris*) is the only member of the family Canidae that can be fully domesticated and is the oldest domesticated animal in the world. According to archeological research, domestication of dog is strongly connected with human population in pre-agricultural age (Turnbell and Reed, 1974). Nowadays, there are more than 400 breeds of dogs existing all over the world, which are very variable in their size, color, shape and use. When we compare the variability of dogs (for example the very small breeds such as Chihuahuas and breeds of very big sizes such as St. Bernards dogs) it is almost unbelievable that there is just one mutual ancestor for all these breeds (Morell, 1997). Studies based on morphological (Lorenz, 1975; Zimen, 1981), behavioral and genetic aspects showed that there is only one ancestor of domestic dogs - the wolf (*Canis lupus*) (Wayne, 1993; Clutton-Brock, 1995). Origin of dogs had been a big question of many debates (Verginelli, 2005). There were opinions suggesting that also other canids, for example jackal or coyote may have influenced forming of the dog (Wayne, 1993; Clutton-Brock, 1995). But previously mentioned studies showed, that the wild ancestor of the domestic dog is the only wolf (*Canis lupus*).

Farm animals and crops domestication is highly affected by human impact and these animals and plants were first used as food providers. Wolf domestication began in Mesolithic period by hunting and picking nomadic inhabitants. Especially selection of advantageous for the human needs had evolved domestication in historic centers of domestication. Cats and dogs domestication can be viewed as an exception because of their use was as a human companion. Molecular evidence supported by archeological findings indicates Near East as a place where domestication started (Driscoll et al., 2009).

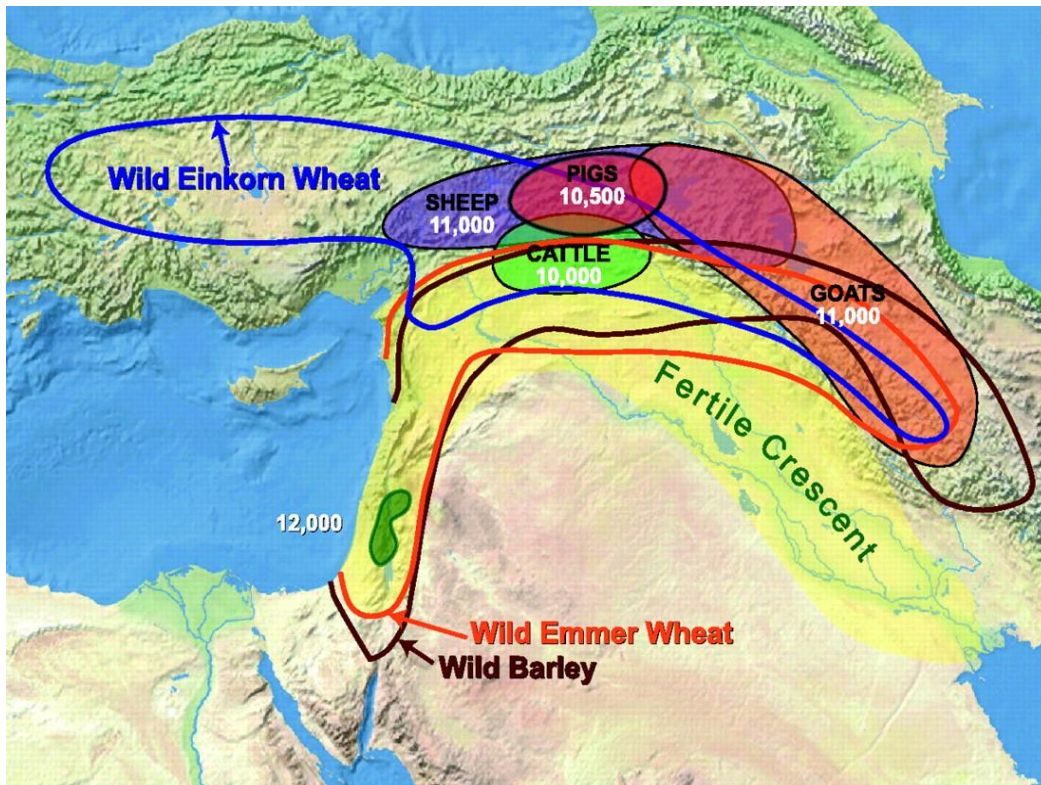


Figure 1: Map of the Near East, approximate areas of domestication of certain cattle and crops (Driscoll et al., 2009)

Domestication time period and the location are still uncertain and intensively studied. Dog domestication can be apperceived as the longest and largest human controlled experiment ever (Shearman and Wilton, 2010). There are more opinions about the date of the domestication. One of the phylogenetic studies is using amino acids substitution rates of mitochondrial D-loop sequences. This analysis estimates the dog origin as early as 100 000 years ago (Vila et.al., 1997). Other opinion based on different research used mtDNA sequence variation among 654 domestic dogs, representing all major dog population worldwide, estimates the domestication period of 15 000 years ago (Savolainen et al., 2002). Archeological findings show that the domestication proceeded 14 000 years ago (Savolainen, 2007). Bones of wolves are often found close to human inhabitance from Middle Pleistocene period. Probably some of their pups could be tamed and get used to human presence. These animals could be assumed as precursors of domesticated dogs (Clutton-Brock, 1995).

Samples of five prehistorical Italian canids (oscillating between 15 000 and 3 000 years ago) were taken from National Prehistoric-Ethnographic Museum in Rome. Their pool sequences were compared to sequences of 341 extant wolves and to 547 purebred dogs. Amova was used for hierarchical analysis of molecular variance. Based on locations of dog and wolf sequences was specified the area of origin of each breed. Genetix software was used for necessary calculations. Samples from Europe, West Asia, East Asia, Africa, North America and Central America were analyzed in the study. No significant relationships between genetic and geographic distances were found (Verginelli et al., 2005).

Tsuda et al. (1997) performed a sequence comparison of the mtDNA D-loop region among 24 breeds of domestic dogs (34 individuals of dogs – blood samples were used) and 19 individuals of 3 subspecies of wolf (*C.l.lupus*, *C.l.pallipes* and *C.l.chanco* – from captivity). As outgroups for comparison were used the samples of raccoon dog and 4 foxes. No significance in sequence between dogs and wolves was found that it follows there is no genetic differentiation between dogs and wolves. Their research showed that the domestication of dogs has not arisen just in one place, but there were more domestication centers from wild wolves. Also inbreeding occurred quite often among the multiple matriarchal origins (Tsuda et al, 1997).

Genetic studies suppose multiple dog origin or single origin in East Asia (Verginelli et al., 2005).

Different study used for domestication location 48 000 single nucleotide polymorphism (SNP) of 912 dogs from 85 breeds and samples of 225 grey wolves from 11 globally distributed populations. The East Asian origin estimated by mtDNA sequence study, suggesting dogs have an origin in East Asia because genetic diversity was highest in East Asian dog breed, was tested. However was found that genetic diversity of dogs does not vary with geography in an accordant pattern. Haplotypes shared between European, Chinese and Middle Eastern dogs and wolves populations were analyzed. As a negative control were analyzed samples of North American dogs and wolves, because is generally known dogs do not originate in America. It was found that haplotype sharing of the majority of breeds was uniformly higher between modern dog breeds and Middle Eastern wolves than between other wolf populations. Just two breeds originally from Asia (Akita

and chow chow) were closer to Chinese wolves, but the result was not significant. Other breed, originated in Middle East - basenji, had a highest amount of shared haplotypes with Middle Eastern wolves than other breeds. This means that basenji population was very effective in early domestication and that they have recently backcrossed with wolves more than other breeds. Also it is probably one of the most ancient extant dog breeds. This study confirms the Middle East as a primary source of genetic variation in the dog, with potential secondary sources of variation from Europe and East Asia. Together with previous microsatellite results, three well supported groups of ancient breeds can be distinguished: an Asian group (dingo, New Guinea singing dog, chow chow, Akita and Chinese Shar Pei), a Middle Eastern group (Afghan hound and saluki) and a northern group (Alaskan malamute and Siberian husky). Cladogram based on allele sharing of SNPs showing genetic diversity of domesticated breeds and wolves seen on Figure 2 and haplotype shering phylograme seen on Figure 3 (vonHoldt et al., 2010).

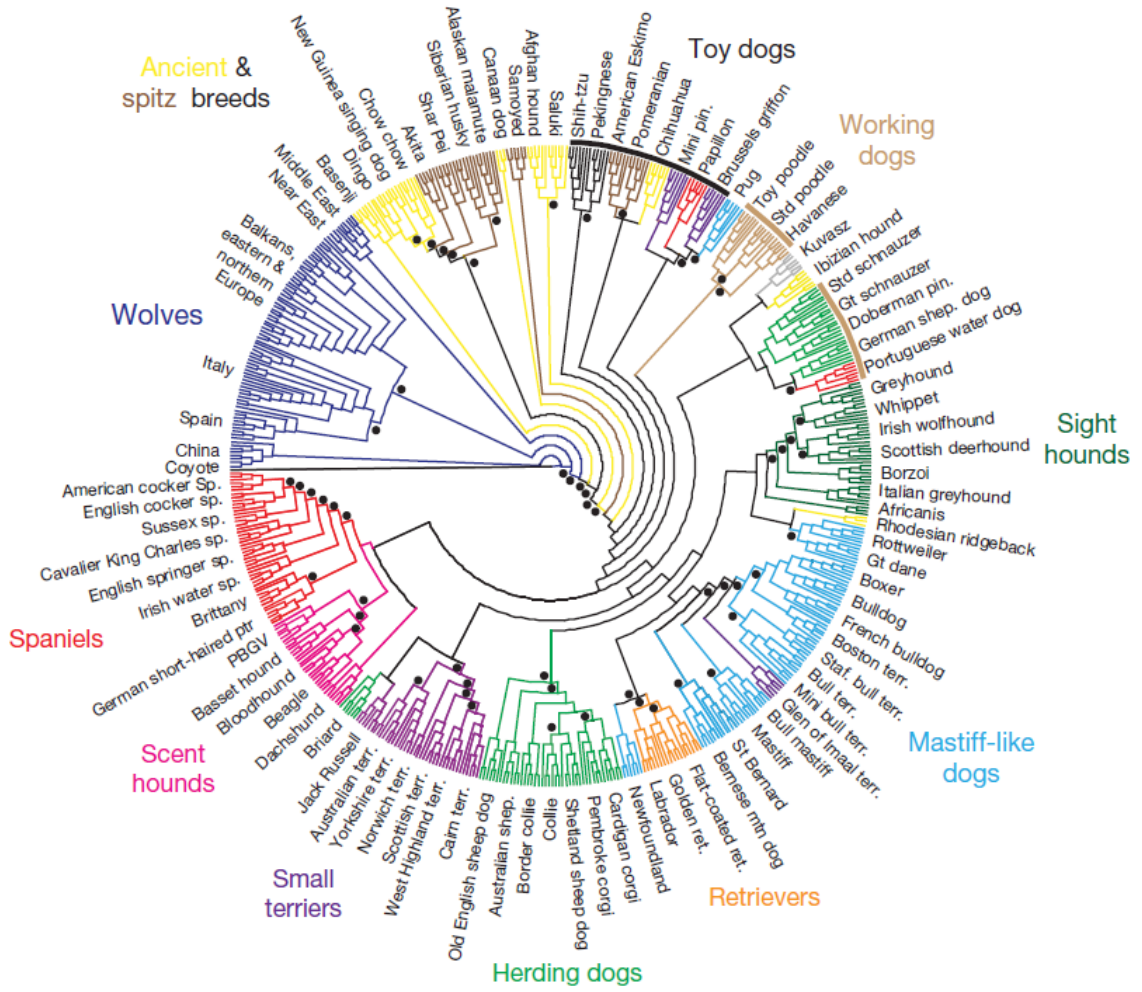


Figure 2: Allele-sharing cladogram of individuals based on individual SNP loci (vonHoldt et al., 2010)

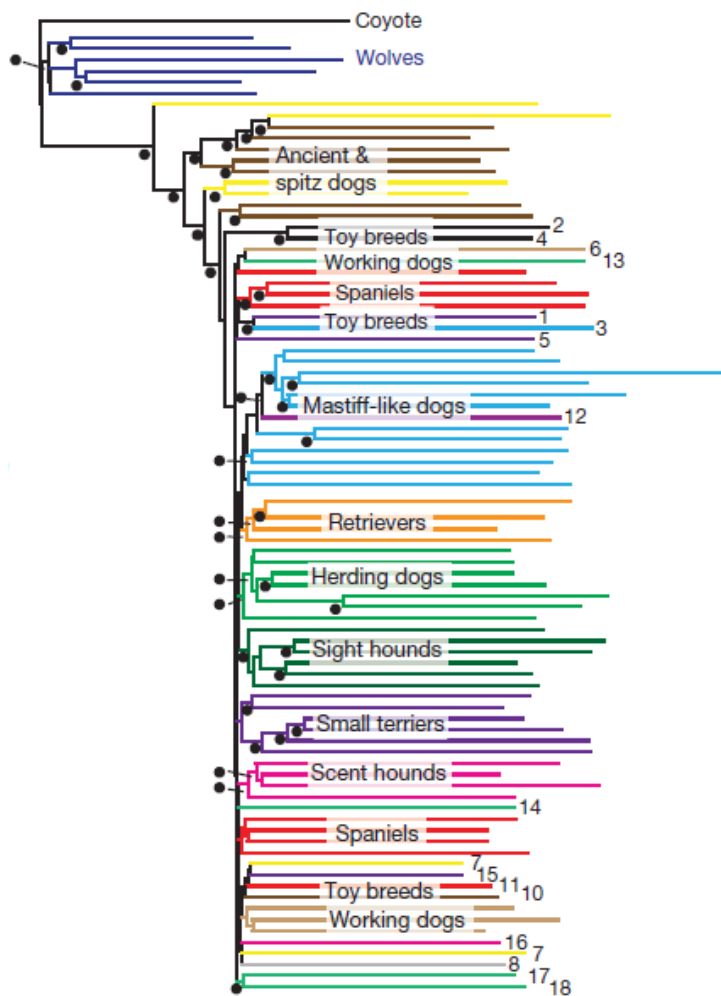


Figure 3: Allele-sharing phylogram of individual SNPs for breeds and wolf populations ; 1, Brussels griffon; 2, Pekingese; 3, pug; 4, Shih-tzu; 5, miniature pinscher; 6, Doberman pinscher; 7, Kuvasz; 8, Ibizian hound; 9, chihuahua; 10, Pomeranian; 11, papillon; 12, Glen of Imaal; 13, German shepherd; 14, Briard; 15, Jack Russell; 16, dachshund; 17, great schnauzer; 18, standard schnauzer (vonHoldt et al., 2010)

Modern systematic disciplines are mainly using genetic data (based on nucleic acids sequences) for phylogenetic relations solution. One of the main reasons of its using is lower occurrence of genotype convergences, convergence in phenotype is higher, where relationships between the relatives can be covered by adaptive evolution (Hulva 2008). On the base of DNA analyses carnivores are sorted to the group *Laurasiatheria*. It is northern part of perished continent named Pangea. Together with bears (*Ursidae*), pinnipeds (*Pinnipedia*), weasels (*Mustelidae*), (*Procyonidae*) belong to *Caniformia* (Yu et al. 2004).

1.2 Hybridization occurrence in the wild

Populations of wild grey wolves were rapidly decreasing during last centuries. Wolves were found as dangerous and predatory animals. They were often hunted as trophies. During the end of 19th century the populations of grey wolves were surviving just in the area of Portugal, Spain, Italy, Balkan and eastern region. Protection status for wolves was confirmed during the late 20th century (Boitani, 2003). Nowadays it is obvious that population sizes are increasing and even shooting of 150 wolves per year was allowed in Slovak republic. This permission is a bit controversial and not good accepted by conservational trusts. It is the only state in Europe with such a regulation.

Interspecies hybridization is a natural process that can occur in the wild. Hybridization between different species nowadays can be detected using many methods. Mainly the genetic markers are used for determination of various taxons and number of studies about hybridization is increasing. Even though identification of hybrid individuals or populations can be very problematic because of formation of allele frequency differences more than appearance of private alleles.

Vila et. al (2003) described in their article possibility of hybridization between wolves (*Canis lupus*) and dogs (*C. lupus familiaris*) in Scandinavia. They analyzed samples of urine and blood which they found in snow and one blood sample from a young individual killed by car. These samples were compared to the pure wolf samples taken from muscular tissues collected in Scandinavia and also compared to pure-bred dog samples. Analyzed samples were recognized as pure wolves or hybrids of wolf ancestry in the maternal line using mitochondrial DNA sequences. Using Y chromosome microsatellite it was detected that the first sample (taken from urine and blood) was a female individual and therefore the Y chromosome was not detected. In the sample collected from a muscle it was suggested that the father of sampled individual was not a wolf. Last used markers were 18 autosomal microsatellites. The first sample was detected as pure wolf individual and the second had a highest likelihood as wolf-dog hybrid according to these nuclear markers. As the populations in whole Europe had dramatically decreased during last centuries, some opinions say that all current wolf populations might be developed from the wolf hybrids.

This article proves an evidence of hybridization occurrence between wolfs and dogs in Scandinavian wolf population (Vila et.al, 2003).

In Italy urgent need of research and genetic monitoring of wolf population is needed. There are some qualms that population developed after World War II. might be affected by hybridization. Populations of free ranging dogs are extended in the wolf range area without any human control. In the morphological point of view, anomalous carcasses were found on the periphery of the wolf range. The dependence between increasing number of wolves and stray and feral dogs should be noticed (Boitani, 1984). 220 presumed Italian wolves and 85 dogs (from feral individuals collected in areas of the Central Apennines, where they are sympatric with wolves) were tested in another study from Italy. Wolf's tissue biopsies were taken from carcasses and dog's samples were from blood taken through veterinary practices, dog pounds and private owners and 7 known hybrids (also 3 Czechoslovakian wolfdogs were expected as hybrids by authors). The testing of individuals was done on 16 microsatellites of four different linkage groups and also 4 other unlinked microsatellites were used. Genetic determination was done in programme Genotyper. Used 16 loci belonged to 4 different linkage groups from four different chromosomes. Programme Amova was used to detection of hierarchical distribution of genetic diversity. Genetic structuring of all number of sampled individuals was counted in Structure. Optimal clustering was for K=3. All the known hybrids were sorted to their own cluster. In this article authors proposed that dog and wolf gene pools stay sharply distinct and suggest that hybridization does occur quite rarely in Italy (Verandi et.al., 2006).

Next study that is focused on studying hybridization between wolves and dogs is in the area of Iberian Peninsula. 208 samples of assumed Iberian wolves (tissues mainly from road kills and hunted animals), 196 dogs (54 feral dogs from wolf area, 152 purebred individuals composed of Iberian Molossoid cattle dog breeds and German shepherds) and 4 assumed hybrids (detected on morphological and behavioural aspects) were collected. Samples consisted of blood, tissue or buccal swaps. Authors used 42 autosomal microsatellite loci for their analyses. Excluding hybrids, autosomal microsatellite diversity was evaluated individually for dogs and wolves. A set of Bayesian analyses was used for detection of differences between Iberian wolves and dogs, the frequency and geographical distribution of hybridization and directivity of hybridization. Programme Genetix was used

to see the structure of genetic differences between dogs and wolves. Strict difference between dogs and wolves was detected using the program Structure. There were also 4 individuals presumed to be hybrids and were detected 4 more which were assigned to both clusters. All 8 hybrid individuals showed typical wolf mitochondrial lineage based on study Villa et al. (1999). Genetic differences were recognized between Iberian wolves and dogs. While linkage disequilibrium for all pairs of loci was tested, 188 pairwise significant combinations in wolves and 113 pairwise significant combinations in dogs were found. However just 75 combinations were common for both, dogs and wolves. From this result can be suggested that all hybrids had wolf mothers mating with male dogs. Hybridization between wild individuals of single species and their domesticated equivalents may become a biggest threat of wild animal populations. The highest number of hybrids evidence is restricted to peripheral areas (Godinho et al., 2011).

Presence of vestigial first toes (dewclaws) on the hind legs is common just for large dog breeds. In Tuscany in Italy wild wolves with dewclaws have been observed. These individuals might be considered as potential hybrids of wolf and dog. A research based on 18 microsatellite markers was made by Ciucci et.al. (2003). It was discovered that the presence of dewclaws is not originally common in the population of wolves. It starts to appear in second or later generation of wolf-dog hybrids. Admixed ancestry was confirmed by alleles uniquely shared with dogs, mtDNA and Y haplotypes identical to Italian wolves. Presence of dewclaws now is concluded as a result of the large dogs breed origin involved in cross mating. Also other morphological abnormalities may occur – white nails, atypical colors and body proportions, dental abnormalities. All these expressions can be considered as a result of admixed ancestry (Ciucci et.al., 2003).

There are also some misgivings that hybridization between wild animals and their domesticated relatives could influence local genetic adaptation or outbreeding depression. DNA samples (wolves tissues collected from found-dead, shot or trapped wild-living wolves, pure dogs samples taken in veterinary practices mainly) were tested to analyze hybridization occurrence in mainly in Italy but also in other European countries. Multilocus microsatellite genotypes were analysed using a Bayesian clustering procedure implemented in Structure programme. All wolves and dogs were genotyped using a panel of 18 unlinked canine microsatellite loci (Randi et al., 2000) or by 16 microsatellites

belonging to 4 different linkage groups from 4 different chromosomes (Verardi *et al.*, 2006). Both populations were sharply divided into two clusters (Figure 4). Two individuals were identified as probable backcrosses of wolf or dog parental. Genetic data prove that, even though hybridization can occur in nature, wolf and dogs keep their genetic distinction, suggesting that introgression in nature might be strongly counteracted by selection (Randi, 2008).

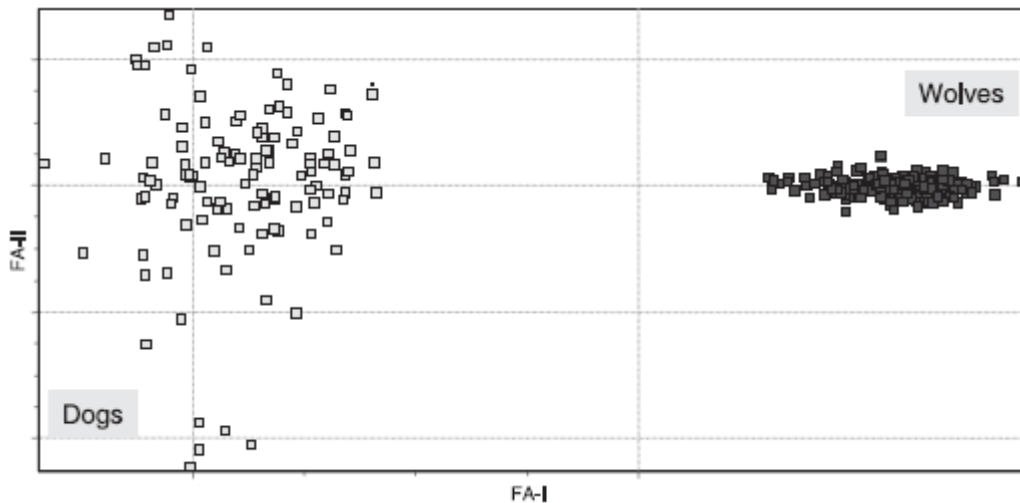


Figure 4: Relationships between Italian wolves and dogs computed and displayed in Genetix programme (Randi, 2008)

There is no documentation available about hybridization between wolf and dog in the area of Czech Republic. But some people keep wild wolves or F1 hybrids in captivity. On these animals it can be study their behavior, food and nutrition needs or sexual behavior. One of these people are for example Ing. Naděžda Šebková and František Hrach who successfully crossbred dog and wolf. Female wolf individual named Lupina who was born in 1993 in Brno Zoo was used in their experiment. She was kept in 30x30m large paddock together with a male individual of GS. During several years they never mate. All his attempts were hardly refused by female wolf. Finally in March 2002 during the rut these two, wolf and dog, mated. It happened on the walk, when female wolf was kept on the long lead. Two months later 3 offspring, one male and two females, were born to Lupina. They stayed with their mother till the age of 18 days. Then the puppies were taken by Jindřich Jedlička and Naděžda Šebková.

The result of this experiment was that the F1 generation individuals were not uniform in color nor in character. Their size was intermediate between dog and wolf, whereas the wolf even though it was female, was 10cm higher than male German shepherd. Different was also their heat period. Females came to first heat at the age of 18 month compared to GS females who usually come to heat at the age of 6-8 month and they are in heat twice a year. F1 females were in heat just once a year (in autumn) which is more similar to wolves. The conclusion of this article says that all individuals at any time would prefer individual of its own species for mating (Šebková et.al., 2008).

1.3 Czechoslovakian wolfdog:

Czechoslovakian wolfdog is one of the couple of Czech national breeds. It is a new breed and I think it has got a perspective future. More and more people start to like these dogs. They can be used for sport cynology, dog trekking and other dog sports, for dog exhibitions or just as a family pet.

1.3.1 First mating:

One year old female of wolf, Brita, was received for an experiment of crossing wolf and dog. This female wolf was placed into the kennel Pohraniční stráž in Libějovice in south of Czech Republic where the whole experiment was done. She was stabled in the coop with kennel. Two high quality male individuals of German shepherd breed were chosen for mating. They were presumed as good breeding founders. First one was calm, very well trained, second was more aggressive, also very well trained, both animals had sable gray color. In 1957 the mating was not successful because the female wolf was most of the time hidden in the kennel and she didn't let the dogs mate with her. Next year, in 1958, was assured the period when the wolf was on heat. Twelfth day of being on heat the calm dog was putted to the cage of wolf. She beat him hardly. Next day the aggressive dog (César z Březového háje) (Šebková et.al., 2008) was let in the cage and the mating was successful (Figure 5). The mating was repeated every day till 20th day of her period. The gravidity was not well recognizable. Five offspring were born 61th day after first mating. Due to big aggressiveness of the wolf, offspring were controlled and weighed 10 days after they were born. The difference in weight was -90g compared to same age offspring of German shepherd (Hartl, Jedlička, 2002).

All F1 hybrids are in phenotype and also in behavior more similar to wolves when crossed female wolf and male GS. When crossed female GS and male wolf young are also similar to wolf but bigger phenotype variability can be observed in the litter. Wolf is more dominant in both cases of crossing. F1 generation individuals and also hybrids of next generations are fertile and can mate with dog and also with wolf (Hartl, Jedlička, 2002).

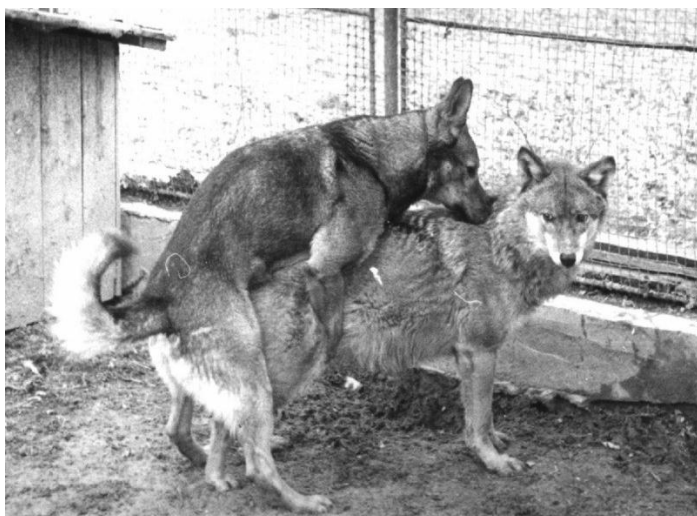


Figure 5: Mating of wolf Brita and German shepherd César z Březového háje (Šebková et.al., 2008)

Formation of the breed:

Four filial generations were born after Brita and César z Březového háje and other two filial generations were born after Brita and another GS Kurt z Václavky. The registration of the new developed breed was rejected due to the small amount of individuals. In 1968 new crossbreeding was made in kennel in Býchory. Male wolf Argo mated with GS female Asta z SNB. Request for confirmation of a new breed was again rejected in 1970 and one more time in 1976. Breeding was moved close to Bratislava where experiments continued. Third wolf – Šarik, was used for mating. Šarik mated with F3 hybrid Xela z Pohraniční stráže.

Club of breeders of Czechoslovakian wolfdog was established 20.3.1982 in Brno. Name of new breed was confirmed there. Also breed program and its conception was set. However during two following years were Slovakian breeders breaking these rules and 77% of litters were just after one male founder – Rep z Pohraniční stráže. It follows that most of the individuals were highly relative and it is probably a big bottleneck in whole population and its development has been influenced (Hartl, Jedlička, 2002).

Later, in eighties, new crossbreeding was done to avoid the degeneration of the gene pool. Stanislav Maršálek mated female wolf with male GS. In 1983 the first litter was born to wolf Lejdy and to GS Bojar von Schotterhof. Bojar was very well trained, service dog used

as a guide dog. Lejdy was tame and used to human presence. Mr. Maršálek even taught her to travel by train with him. One of their offspring was named Kazan z Pohraniční stráže (Figure 6), which was very well socialized and was able to pass service exams ZM and ZVV1 (tracking, obedience, defense).

Kazan was used directly in breeding of breed Czechoslovakian Wolfdog. He was the only individual from the litter that was useful for service training. The rest of puppies were more similar to wolf in phenotype and also in behavior – they were shy, hardly socialized, not so useful for people (Šebková et.al., 2008).

Standard of CSW was confirmed 28.4.1994 in Helsinki. Also the breed was classified to the 1.FCI group (sheep dogs and cattle dogs) under the number 322. Country of origin is Czechoslovakia, after the separation of federative republic patronage of the breed belongs to Slovakia (FCI online, 2012). During 25 years, four Carpathian wolves were crossbred into the new developing breed – Brita, Argo, Šarik and Lejdy (Hartl, Jedlička, 2002).

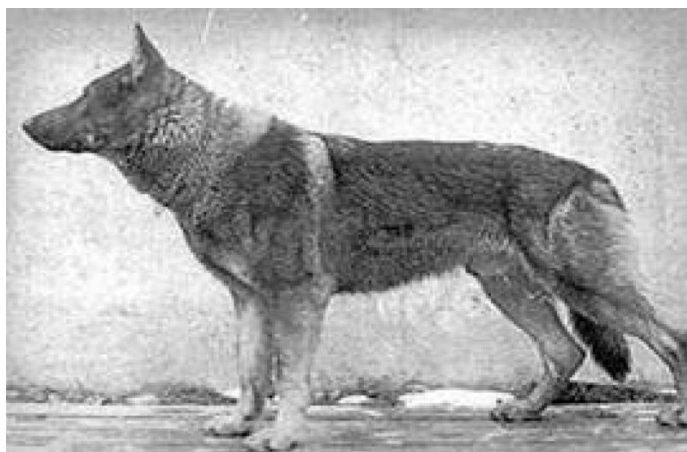


Figure 6: F1 hybrid Kazan z Pohraniční stráže (Šebková et.al., 2008)

1.3.2 Standard of the breed

(According to Czechoslovakian wolfdog breeders club, translated by C.Seidler):

- ORIGIN:** Czechoslovakian Republic.
- PATRONAGE:** Slovakian Republic.
- DATE OF PUBLICATION OF THE ORIGINAL VALID STANDARD:** 03.09.1999
- UTILIZATION:** Working Dog.
- CLASSIFICATION F.C.I.:** Group 1: (Sheepdogs and Cattle Dogs.)
Section 1: (Sheepdogs with working trial.)

GENERAL APPEARANCE:

Firm type in constitution. Above average size with rectangular frame. In body shape, movement, coat texture, color of coat and mask, similar to the wolf.

IMPORTANT PROPORTIONS:

- Length of body: Height at withers = 10 : 9.
- Length of muzzle: Length of cranial region = 1 : 1.5.

BEHAVIOUR / TEMPERAMENT:

Lively, very active, capable of endurance, docile with quick reactions. Fearless and courageous. Suspicious. Shows tremendous loyalty towards his master. Resistant to weather conditions. Versatile in his uses.

HEAD:

Symmetrical, well muscled. Seen from the side and from above, it forms a blunt wedge. Sex should be unmistakable.

CRANIAL REGION:

Skull: Seen from the side and from the front, the forehead is slightly arched. No marked frontal furrow. Occipital bone clearly visible.

Stop: Moderate.

FACIAL REGION:

Nose: Oval shape, black.

Muzzle: Clean, not broad; straight bridge of nose.

Lips: Tight fitting. No gap at corner of mouth. Rims of lips are black.

Jaws/Teeth: Jaws strong and symmetrical. Well developed teeth, specially the canines. Scissor or pincer bite with 42 teeth according to the usual tooth set. Regular teeth set.

Cheeks: Clean, sufficiently muscled, not markedly protruding.

Eyes: Small, slanting, amber colored. Well fitting lids.

Ears: Pricked, thin, triangular, short (i.e. not longer than 1/6th of the height at withers); the lateral point of the set on of the ears and the outer corner of the eyes are in a direct line. A vertical line from tip of ear would run close along the head.

Neck: Dry, well muscled. In repose forms an angle of up to 40 degrees to the horizontal. The neck must be sufficiently long for the nose to touch the ground effortlessly.

BODY:

Topline: Flowing transition from neck to body. Sloping away slightly.

Withers: Well muscled, pronounced. Though pronounced, they must not interrupt the flow of the topline.

Back: Firm and straight.

Loins: Short, well muscled, not broad, sloping slightly.

Croup: Short, well muscled, not broad, falling away slightly.

Chest: Symmetrical, well muscled, roomy, pear-shaped and narrowing towards the sternum. The depth of chest does not reach to the elbows. The point of the sternum does not extend beyond the shoulder joints.

Lower line and Belly: Taut belly, tucked up. Slightly hollow in flanks.

TAIL:

Set on high, hanging straight down. When dog is excited, generally raised in sickle shape.

LIMBS

Forequarters: The front legs are straight, strong, clean and close together with slightly turned out feet.

Shoulders: The shoulder blade is placed rather far forward, well muscled. It forms an angle of nearly 65 degrees to the horizontal.

- Upper arm: Strongly muscled, forms an angle of 120 to 130 degrees to the shoulder blade.
- Elbows: Close fitting, turned neither in nor out, well defined, flexible. Upper arm and forearm form an angle of approximately 150 degrees.
- Forearm: Long, clean and straight. The length of the forearm and pastern is 55% of height at withers.
- Pastern joint: Solid, flexible.
- Pastern: Long, forms an angle of at least 75 degrees to the ground. Lightly springy in movement.
- Front feet: Large, turned slightly outwards. Longish arched toes and strong, dark nails. Well defined, elastic, dark pads.
- Hindquarters: Powerful. The hind legs stand parallel. An imaginary vertical line drawn from the point of the ischium, would run midway through the hock joint. The dewclaws are undesirable and must be eliminated.
- Upper thigh: Long, well muscled. Forms an angle of 80 degrees to the pelvis. The hip joint is sturdy and flexible.
- Stifl: Strong and flexible.
- Lower thigh: Long, clean, well muscled. Forms an angle of about 130 degrees with the hock.
- Hock joint: Clean, solid, flexible.
- Hock: Long, clean. Position almost vertical to the ground.
- Hind feet: Longish, arched toes with strong dark nails.

GAIT / MOVEMENT:

Harmonious, light-footed, ground covering trot in which the limbs skim over the ground as closely as possible. Head and neck incline to the horizontal. Pacing when walking.

SKIN:

Elastic, tight, without wrinkles, unpigmented.

COAT

HAIR: Straight and close. Winter and summer coat differ greatly. In winter an immense undercoat is predominant and, together with the topcoat, forms a thick coat all over the body. It is necessary for the hair to cover the belly, the inside of the upper thigh, the scrotum, the inner part of the ear and the area between the toes. Well coated neck.

COLOUR: Yellowish-gray to silver-gray with a characteristic light mask. Light hair also on the underside of the neck and the forechest. Dark gray color with light mask is permissible.

SIZE AND WEIGHT:

Height at withers:

Dogs at least 65 cm

Bitches at least: 60 cm

Weight:

Dogs at least 26 kg

Bitches at least 20 kg

FAULTS:

Any departure from the foregoing points should be considered a fault and the seriousness with which the fault should be regarded should be in exact proportion to its degree.

- Heavy or light head.
- Flat forehead.
- The absence of two PM1 (premolar 1) or of both M3 (molar 3) is not to be penalized. However, the absence of one M3 in addition to 2 PM1 or the absence of one PM1 in addition to both M3 is to be considered as a fault.
- Dark brown, black or different colored eyes.
- Coarse ear. High or low set-on of ear.
- Neck carried high in repose; low position of neck when standing.
- Unpronounced withers.

- Untypical topline.
- Long croup.
- Tail long, set on low and not carried correctly.
- Too little or too much angulation in forequarters.
- Weak pastern.
- Too little or too much angulation in hindquarters. Insufficient muscle.
- Barely pronounced mask.
- Short, wavy movement.

ELIMINATING FAULTS:

- Aggressive or overly shy.
- Discrepancy in proportions.
- Faults in deportment and temperament.
- Untypical head.
- Missing teeth (except 2 PM1 and the M 3, see § faults), irregular bite.
- Untypical shape and position of eyes.
- Untypical set-on and shape of ears.
- Dewlap.
- Strong slope in croup.
- Untypical ribcage.
- Tail untypical in set on and carriage.
- Faulty and untypical position of front legs.
- Stand-off and untypical coat.
- Colors other than those in the standard.
- Slack ligaments.
- Untypical movement.

Any dog clearly showing physical or behavioural abnormalities shall be disqualified.

N.B.: Male animals should have two apparently normal testicles fully descended into the scrotum.

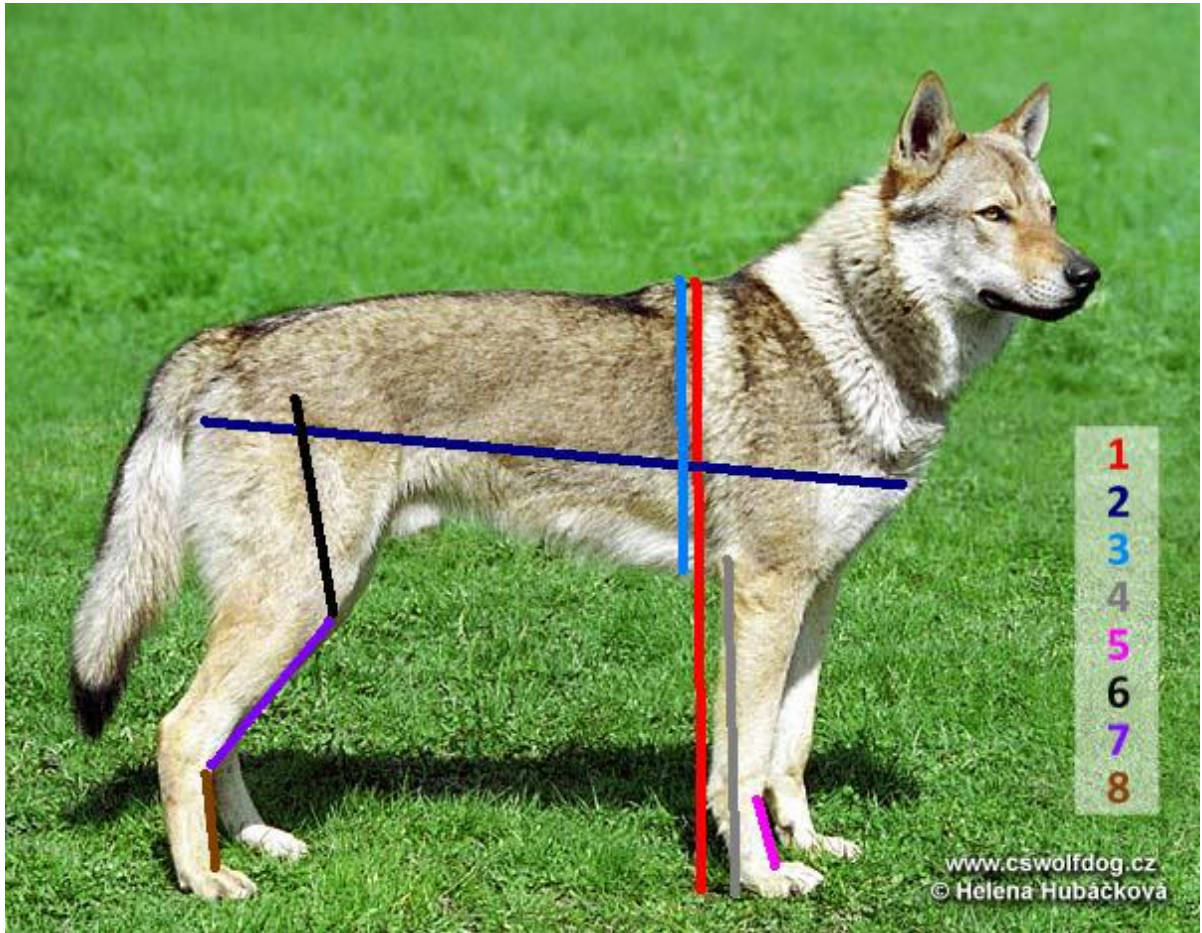


Figure 7: Schematic representation of measured parts controlled on evaluation (Picture taken by Helena Hubáčková): 1- Height at withers, 2- oblique body length, 3- depth, width and girth of the chest, 4- front leg up to elbow distance, 5- pastern (tarsus) length, 6- thigh (femur) length, 7- shank (tibia) length, 8- metatarsus length

1.4 Microsatellites

Microsatellites occur in all living organisms. They are most variable parts of DNA sequences in the genome. They are unique for each individual. They are variable in their length rather than in primary sequence. They are repeating in di-, tri-, tetra- nucleotides motives. After PCR was discovered, microsatellites became the marker of choice in genome mapping, and subsequently also in population genetics studies and related areas (Ellegren, 2004).

2 Material and methods:

Collection of tissue samples (75 individuals) was made during the year 2011. All of the samples were collected by non-invasive cheek swab method (Figure 8) mainly on 7.5.2011 in Roudnice nad Labem (52), where was a dog show of Czechoslovakian wolfdog (CSW) breed. The rest of the samples (23) were collected 22.10.2011 also in Roudnice nad Labem during the animal muster evaluation and evaluation of youngs. 10 individuals of the German shepherd (GS) breed were also collected by the cheek swab method. The individuals of CSW and GS were mainly from the Czech Republic or from the Slovak Republic (Appendix 1). Individuals were similar in age. Maximum taken from one litter was one male and one female dog, to prevent high amount of relative animals in the analyses.



Figure 8: Buccal swab taking from CSW individual

Samples of 6 wolfs were contained to analyses. 3 samples were obtained from Zoological garden in Prague and 3 from Beskydy Mountains. Samples from the Zoo were mainly from the faeces and in one case it was a muscle from a dead animal. Samples from Beskydy

Mountains were only from faeces and it wasn't sure that they are from a pure wolf or a stray dogs. All samples were fixed in 96% ethanol and stored at -18°C.

2.1 Isolation of DNA

Genomic DNA (of cheek swab samples) was extracted by the DNA Blood and Tissue Kit (Qiagen) according to the manual of the producer. In case of the faeces, DNA was extracted by the QIAamp Stool Mini Kit (Qiagen) according to the manual of the producer. In the last step DNA was washed out from the membrane by 100 µl of buffer AE. Concentration of purified genomic DNA was measured on Spectrophotometer ND-1000 (Nanodrop®) in sequence laboratory of biological section on Faculty of Science of Charles University in Prague. Samples that had concentration higher than 10ng/µl were diluted by the water for optimal concentration for PCR. DNA samples were stored in freezer at -20°C.

2.2 Polymerase Chain Reaction (PCR)

We used 19 microsatellite loci that were developed in Finnzymes Diagnostics in Finland. The Canine Genotypes™ Panel 1.1 kit encompasses PCR master mix and the following 19 loci: AHTk211, CXX279, REN169O18, INU055, REN54P11, INRA21, AHT137, REN169D01, AHTh260, AHTk253, INU005, INU030, Amelogenin, FH2848, AHT121, FH2054, REN162C04 AHTh171 and REN247M23 (Table 1). These markers are included in the 'core panel' of loci recommended by the Applied Genetics Committee of Companioning Animals of the International Society for Animal Genetics (ISAG). Size ranges are based on information provided by ISAG and data generated by Finnzymes Diagnostics. The data represents a large selection of dog breeds. However, some breeds may have alleles outside the ranges provided. The list of primers, their size, number of chromosome and their fluorescent markings is shown in the Table 1. Locus Amelogenin is located on XY chromosome. It determines the individual's gender.

Locus Name	Chromosome	Repeat Motif	Size Range (bp)	Dye Color
AHTk211	26	di	79-101	blue
CXX279	22	di	109-133	blue
REN169O18	29	di	150-170	blue
INU055	10	di	190-216	blue
REN54P11	18	di	222-244	blue
INRA21	21	di	87-111	green
AHT137	11	di	126-156	green
REN169D01	14	di	199-221	green
AHTh260	16	di	230-254	green
AHTk253	23	di	277-297	green
INU005	33	di	102-136	black
INU030	12	di	139-157	black
Amelogenin	X	-	174-218	black
FH2848	2	di	222-244	black
AHT121	13	di	68-118	red
FH2054	12	tetra	135-179	red
REN162C04 7	7	di	192-212	red
AHTh171	6	di	215-239	red
REN247M23	15	di	258-282	red

Table 1: List of primers: their size, number of chromosome and fluorescent marking color

The composition of a reacting mixture was: 3µl (c=10 µM) of fluorescently labeled forward primers and nonlabeled reverse primers, 3µl of PCR master mix and 0,7µl (c=1-10 ng/µl) genomic DNA of each sample. PCR run in Mastercycler Eppendorf Gradient thermocycler with temperature protocol according to producer (Table 2).

PCR			
98°C	3 minutes		
98°C	15 seconds	}	30x
60°C	75 seconds		
72°C	75 seconds		
72°C	5 minutes		

Table 2: PCR temperatures protocol used in Mastercycler Eppendorf Gradient thermocycler

2.3 Fragmentational analysis

The mixture for fragmentational analysis consisted of 2 µl of PCR product, 7,5 µl of formamide and 0,5 µl of size standard (Gene Scan™ 500 LIZ Size Standard, Applied Biosystems). This mixture was denaturated for 5 minutes in temperature 95°C and then cold to 4°C or stored in the temperature of -20°C.

Fragmentational analysis was made in sequencing laboratory center of Faculty of Science of Charles University in Prague on sequencor ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems) with polymer POP4 and standard DS-33.

2.4 Data Analysis

The length of each allele was manually scored in GeneMarker V2.2.0 (www.softgenetics.com). Total amount of analyzed CSW dogs was 61 individuals, of GS dogs 9 individuals and 6 of wild wolfs. All the data were rounded in the programme Autobin (<http://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/Logiciels/Autobin>). An error at genotyping due to the occurrence of artefacts in vitro amplification such as large allele drop out, stuttering or presence of null allele was tested in programme Mikro-Checker which is using Monte Carlo simulations of expected difference in allele size (Van Oosterhout et al. 2004). Loci with high frequency of null alleles are showing apparently high number of homozygotes.

For the basic visualization of the relations between the individuals on the base of microsatellites, factorial correspondence analysis was done in programme Genetix (Belkhir et al. 2004). Individuals were sorted to the groups due to the breed. Total amount of the individuals was 76.

Allele frequency distribution and presence of population specific allele at each loci was tested. Further F_{st} (fixation index) and F_{is} (coefficient of inbreeding) were calculated in programme GenePop (Raymond & Rousset 1995). F_{st} shows decrease of heterozygosity of subpopulation in proportion to the total population because of genetic drift in

subpopulations. Values are between 0 (no differentiation) to 1 (total differentiation). It is about the differentiation ratio between the subpopulations. F_{is} measure heterozygosity decrease due to nonrandom mating inside the population. The values of F_{is} are between -1 (no homozygotes) and +1 (no heterozygotes). Inbreeding ratio inside the subpopulation is measured.

Expected (H_e) and observed (H_o) heterozygosity was also evaluated in Genepop.

For detection of pattern in nuclear data, for assigning all individuals to populations and for identification of eventual hybrids was used Bayesian cluster method with model of correlated allele frequencies and admixture analysis in programme STRUCTURE 2.3. (Pritchard et al., 2000). Marcov Chain Monte Carlo (MCMC) repetition number was 1 000 000 steps after 100 000 steps long burn-in period. Number of clusters (K) was set from $K=1$ to $K=6$. Analysis was run for each K ten times. Programme established the probability of belonging of each individual to specific cluster, value q , it is estimate of membership coefficient in each individual in appropriate cluster and the value α which describes rate of convergence Marcov Chain. STRUCTURE results were visualized in STRUCTURE HARVESTER (Earl et al. 2011) implementing the method of Evanno et al. (2005) which is based on computing an *ad hoc* statistic ΔK . The results of multiple runs were combined using Greede algorithm in CLUMPP 1.1.1 (Jakobsson & Rosenberg 2007).

3 Results

Microsatellite dataset analyses in programme Micro-Checker didn't find any error in genotype determinations. No evidence for scoring error due to stuttering was observed between CSW samples. No evidence for large allele dropout was detected and also no evidence for null alleles was detected between samples.

The dataset consisted of 76 DNA samples belonging to 2 dog breeds and wild wolves. All 19 loci were polymorphic. Locus Amelogenin was located on XY chromosome. It determined gender of single individuals. Females are homozygotes with size 160 and males are heterozygotes with alleles 180 and 216. In the whole sample, a total amount of 106 alleles were detected, in average 5,9 per locus. Number of alleles ranged between 2 to 8 per locus.

Inbreeding coefficients (F_{is}) of each locus of the sampled population are given in Table 3. Average F_{is} was 0,0222. F_{is} ranged between -0,0036 to 0,1598.

Average fixation index (F_{st}) was 0,1367 and ranged between 0,0043 to 0,6065. It was measured against the GS population. The reset number means that the differentiation of CSW population against GS is small.

Observed overall heterozygosity (H_o) was 0,5476 ranging from 0,1607 to 0,8182. Expected heterozygosity (H_E) was 0,5553 ranging from 0,1771 to 0,7950. Observed and expected heterozygosity did not significantly differ and population of CSW is in equilibrium.

Locus Name	F_{st}	F_{is}	Number of allele	H observed	H expected
AHTk211	0,6065	-0,0108	7	0,2131	0,2115
CXX279	0,0043	-0,0036	4	0,7213	0,7131
REN169O18	0,3511	0,0034	5	0,3934	0,4004
INU055	0,1259	-0,0005	5	0,7719	0,7268
REN54P11	0,0683	0,1287	5	0,3729	0,4387
INRA21	0,013	-0,0005	6	0,7705	0,7549
AHT137	0,0735	-0,0265	6	0,7667	0,7715
REN169D01	0,0916	0,0286	9	0,6667	0,6918
AHTh260	0,1129	0,0136	4	0,5738	0,5795
AHTk253	0,1677	0,1208	2	0,1607	0,1771
INU005	0,1753	0,1598	6	0,2623	0,3083
INU030	0,0254	0,0080	6	0,6557	0,6438
Amelogenin	-	-	-	-	-
FH2848	0,0619	0,0528	8	0,5741	0,5616
AHT121	0,2786	0,1248	8	0,6066	0,7067
FH2054	0,0204	-0,0290	8	0,8182	0,7950
REN162C04 7	0,1344	0,0375	8	0,6182	0,6674
AHTh171	0,013	-0,0195	6	0,6230	0,5943
REN247M23	0,0798	-0,1415	3	0,2885	0,2535
Total	0,1367	0,0222	5,8888	0,5476	0,5553

Table 3: F_{st} (fixation index), F_{is} (coefficient of inbreeding), number of allele and observed and expected heterozygosity of CSW population

Factorial correspond analysis was done in programme Genetix. This approach is providing very useful visualization of relations between populations and individuals. Individuals of all populations were considered to first analysis (Figure 9.). All genetic distances can be very well seen. Wolf individuals are in the biggest distance to the individuals of other two populations and also the wolf population is not uniform. It can be affected by different samples origin, 3 were from Prague ZOO and 3 were from Beskydy Mountains.

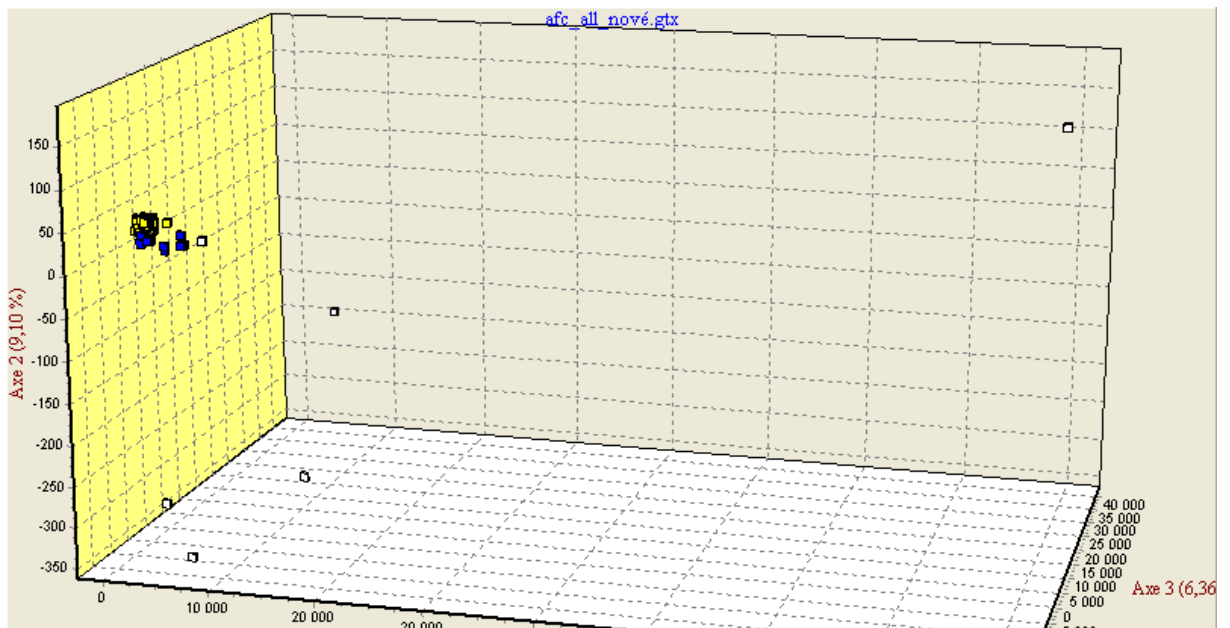


Figure 9: Individuals of single populations are recognizable by different colors: CSW – yellow, GS – blue, wolves – white

Factorial correspond analysis made for sampled population of CSW and GS is visualized on Figure 10. Populations are separated to well distinguish not overlapping clusters. Distances between CSW individuals are smaller than between GS. This is because the affinity between CSW individuals is after all higher than between GS individuals where the total population number is very high and the breed is expanded all over the world. Samples of GS were on purpose picked just unrelated animals.

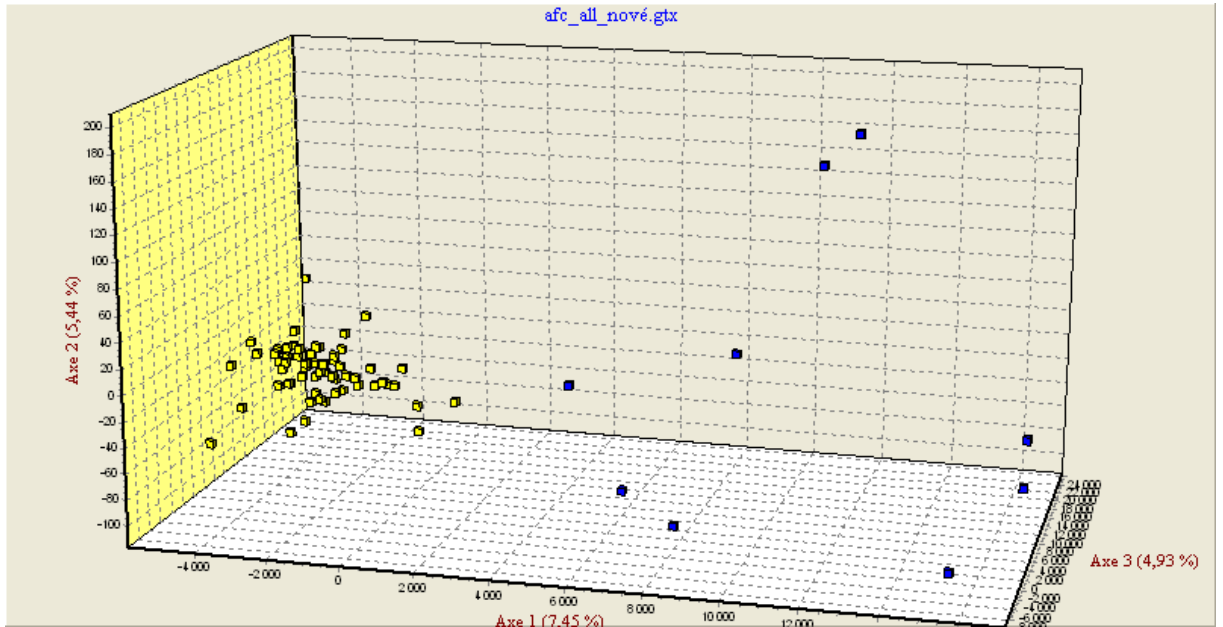


Figure 10: Visualization of relationships between CSW (yellow), GS (blue)

In following graph (Figure 11) there are visualized just individuals of CSW breed. Distances between individuals are well recognizable.

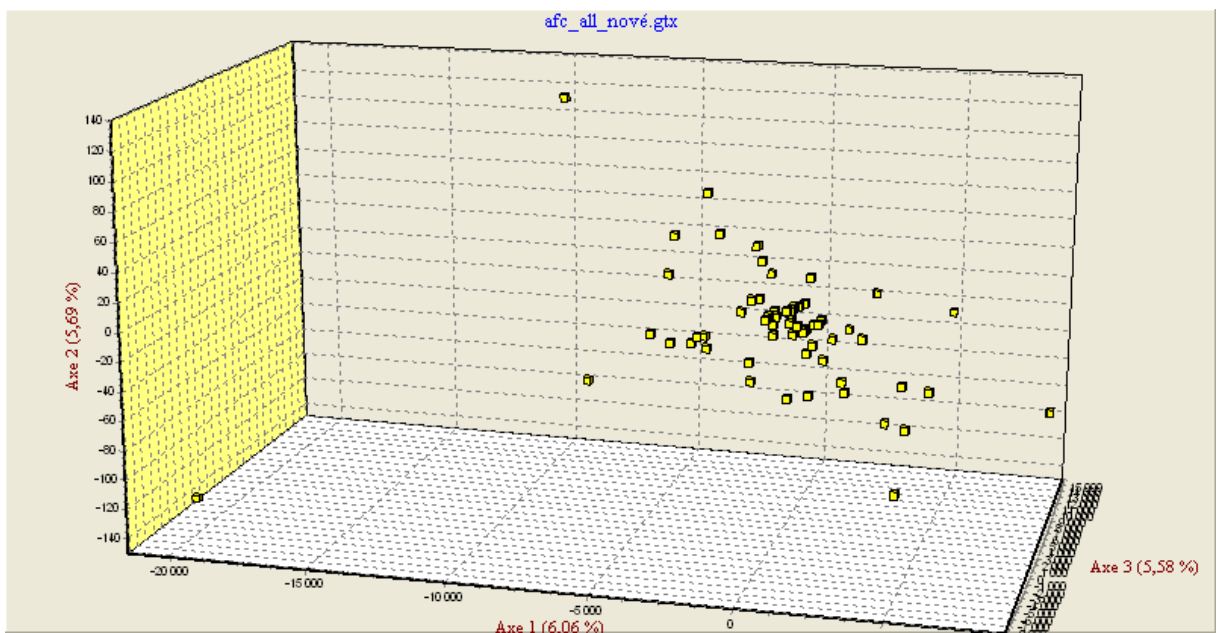


Figure 11: Visualization between individuals of CSW population

Programme Structure was used for assignment of single individuals to each cluster. A priori determination on the base breed was used for admixture analyses. In all CSW

individuals was highest likelihood for $K=1$, which means that all individuals were assigned to one cluster.

Testing just individuals of CSW (Figure 12.) the determination showed the sampled population was uniform and well recognized. Even when we tried to put in programme the number of $K=2$ or $K=3$, the uniformity of population was well recognized.

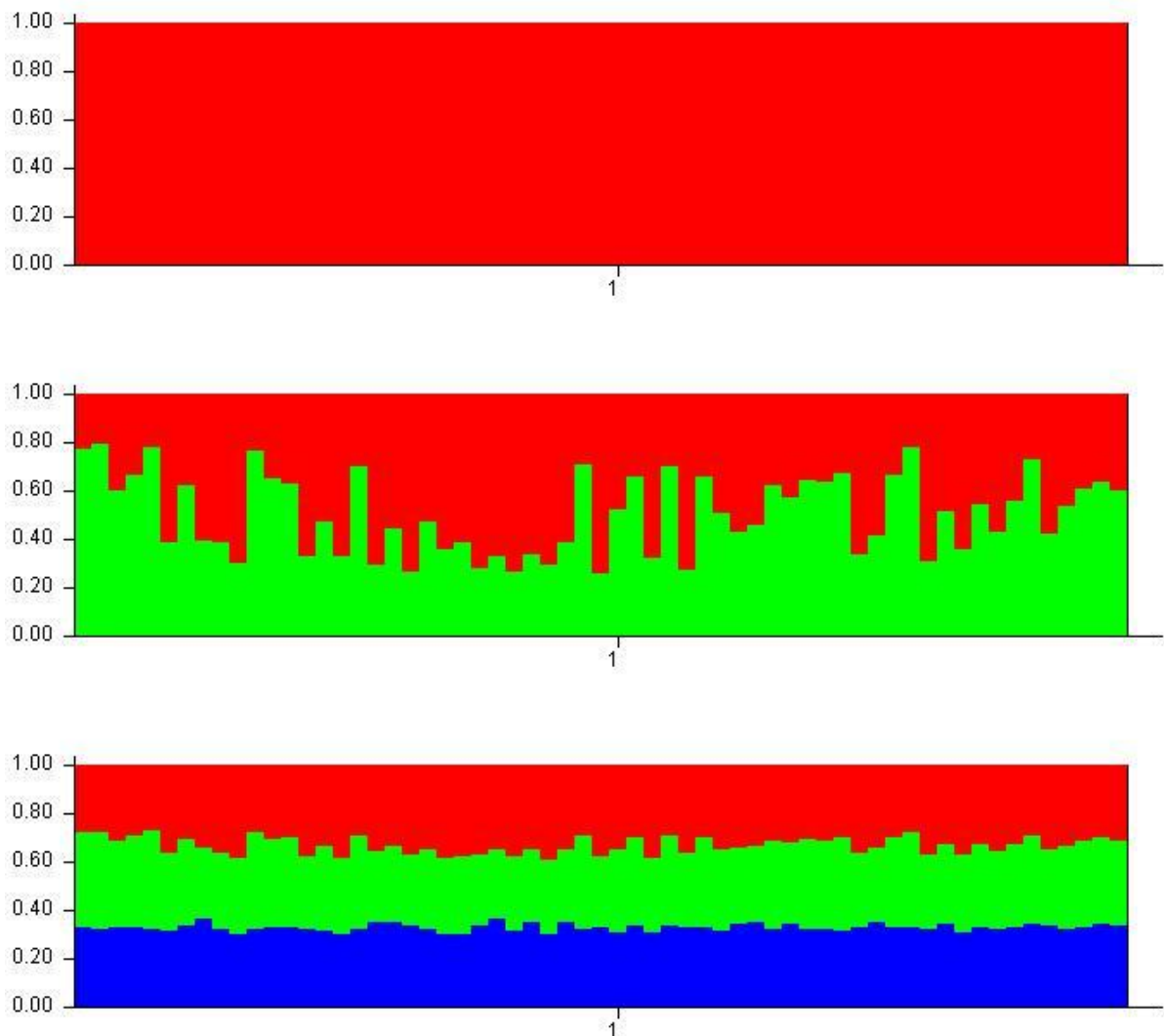


Figure 12: Admixture analyses of samples of CSW population, for $K=1$, $K=2$, $K=3$

When all individuals tested in programme Structure harvester online, the biggest likelihood was for 2 clusters (Figure 13).

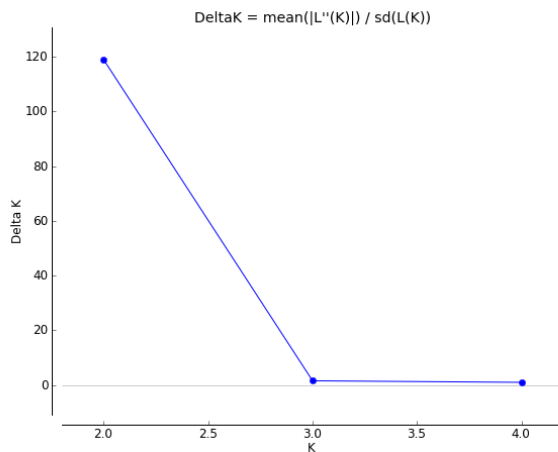


Figure 13: Structure harvester, behalf of K, by Evanno

K	Reps	Mean LnP(K)
1	3	-3530,033333
2	3	-3302,333333
3	3	-3288,033333
4	3	-3251,966667
5	3	-3164,533333

Table 4: Determination of the likelihood

On the bar graph is detected separation between CSW and GS+wolves (Figure 14) but even though that samples are separated just into two clusters the difference between three populations is very well recognizable. When we put all individuals of all three populations to the programme under the number of 3 clusters, all individuals were correctly classed to their own cluster (Figure 15).

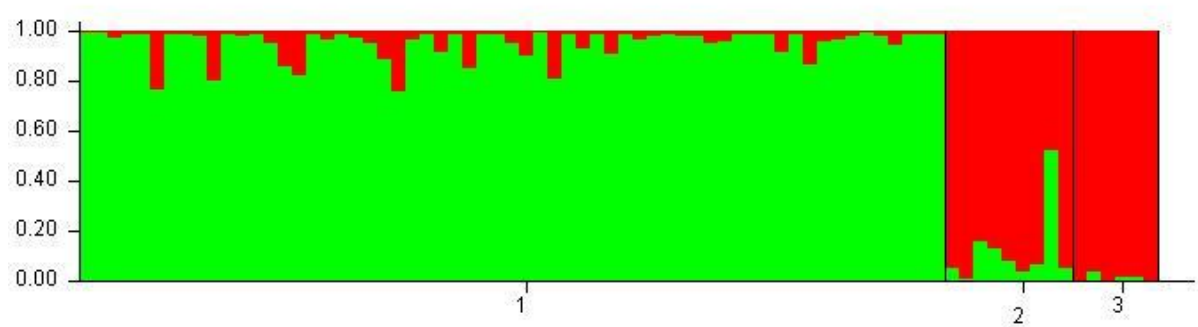


Figure 14: Highest likelihood for separation into two clusters, 1-CSW, 2-GS, 3-wolves

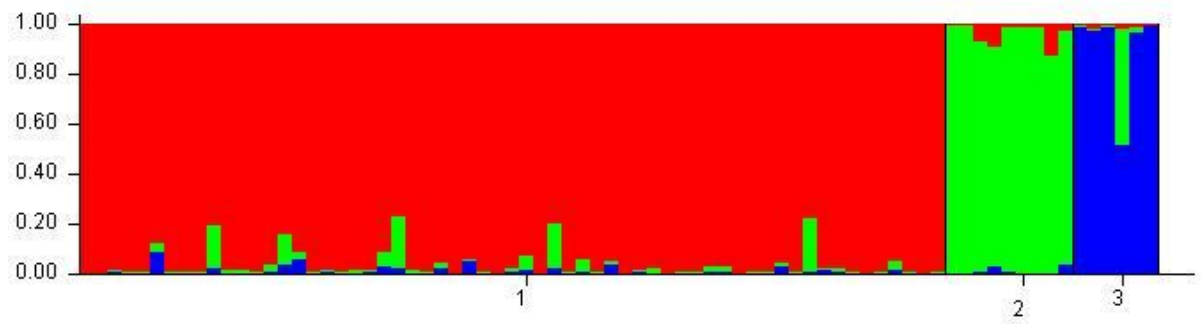


Figure 15: All individuals clustered into three clusters: 1-CSW, 2-GS, 3-wolves

4 Discussions

There are still different opinions about dating of domestication based on different studied supplies. For example study using amino acids substitution rates of mitochondrial D-loop sequences estimates the dog origin as early as 100 000 years ago (Vila et.al., 1997). Other opinion based on different research using mtDNA sequence variation of domestic dogs of majority dog breeds estimates the domestication about 15 000 years ago (Savolainen et al., 2002). Study based on archeological findings supports the domestication 14 000 years ago (Savolainen, 2007). Another study based on research of bones of wolves found close to human inhabitation was dated to Middle Pleistocene period (Clutton-Brock, 1995). Also the location of domestication remains uncertain. After presumption also coyote, jackal (Wayne, 1993, Clutton-Brock, 1995) could be ancestor of dogs, finally only wolf (*Canis lupus*) was proved as the only dog ancestor (Wayne, 1993; Clutton-Brock, 1995). Even though the very big phenotype variability of dogs it is not considered as an autonomous species. Single breeds are able to crossbreed between each other because the natural reproductive insulating mechanisms do not exist between them. Their pure lines are sustained artificially by human. But also uncontrolled crossbreeding between pure wolves and fertile dogs can occur and was detected in the wild. For example hybridization occurrence between wolves (*Canis lupus*) and dogs (*C. lupus familiaris*) in Scandinavia was described by Vila et. al (2003). Also in other countries hybridization between feral wolves and dogs was detected (Verandi et.al., 2006, Godinho et al., 2011, Ciucci et.al., 2003). This kind of study is completely missing in the Czech Republic. Our study provides new inside into the problematic of the hybridization as the new breed has been bred as a consequence of experimental crossbreeding of wild female wolf and male German shepherd. CSW by feature and also by behavior remains wolf more than dog. The breed is still new and it is still developing. Single animals are not very uniform in phenotype nor in behavioural aspects. After genetic variability testing genetic uniformity of sampled individuals was proved.

Our CSW samples were taken from similar age animals. From one litter we tried to take not more than one of the same gender. As we know from the pedigree of the breed, inbreeding is occurring between the individuals even that the CSW breed has got a

controlled breeding programme. Breeding advisor of Club of breeders picks for each individual the most suitable mating partner. CSW breed consist of limited number of animals. However between sampled animals F_{is} was lower than expected. Surprisingly, low inbreeding coefficient was observed in CSW population (0,0222), even though the population was based on a four wolf individuals and few GS individuals. However even the main breeders in Czech Republic have no idea how many GS were crossbred in the beginning of the experiment. Compared to study of Verandi et al. (2006), wolfs showed higher value of overall F_{is} (0.127). But authors used different primers than we did. Many other modern dogs are also showing low F_{is} values (Kim et al. 2001, Koskinen and Bredbacka 2001, Přibáňová et al. 2008, etc.). This data can also show advantage of using real genetic data across pedigree-based method for estimation of inbreeding.

In the graph made by Genetix programme, distances between CSW, GS and wolfs were very well detectable. The biggest distances were between wolf individuals and domesticated individuals.

The result of admixture analyses of all individuals supported separating just into 2 clusters, even though we know samples were taken from animals of 2 different dog breeds and wild wolfs. It may be affected by small amount of wolfs samples. And also individuals of wolfs were not from one population of wolfs (3 were from captivity from ZOO Prague, 3 from the wild from Beskydy Mountains). Even that all three groups were well recognized by the analysis, when the number of expected clusters was set to three. These results are showing that our microsatellite panel is very good tool for recognition of pure free living wolfs in nature and they will not be mistaken with dogs.

Structure analysis classified sample Z2 as a mixture of GS and wolf. It may be undetected and unknown F1 hybrid kept in ZOO as a pure wolf. However, PCR from faeces samples should be at least three times repeated to reduce genotyping errors (Creel et.al, 2003). Probably isolation of DNA should be also repeated to avoid mistakes caused by contamination.

Surprisingly, we did not see fingerprints of wolfs and GS genomes in the genome of CSW. This points out an important question. Are we really able to recognize backcrossed individuals of dogs and wolfs? How is possible, that relatively young breed is genetically

distinct from the parent organisms? Probably more wolfs samples are needed for further conclusion. Microsatellite data perhaps cannot answer all these questions and we propose to use some more approaches such as SNP (single nucleotide polymorphism) or next-generation sequencing methods for resolving details in evolution of this unique breed. CSW is important model organism which can be used in researches focused on hybridizations.

5 Conclusion

Inbreeding coefficient between CSW individuals was low. That means that the breed is not very endangered by inbreeding depression and potential losses of genetic variability. Even though inbreeding occurs often between individuals, the CSW breed is enough genetically variable.

No interior structure of the breed CSW was found. Individuals are in all areas unstructured and the population is genetically uniform.

Even though the origin of CSW breed is based just on four wolf individuals, genetic fingerprinting is not well seen in genome. Despite the fact CSW is young breed there are differences in genome between its parental organisms and CSW individuals.

We truly distinguished all three populations – CSW, GS and wild wolves. Based on our analysis, domesticated breeds and wild wolves can be distinguished, also single breeds can be distinguished. These methods are very suitable for distinction of wild wolves and domesticated dogs. It can be later used for confirmation of wolf occurrence in the wild or for detection of wolf territory in the wild. Also it can be useful for eventual wolf-dog hybrid detection.

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7 Figure sources

Figure1: Dricsoll, C., A., Macdonald, D., W., O'Brien, S., J., O. 2009. From wild animals to domestic pets, an evolutionary view of domestication. *Proceedings of the National Academy of Sciences of the United States of America*. Vol. 106. pp. 9971-9978. doi:10.1073/pnas.0901586106

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Figure 6: picture taken from Helena Hubáčková personally

Apendix 1:

NUMBER OF SAMPLE	DOG NAME	GENDER
POPULATION:	CZECHOSLOVAKIAN WOLFDOG	
1	Firsin Hell Z věrné smečky	M
2	Corina Sotis	F
3	Casey Karpatský Bandita	F
4	Asta Jasmine Eyes	F
5	Daily Wolf Rhoderick Sodar	F
6	Bred Ze Studeného	M
7	Houdini z Věrné Smečky	F
13	Xitta z Molu Es	F
14	Caltoo Waawanyanila	M
15	Chilli Polární Vlk	F
17	Benjamin Ben Srdcerváč	M
20	Akira u Údolí komárů	F
21	Kelt z Molu Es	M
22	White Snowking	M
23	Astra Zora Jasmine Eyes	F
24	Fantazie z Vlčí Chaloupky	F
25	Christa Z Věrné Smečky	F
26	Dream of Darkness	F
27	Agar z Březové Samoty	M
28	Argo od Vonoklaské Skály	M
29	Xi od Úňhoště	M
34	Ares z Heřmic	M
35	Colt Kuklík	M
36	Fagus z Dešné hory	M
37	Amálka od Vonoklaské Skály	F
38	Cybelg Karpatská Bandita	M
39	Ambra z Heršpic	F
40	Auronita Bohemia Cuatro	F
41	Belona Arqeva	F
42	Ruby Pasa de Lupo	M
43	Farley z Dešné Hory	M
44	Seri z Vlčí Chaloupky	M

NUMBER OF SAMPLE	DOG NAME	GENDER
POPULATION:	CZECHOSLOVAKIAN WOLFD OG	
46	Ziggy-Lee z Molu Es	M
47	Clif Sotis	M
48	Arina Sotis	F
49	Felone z Molu Es	F
50	Dina Doubravka z Vlčí chaloupky	F
51	Barg Stříbrný Úplněk	M
52	Bard Jantarowa wataha	M
53	Atti Z Údolí Komárů	F
54	Arya Arqeva	F
55	Dante od Voldušské Nivy	M
56	Argus Jasmine Eyes	M
57	An Bizzare Amant gris	F
58	Florita Z Věrné smečky	F
59	Ameli Sofia de Molai	F
60	Desire Sotis	F
61	Bref od Tišínského potoka	M
63	Cybele Karpatský bandita	F
64	Danae Karpatský Bandita	F
65	Ypsi Eden Severu	F
66	Ceyen Polární Vlk	F
67	Cairo z Blatnických Vinic	M
68	Dantea Srdcerváč	F
69	Akim od Voldušské Nivy	M
70	Beauty z Blatnických vinic	F
71	Elado z Deštné hory	M
72	Amira z Bělského Háječku	F
73	Bryan Sotis	M
74	Belfedor Arqeva	M
75	Zephiré z Molu Es	F

POPULATION:	GERMAN SHEPHERD	
I	Cameron Mir-Čer	M
II	Yasko Sinensis Bohemia	M
III	Orin Lerika	M
IV	Faki Bořický Jez	M
V	Qaira z Labského přívazu	F
VI	Nick Barabak	M
VIII	Apokalypsa Holli Ze Suché	F
IX	Princ z Labského přívazu	M
X	Rena vom Strem	F