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Genetic analysis of bush babies (*Galago* spp.) in European Zoos

Diploma thesis

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Declaration

I hereby declare that this thesis entitled “Genetic analysis of bush babies (*Galago* spp.) in European Zoos“ is entirely result of my own work and effort. I have only used the resources given in the list of references.

In Prague
10th of April, 2012 .....

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Abstract

Genetic analysis of bush babies (*Galago* spp.) in European Zoos

Galagos are not very common in zoological gardens, mostly due to their nocturnal activity. Species recognition based on morphological characters is very problematic, therefore the real species determination remains unknown in many Zoos.

The aim of this thesis was the determination of galagos of the genus *Galago* from Czech and other European Zoos based on comparison of sequences of selected genetic markers and to reveal intraspecific variability. Two fragments of the cytochrome *b* (mtDNA) of a length of 680 bp were sequenced from animals from three Czech (Prague, Plzeň, Zájezd) and five European (Poznan (PL), Frankfurt (DE), Copenhagen (DK), Amneville (FR), Amersfoort (NL)) Zoos. Subsequently, our data were compared with already published sequences deposited in GenBank enabling definite species determination of all Zoo animals.

Genetic data confirmed preliminary determination based on morphological traits – animals from the Zoo in Frankfurt, Copenhagen and Amneville belong to the lesser galago (*G. moholi*), whereas all the other studied Zoos keep the Senegal galago (*G. senegalensis*). Three independent mitochondrial lineages separated by surprisingly high genetic distances (2,3 – 4,2%) were revealed within *G. senegalensis*. One consists of animals from the Zoos Zájezd, Plzeň and Amersfoort brought in several imports from Guinea, second is formed by individuals from the Zoo Prague and Poznan originally from Ghana and Togo. The third included the GenBank data. The level of genetic differentiation corresponds with intraspecific variability described in other primate species and indicates a long time isolation of animals from different parts of the species range and thus suggests reproductive isolation between them. Therefore, in case of mixing different breeds it is essential to keep in mind their origin and not interbreed animals from different areas.

Keywords: *Galago*, Zoo, cytochrome *b*, phylogenetics

Abstrakt

Genetická analýza komb (*Galago* spp.) v evropských zoologických zahradách

Komby patří díky své noční aktivitě k ne příliš častým chovancům zoologických zahrad. Určování jednotlivých druhů na základě morfologických znaků je značně problematické, proto v mnoha Zoo zůstává druhová příslušnost komb často neznámá.

Cílem diplomové práce byla druhová determinace a odhalení vnitrodruhové variability komb rodu *Galago* z českých a evropských zoologických zahrad založená na porovnání sekvencí vybraných genetických markerů. U zvířat z celkem tří českých (Praha, Plzeň, Zájezd) a pěti evropských (Poznaň (PL), Frankfurt (DE), Kodaň (DK), Amneville (FR), Amersfoort (NL)) zoologických zahrad byly osekvenovány dva fragmenty genu pro cyt *b* (mtDNA) o celkové délce 680 bp. Ty byly následně porovnány s dříve publikovanými daty a umožnili definitivní stanovení druhové příslušnosti zvířat z jednotlivých chovů.

Genetická data potvrdila dřívější determinaci založenou na morfologických znacích – zvířata ze Zoo ve Frankfurtu, Kodani a Amneville jsou komby jižní (*Galago moholi*), ve všech ostatních zahradách je v chovech komba ušatá (*G. senegalensis*). U druhu *G. senegalensis* byly objeveny tři separované mitochondriální linie s překvapivě velkou genetickou diferenciací (*p* distance 2,3 – 4,2%). Jedna je tvořena zvířaty ze Zooparku Zájezd, Zoo Plzeň a Amersfoort pocházejícími z několika importů z Guinei a druhou představují jedinci ze Zoo Praha a Poznaň původem z Toga a Ghany, třetí tvoří data z GenBank. Míra genetické variability mtDNA koresponduje s vnitrodruhovou variabilitou zjištěnou u jiných primátů a tím indikuje dlouhodobou separaci populací z různých částí areálu a naznačuje reprodukční izolaci jednotlivých linií. Proto je v případě slučování více chovných skupin nebo třeba dbát na původ chovaných zvířat a nekřížit jedince pocházející z různých oblastí.

Klíčová slova: *Galago,* komby, Zoo, cytochrom *b*, fylogenetika

CONTENTS

1. INTRODUCTION

With increased impact of rapidly growing human population on native ecosystems and wildlife, the importance of zoological gardens as protective institutions saving endangered species becomes even highlighted. Nowadays, zoological gardens serve as last refugia for many animal species on one hand, on the other they should guarantee that species will keep their identity and maintain their original and unique gene pool. Hybridization of two different species in captivity can be acceptable only in case of highest need, for example when there is a direct risk of extinction of one species. Than it is possible to breed it with some closely related species to save at least half of the original gene pool. Increased inbreeding of small populations is another, and often unavoidable, problem of animals bred in Zoos. It leads to accumulation of negative mutations which can affect the health and fitness of the animal. Taking into account that most species in Zoos are kept in much lower numbers than would be required for long-term viability of the population (Marešová and Frynta 2008), the temptation of mixing non-related species is understandable, moreover in situations when we are not aware that the animals are actually not related. Especially today, when the boom in molecular systematics and taxonomy caused fusion of some species into one or split of others into even more, these question are no longer unresolveable. It is not a problem anymore to determine species on the basis of DNA barcoding without even knowing any taxonomic affinities. Animals of different origin but recognized as one species are normally kept together and can breed, the origin of the animals sometimes being taken as unimportant. But it is the geographic origin what can be the first indication not to breed them. Unless we are absolutely sure we have the same evolutionary lineage (or subspecies or form or however we want to call it) we should bear in mind these breeding difficulties. Galagos are not an exception.

1.1 Genus *Galago*

Bush babies of the genus *Galago* are small primates distributed in the Sub-Saharan Africa. Their large and forward directed eyes equipped with an enhancing layer of tapetum lucidum clearly indicate their adaptation for nocturnal life. Galagos eyes and rhinarium are surrounded by a dark mask that is species-specific and is the most common cue to species determination. Hind limbs are longer than front limbs, strong musculature supporting them allows galagos make long-distance horizontal leaps to a distance exceeding 14-fold the length of their body (i.e. leaps up to 4 m long). Long furry tail serves as a rudder while the animal glides through the air (Maina 1990).

Biotope preferences of bush babies span from open savannas and thorny bushveld across forested miombo habitat with dominant *Acacia* and *Brachystegia* trees, to continuous primary and secondary forests. In such dense biotopes they inhabit ground and lower floor as well as high canopy of the tallest trees. Galago´s diet consists mostly of gum and various species of insects. Diet can also be adjusted according to seasonal fruit offer (Kingdon 1997). All species forage singly. Most frequently, especially in areas with high density of tree hollows, galagos use these hollows as day shelters. Otherwise they rest in tree forks or build leaf nests (Kingdon 1997).

Whereas adult males live solitarily, females form social groups with their offspring. Territory size depends on food offer as well as on overall population density of galagos in the neighborhood. The highest population density reported by Butynski and de Jong (2004) for East African *G. gallarum* reached four animals per hectare. However, *G. gallarum* is the most xerophilous species of all bush babies inhabiting the driest environment with prevalence of thorny shrubs and semideserts, therefore population densities of other species living in densely forested habitats are likely to be even higher. Both males and females advertise their territories by loud calls and by scent marks. Scent is laid indirectly by urinating into the palms of the hands and feet and then left on branches while walking around. The territories of males and females can overlap widely, in some cases more animals can share the same day shelter as a refuge (Kingdon 1997).

1.2 Biogeography

Galagos (family Galagonidae, sometimes referred to as Galagidae as well) are one lineage of strictly Old World primates radiations – Strepsirrhini. Strepsirrhini are considered as evolutionary primitive or lower primates, total diversity does not reach that of Haplorrhini (the true primates), their sister clade. When compared with Haplorrhini, lower primates have relatively smaller brain with strongly developed olfactory bulbs. Biogeographically Strepsirrhini are restricted to the tropical regions of Asia and Africa including Madagascar as a centre of their recent distribution. There are only two families living out of Madagascar: Lorisidae, with patchy distribution in equatorial Africa, Indian subcontinent and southeast Asia and Galagonidae inhabiting Sub-Saharan Africa. Galagos are absent only from the driest parts of the Horn of Africa, the Cape province and the wettest regions of the Congo river basin (Fig. 1) (Kingdon 1997; Roos 2003). Masters et al. (2005) tried to solve the biogeographical riddle of vicariant distribution of the family Lorisidae. Expansion of the ancestors of this group from Africa into Asia is estimated to happen 70 – 50 millions years ago when both continents were still connected by a landbridge linking southeast Arabia and northwest India. A robust analysis of 13 protein-coding genes performed by Matsui et al. (2009) supported this hypothetical scenario together with the same age estimate. This hypothesis, however, does not explain why a group of slow moving sedentary lorisids, on top restricted to dense forest biotopes, was able to expand such extensive territory, whereas their agile allies with no such strict biotope preferences have actually never left Africa.

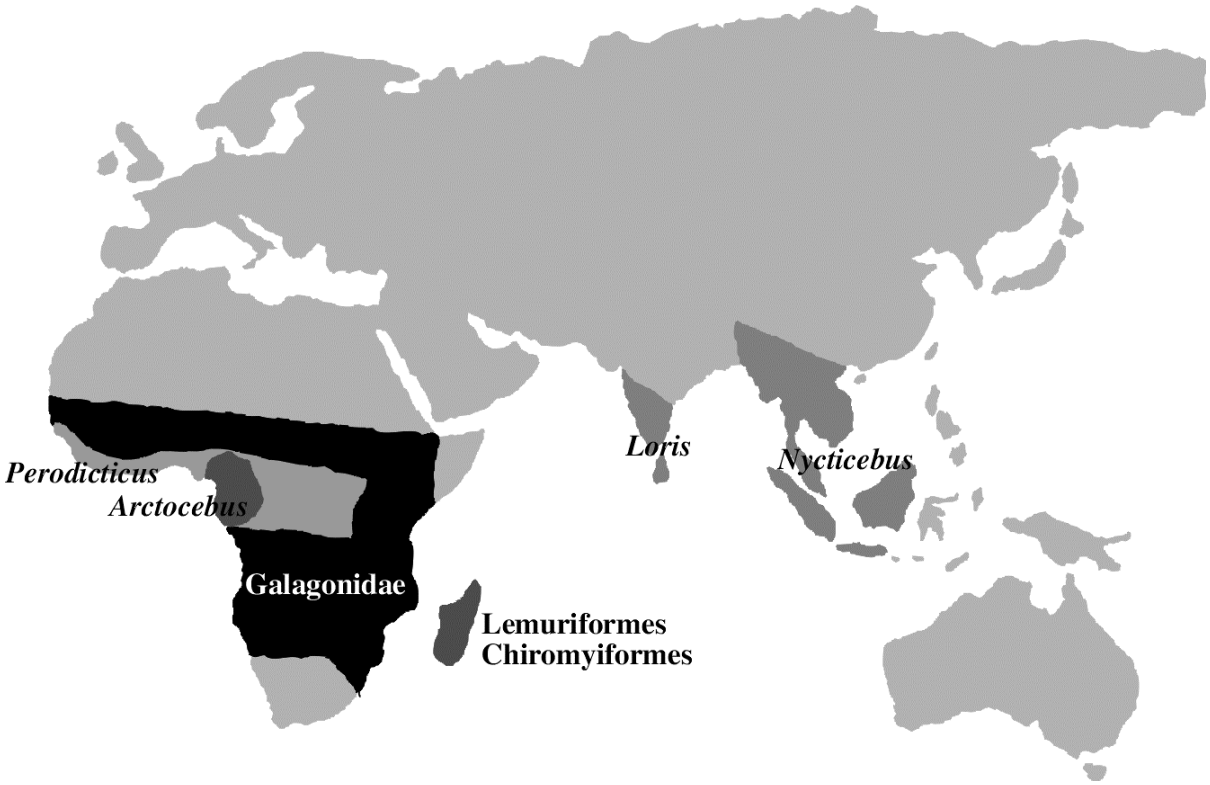

Fig. 1. Geographic distribution of Strepsirrhini. Genera *Perodicticus*, *Arctocebus*, *Loris* and *Nycticebus* form the family Lorisidae, which together with Galagonidae form the infraorder Lorisiformes. From Roos (2003).

Family Galagonidae currently contains 19 described species. Their taxonomy varies significantly, various authors have different opinion on the number of genera as well as species composition of individual genera (Purvis 1995; Kingdon 1997; Roos et al. 2004; Wilson and Reeder 2005; Masters et al. 2007; Chatterjee et al. 2009). The classification shown here is based on Kingdon (1997), although I am well aware that it may be outdated. On the other hand, modern primatologists have not been able to reach consensus concerning galagos taxonomy and nomenclature yet. Galagos consist of five genera: *Euoticus*, *Galago*, *Galagoides*, *Otolemur* and *Sciurocheirus*. Distribution of the genus *Euoticus* (*E. elegantulus*, *E. pallidus*) and the genus *Sciurocheirus* (*S. alleni*, *S. gabonensis*) is restricted to the central Africa and the Congo Basin, the latter mostly occur along the coast of the Gulf of Guinea. *Otolemur* (*O. crassicaudatus*, *O. garnettii*,

O. monteiri) live in the south Africa along the 10th parallel of latitude all across the continent. Representatives of the species-richest genus *Galagoides* (eight species) have usually very small distribution ranges that sometimes encompass only the nearest neighborhood of the type locality. It may be caused by problems with species identification or by only recent descriptions (as in *G. rondoensis* described in 1997) restricting the time needed to confirm this species from new localities. Overall distribution of the genus *Galagoides* spans from Guinea in the west along the Gulf of Guinea coast eastward up to Mozambique.

The genus *Galago* contains recently four species: *G. gallarum, G. matschiei, G. moholi* and *G. senegalensis*. *G. gallarum* inhabit arid semidesert biotopes in the northern Kenya, Ethiopia and Somalia. Especially its northern distribution extent is still poorly known mostly due to insufficient zoological surveys in eastern Ethiopia and Somalia. *G. matschiei*, a species best adapted for a life in continuous tropical forests, live in mountainous areas of eastern Congo (DRC) and neighboring Rwanda and Burundi. Isolated populations are known from the central and east Uganda. This patchy distribution pattern can be explained by a lack of information, it also can, however, signal vanishing of its natural habitats and a reduction of the original range. Distribution of the lesser bush baby (*G. moholi*) overlaps largely with that of the genus *Otolemur*. It spans from Angola in a wide belt across Africa up to the Indian Ocean. The best known representative of the family, Senegal bush baby (*G. senegalensis*), has the largest distribution range. It goes from the west Africa (Senegal) through the Sahel to the Red Sea coast in Eritrea, in the east Africa it continues south crossing the equator down to Zambia.

G. senegalensis currently contains four subspecies: *braccatus* Elliot 1907, *dunni* Dollman 1910, *sotikae* Hollister 1920 and the nominotypic *senegalensis* Geoffroy 1796. Subspecies *braccatus* was described from Tanzania from the Mount Kilimanjaro region (Elliot 1907), *sotikae* is restricted to Tanzania, Kenya and Uganda, *dunni* is known to live in the Ethiopian highlands and finally *senegalensis* covers the rest of the species distribution, i.e. Sahel, with the type locality in Senegal (Wilson and Reeder 2005). Two subspecies are recognized within *G. moholi*: *bradfieldi* Roberts 1931 with an unknown type locality and the nominotypic *moholi* Smith 1834 with the type locality by the Marico-Limpopo confluence, South Africa (Wilson and Reeder 2005). Geographic distribution of all *Galago* species is depicted in Fig. 2.

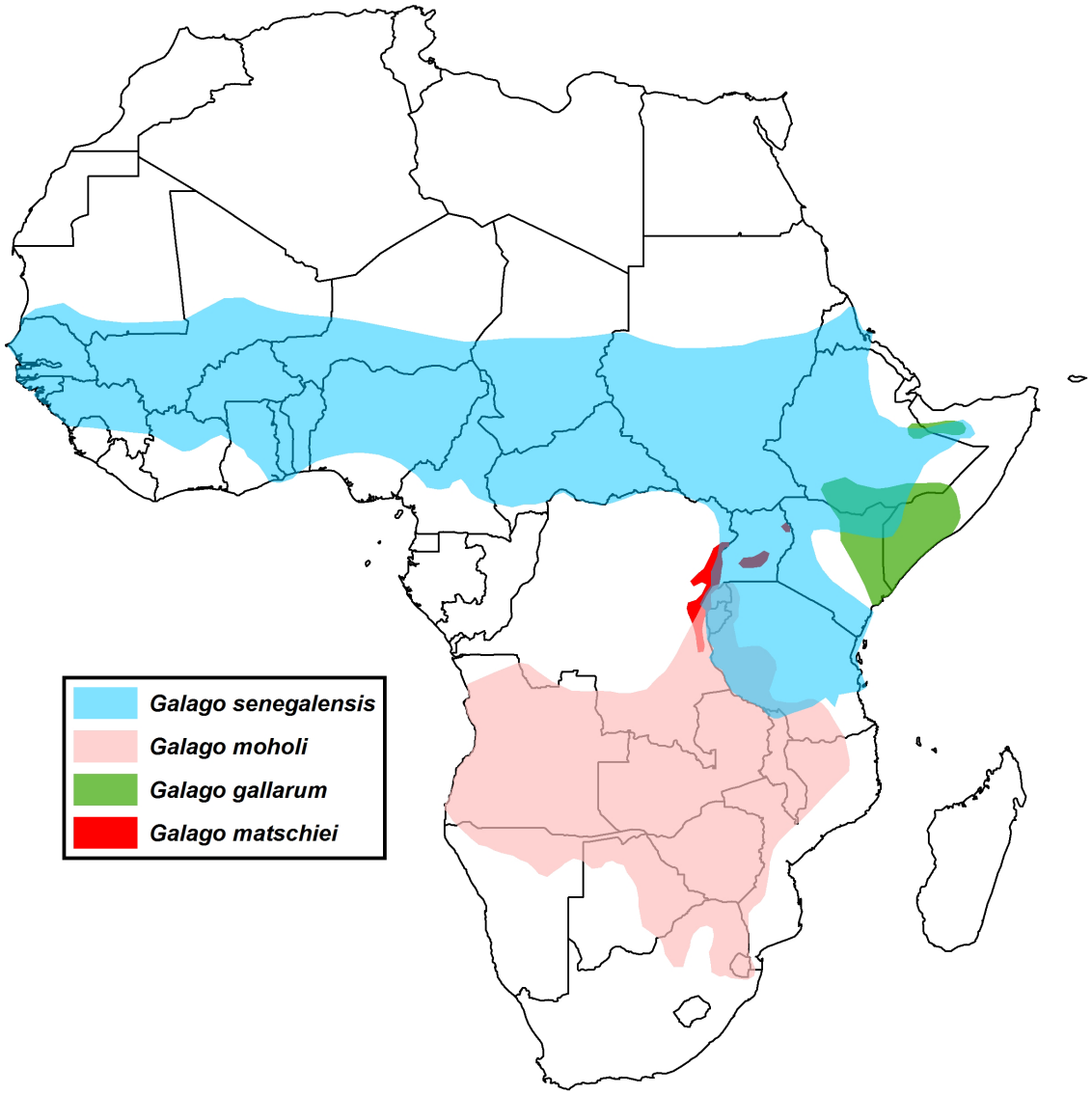

Fig. 2. Geographic distribution of the genus *Galago*. Based on data from IUCN; Courtenay and Bearder 1988; Kingdon 1997; Anderson et al. 2000; Butynski and de Jong 2004.

1.3 Systematics and taxonomy

As already mentioned above, the order Primates consists of two lineages: Strepsirrhini (lower primates) and Haplorrhini (higher primates). Strepsirrhini are further divided into three branches (infraorders; I will, however, avoid using this taxonomic

designation in the text): 1) strictly Madagascar lemurs (Lemuriformes), 2) aberrant monotypic group Chiromyiformes containing only the Aye-aye (*Daubentonia madagascariensis*) restricted also to Madagascar and 3) lorises, pottos and galagos (Lorisiformes). Phylogenetic position of an enigmatic group of tarsiers (Tarsiiformes) who remarkably resemble strepsirrhines (and especially galagos) remained dubious until the entrance of modern genetic methods. Before that, some primatologists reckoned them to be an inner clade of Strepsirrhini (Napier and Napier 1967; Schwartz 1984). After the genetic revolution in DNA sequencing their position became stable as a basal lineage of Haplorrhini, closer relationships to galagos were definitively rejected (Koop et al. 1989; Purvis 1995; Shoshani et al. 1996; Cowan 2006). The phylogeny of Lemuriformes will not be discussed here in details, they are a sister group of Aye-aye and mutual relationships among their families have already been satisfyingly resolved (Yoder and Yang 2004; DelPero et al. 2006; Perelman et al. 2011). The third branch of strepsirrhines, lorises and galagos, is formed by two families: Lorisidae and Galagonidae.

Whereas the systematics of higher primate taxa has always been based on "traditional" techniques such as craniology, relationships of lower taxonomic levels relies more on several more variable morphological characters (penile or baculum anatomy, placement and the shape of hand pads, hair ultrastructure, colour) or bioacustic data (Anderson 1999, 2000, 2001; Perkin 2007). On the basis of morphological differences Olson (1979) divided Galagonidae into three genera: *Otolemur*, *Galagoides* a *Galago*, the latter two formed by two subgenera: *Galagoides* by *Sciurocheirus* and *Galagoides*, *Galago* by *Euoticus* and *Galago*. I will now focus only on the species belonging to the genus *Galago*, i.e. *gallarum*, *matschiei*, *moholi* and *senegalensis*. Olson (1979) recognized only *senegalensis* and *gallarum* as members of the genus *Galago*, the other two species (*moholi* and *matschiei*) considered as members of the genus *Euoticus*. According to Olson´s morphological analysis *senegalensis* was a sister form to *gallarum*, *moholi* was supposed to be a sister lineage to these two. Another work of Zimmermann (1990) focused on a study of galagos relationships based on bioacustics. He considered *alleni* being a sister to *matschiei*, although *alleni* belonged at that time to the genus *Galagoides* (nowadays is this species a member of the genus *Sciurocheirus*). As a sister lineage to these two was considered *senegalensis*, the species *moholi* was sister to all these three forms – for a better intelligibility: (((*matschiei*, *alleni*) *senegalensis*) *moholi*). This

topology, however, resulted in paraphyly of the genus *Galagoides* with respect to *Galago*. Moreover, Zimmermann did not have bioacustic data of *G. gallarum* at his disposal what could affect the final topology. Neither had Purvis (1995) data for all *Galago* species on hand and could not provide more detailed picture of the phylogeny of the genus, although his composite primate phylogeny was otherwise based on a broad and robust karyotype, behavioral and molecular data. With *matschiei* and *gallarum* missing in his dataset he determined *senegalensis* being sister to *moholi* and removed *alleni* from their vicinity. Yet another publication based on sequences of three mitochondrial genes (12S rRNA, 16S rRNA, Cytochrome *b*) by DelPero et al. (2000) retained *senegalensis* in the vicinity of *moholi*, the authors, however, placed *gallarum* as a nearest relative to *senegalensis* and thus revived the original idea of Olson (1979). The same topology was provided by Chatterjee et al. (2009), furthermore supported by an analysis of four more mtDNA genes (COI, NADH3, NADH4L, NDH4) (Fig. 3) In a majority of above mentioned works we find most of galagid species belonging to the genus *Galago* with the genera *Euoticus* and *Otolemur* nested within. This situation renders *Galago* paraphyletic and taxonomically unacceptable in consequence. Resurrection of *Galagoides* and *Sciurocheirus* helped to improve this issue, nevertheless, the problem of paraphyletic taxa within Galagonidae was only shifted onto *Galagoides*.

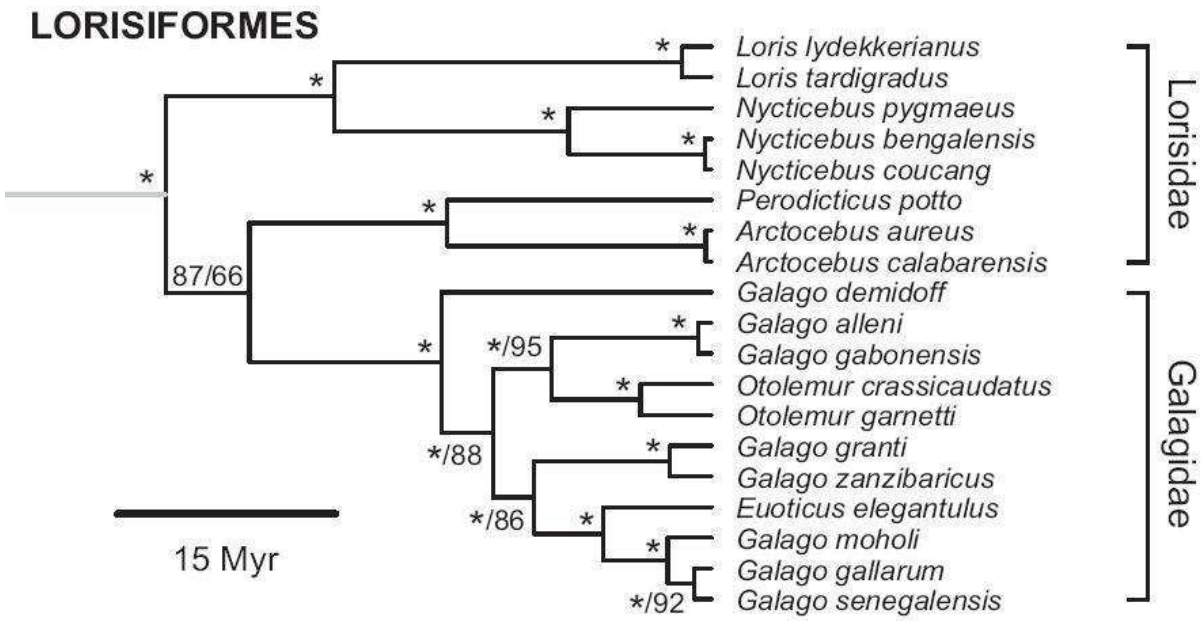

Fig. 3. Phylogenetic tree of Lorisiformes based on 7 mtDNA genes. The numbers by nodes represent bayesian posterior probabilities (* when 100%)/ percentage of ML bootstrap support. The scale shows an estimate of divergence times (Myr = million years). Redrawn and modified from Chatterjee et al. (2009). Note: Some depicted species from the genus *Galago* form currently separate genera (*alleni* and *gabonensis* are *Sciurocheirus; demidoff*, *granti* and *zanzibaricus* are *Galagoides*).

1.4 Phenotypic differences between *Galago* species

Individual species of the genus *Galago* are not easy to differentiate at the first sight. The fur coloration as one of the diagnostic traits can vary largely even within one species. The size and shape of the face mask as the most used character can be used only with certain practice and even then is the species determination rather unreliable. On the basis of a general hair coloration and face mask can be distinguished only *G. gallarum* with certainty, the hair colour is sandy brown to yellow-brown as an adaptation for life in arid semidesert conditions (Kingdon 1997, Butynski and de Jong 2004). On the other hand, *G. matschiei* restricted in distribution to tropical rainforests is the darkest of them all. *G. senegalensis* and *G. moholi* are both dim greybrown animals indistinguishable from each other at first glance. Anderson (2001) in his study of microscopic hair structure found out that only *G. gallarum* can be recognized from the other species on the basis of

frequency of hair scales. *G. gallarum* has the highest density of these scales on hair, the three remaining species are indiscernible by this character. In the same work Anderson also evaluated the ratio between bifurcated and non-bifurcated hair scales. The highest number of bifurcated was found in *G. senegalensis* (70% scales bifurcated) followed by *G. moholi* (18%) and finally *G. gallarum* (0%, all scales non-bifurcated). As the author points out, this character seems suitable to be used for species determination (Anderson 2001).

Apart from hair structure and coloration, the size and shape of the hand pads is another character used for species recognition. Hand pads differ between galagos depending on the environment inhabited and the type and size of preferred food source (Anderson 1999; Anderson et al. 2000). *G. gallarum* is significantly different from all other species in terms of the size of the first hand pad (that one by thumb), *G. senegalensis* is distinguishable from *G. moholi* by differences in size and shape between the fourth and the fifth hand pad (Fig. 4) (Anderson 1999). Within the last two species no intraspecific variability in the hand pads shape and size was found. *G. senegalensis* should be therefore easy to recognize from *G. moholi* by this method (Anderson et al. 2000). Both species differ also in the metacarpal width (Anderson 1999). The two-millimeter difference between an average hand of *senegalensis* (12.92 mm) and *moholi* (10.8 mm) can nevertheless hardly help in species identification. Moreover, there is no need to highlight that without a certain practice and a sufficient comparative material relying on the hand pad method is rather doubtful.

If the list of characters mentioned so far has aroused suspicion of their very limited usefulness and applicability, than the next one will definitely not be very helpful either. Research of the morphology of the copulatory organs is in primates driven primarily by the aim to study social systems, territoriality or parental success rate (Dixson 1987; Verrell 1992). However, it has also found its utilization in the taxonomy of galagos. Detailed study of the surface area and the glans penis structure performed by Anderson (2000) proved that the glans of *G. matschiei* is significantly bigger and covered with higher number of keratinized spines than those of *G. senegalensis* and *G. moholi*. These two species are impossible to be distinguished by the penile structure traits (Fig. 4) (Anderson 2000). Indisputable disadvantage of this method lies in its limited applicability to only half of the material available. Alternative study of females has not been invented yet.

Vocal communication reaches diverse degrees of development among primates. Its research is usually at a very developed level. It allows to study animals on greater distances often without even the necessity of their direct observation. Especially galagos, nocturnal creatures living in complex impenetrable environment, are much more dependent on vocal signals than other diurnal primates. There was 18 different sound signals detected in *G. senegalensis* so far (Zimmermann 1985). They are mostly related to various behavioral stimuli or are sexually or daytime specific. Ambrose (2003) discovered variation in vocalization of *Sciurocheirus alleni* related to geographic origin of the animals. Different populations use own dialects for communication and thus form culturally separated groups. On the other hand, Anderson et al. (2000) did not detect any intraspecific variability in *G. moholi* all over its distribution range. But in search of divergences between *G. moholi* and *G. senegalensis* Anderson et al. (2000) found out, that all homological signals differ significantly (Fig. 4). Since vocal communication is also used in courtship and mating behaviour (Zimmermann 1985), this interspecific separation of *senegalensis* and *moholi* clearly points out that strong prezygotic reproductive isolation mechanisms impeding hybridization must have already evolved.

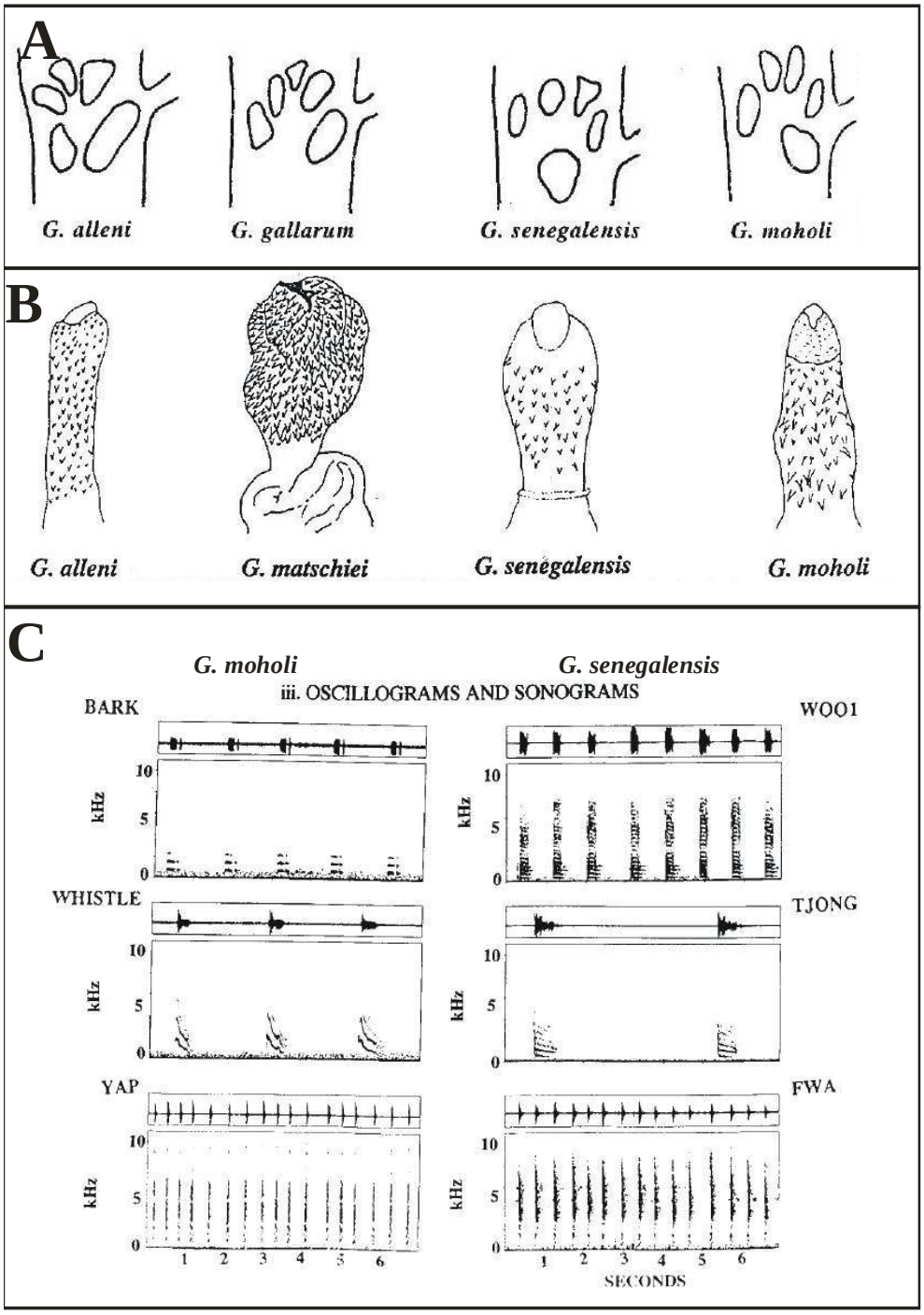

Fig. 4. Comparison of morphological and bioacustic characters of *Galago* species. **A** Hand pads, **B** Detail of penile structure, **C** Oscillograms of six vocal signals in *G. moholi* and *G. senegalensis*. In **A** and **B** is *G. alleni* considered a part of the genus *Galago*; *G. matschiei* is missing in **A**, *G. gallarum* in **B**. Redrawn and modified from: (Anderson 1999, 2000; Anderson et al. 2000).

1.5 Galagos in Czech Zoos

Prague Zoological garden (http://www.zoopraha.cz) keeps a group of galagos obtained from the Zoo in Moscow in 2002. The whole colony is formed of descendants and grand-descendants of one couple born in Moscow. Parents of this couple were caught in the wild in Ghana. Galagos breed in the Prague Zoo regularly, the group currently consists of ca 10 animals (galagos are nocturnal and very swift and since there is no need to capture them regularly, concrete number is not known exactly). According to morphological characters (penis shape), this animals are right inbetween *G. senegalensis* and *G. moholi* (Brandl pers. com.). According to the face mask pattern these animals resemble more *senegalensis*. Taking into account where parents of the original couple were imported from and where both species occur, geographic origin indicates that these animals belong to *G. senegalensis*. There was also one group of galagos in the possession of Zoo Prague in the past, but these animals did not breed and died out without leaving any descendants. The Zoo in the city of Plzeň keeps two species of galagos, the Garnett´s galago (*Otolemur garnetti*) and the Senegal bush baby determined even to a subspecific level as *G. senegalensis senegalensis* (www.zooplzen.cz). Senegal bush babies were for Zoo Plzeň captured in the wild in Guinea. Their origin and also morphological features confirm the species determination as *G. senegalensis* (Brandl pers. com.). From the same import are animals deposited now in the Zoo Zájezd (http://zoopark-zajezd.cz/). Zoo Ostrava keeps the two same species of galagids as are in Plzeň (*O. garnetti*, *G. senegalensis*). Their Senegal galagos are animals and their ancestors obtained from the Prague breeds. *G. senegalensis* from Prague and Plzeň as well as one *G. moholi* from Frankfurt are depicted in Fig. 5.

The Zoo Prague leads the European stud book for the genus *Galago*. The stud book serves as a register where any Zoo keeping galagos (or any other animals) can easily find the origin of its animals. It has the essential role in maintaining the global integrity of the breed. Moreover, the stud book can be used for predicting population development and thus help to manage breeding. The curator, namely Pavel Brandl at the moment in the Prague Zoo, leading the book can give recommendations regarding future plans with the breed, population viability or inbreeding avoidance. Altogether, the Zoo with a stud book

for some species or genus should be the responsible authority and as such it should have as many information as possible about these animals.

G. moholi - Frankfurt

G. senegalensis - Prague

G. senegalensis – Plzeň

Fig. 5. Face masks of *G. moholi* and *G. senegalensis* from different Zoos.

2. AIMS OF THE STUDY

- to provide a definite species determination of galagos from the European Zoos by comparison of their sequential data with already published sequences

- to confirm/disprove reliability of morphological traits used to distinguish individual species

- to find out whether animals belonging to one species are genetically uniform or exhibit any degree of intraspecific variability

- in case of presence of intraspecific variability assess whether the differences between recovered lineages should be reflected in separation of these lineages and prevention of keeping them together

3. MATERIAL AND METHODS

3.1 Material

For the purpose of genetic determination of galagos from various European ZOOs into species, animals from as many founder populations as possible were sampled. All animals were preliminarily determined on the basis of morphological characters as *G. senegalensis* and *G. moholi*. The complete list of the material, the ZOOs of breeds and original countries of import are listed in Table 1.

Samples were taken using an uninvasive method of buccal swabs. Animals were fixed tightly in one hand and the inner side of their cheeks was wiped by a sterile swab. Two samples were taken from each animal to provide the chance to repeat DNA extraction if needed. Swabs were subsequently put into the Eppendorf microvials and immersed in 96% non-denaturated ethanol and stored at – 18°C.

Tab. 1. Material used for genetic analyses. Names of the ZOOs of individual breeds are followed by the international country code.

Species	**ZOO**	**Country of origin**	**Analyzed animals**
G. senegalensis	Prague, CZ	Ghana	5
	Zájezd, CZ	Guinea	3
	Poznan, PL	Togo	2
	Plzeň, CZ	Guinea	1
	Amersfoort, NL	Guinea	2
G. moholi	Amneville, FR	Unknown	1
	Frankfurt, DE	Unknown	3
	Copenhagen, DK	Unknown	1

3.2 DNA extraction

To extract DNA from the swabs, the ethanol had to be removed from the vials first. The microvials were centrifuged for a short time at high speed so the tissue cells were settled down on the bottom of the vial. The supernatant was pipeted out. In order to dry the samples entirely and get rid of all the ethanol, the vials were let in a heating block at 56°C until the swabs were dry. As the next step, 180 µl of the ATL buffer (a component of a commercial isolation Qiagen DNAeasy® Tissue Kit) was added and the solution was left macerating for 10 min in order to loosen the cells from the swab. Subsequently the swab was removed from the vial and 20 µl of proteinase K was added to the mixture to lyse the cells. The next steps followed the DNA extraction protocol provided by the manufacturer (Qiagen). Extracted DNA was stored at -18°C.

3.3 PCR, sequencing

To amplify selected fragment of the mtDNA cytochrome *b* gene two pairs of primers amplifying 307 and 373 bp were used (Fig. 6) (Kocher et al. 1989; Irwin et al. 1991):

L14841	5' - AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA - 3'
H15149	5' - AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA - 3'
L15513	5´- CTAGGAGACCCTGACAACTA – 3´
H15915	5´- AACTGCAGTCATCTCCGGTTTACAAGAC – 3´

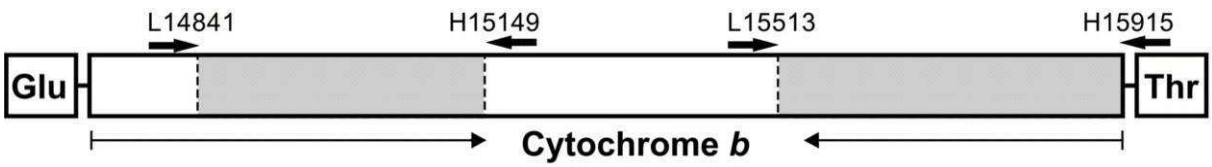

Fig. 6. Targeted regions (grey) of the cyt *b* gene amplified in the study.

Polymerase chain reaction (PCR, Sambrook et al. 1989) was performed in the termocycler QB-96 (Quanta Biotech) operated by Acer n300 android. Table 2 shows composition of chemical mixture used for PCR. Negative controls (i.e. without DNA added) were employed in every amplification to detect possible contamination. PCR program is listed in Table 3. Presence and the length of amplified DNA fragment was controlled using 1% agarose gel and a length standard (GeneRuler™ 100bp DNA Ladder, Fermentas). Quality of PCR products was checked by an eye under the UV light. Successfully amplified samples were purified by a commercial Qiaquick® PCR Purification Kit (Qiagen) following manual therein. Finally, a mixture of PCR product, 1M primer and H_2O was prepared for sequencing. Sequencing was conducted on ABI PRISM 3100 Avant Genetic Analyzer at the Laboratory of DNA sequencing, Faculty of Science, Charles University in Prague.

Tab. 2. PCR mixture composition

Chemical compound	**Ammount (µl)**
Nucleotide free H_2O	8 – 8.5
PCR Master Mix (Fermentas)	12.5
Primers 10pmol/µl	1 – 1.25
DNA	2
total	25

Tab. 3. PCR program

Step	**°C**	**time**
1. Initial denaturation	94	7´
2. Denaturation	94	30´´
3. Annealing	47	45´´
4. Extension	72	1´
5. Final extension	72	10´
Number of cycles	35	
7. Storage	4	

3.4 Alignment

The quality of DNA sequences obtained from the sequential laboratory was controlled in Geneious 5.3.6 (Drummond et al. 2011). The same software was used to assembly corresponding sequences, alignments of concatenated sequences was performed with Clustal W (Thompson et al. 1994) as implemented in Geneious. Prior to analyses, sequences were translated into amino acids in the Mega5 software (Tamura et al. 2011) using the vertebrate mitochondrial translation code. This did not reveal any stop codons or gaps suggesting that all protein coding sequences were functional and no pseudogenes were amplified. The second targeted region terminated by a stop codon confirming that we have reached the end of the cyt *b* gene. To nest our samples of galagos within the family Galagonidae, sequences of all other family members available in the GenBank were downloaded. Their complete list is given in Table 4. *Lemur catta* sequence was used to root the tree.

Tab. 4. List of the GenBank sequences used to nest our data within the phylogeny of Galagonidae.

GenBank accesion no.	Species	Sample origin (*Galago* only)
AF212970	*Galago gallarum*	Somalia
AF271409	*Galago matschiei*	Burundi
AF271410	*Galago moholi*	Duke University, USA
AF212971	*Galago moholi*	University of Witwatersrand, RSA
AY441470	*Galago moholi*	Unpublished data
AY441471	*Galago senegalensis*	Unpublished data
AF212969	*Galago senegalensis*	Stuttgart Primate Facility, DE
AY897401	*Euoticus elegantulus*	
AY897400	*Euoticus elegantulus*	
AF271411	*Galagoides demidoff*	
AY441468	*Galagoides granti*	
AF212964	*Galagoides zanzibaricus*	
U53575	*Lemur catta*	
U53581	*Loris tardigradus*	
U53579	*Otolemur crassicaudatus*	
AF271412	*Otolemur garnettii*	
AY441467	*Sciurocheirus gabonensis*	
Z35095	*Sciurocheirus alleni*	

3.5 Phylogenetic analyses

The best-fit model of sequence evolution was selected by jModelTest 0.1.1 (Posada 2008) under the Akaike Information Criterion (AIC). This was the general time reversible model taking into account the shape of the gamma distribution parameter (GTR + G). Two fundamental methods were employed for phylogenetic computations: Maximum likelihood (ML) and Bayesian inference (BI). ML analyses was conducted using PhyML 3.0 (Guindon et al. 2010) with the nearest neighbor interchange (NNI) and the subtree pruning and regrafting (SPR) tree improvement, and adopting the best fitting model as chosen by jModelTest. Nodal support for the ML tree was assessed by 1000 bootstrap pseudoreplications (Felsenstein 1985). BI was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) implementing the best-fit model, with two runs and four chains for each run for $5x10^6$ generations. Sampling frequency of every 100^{th} generation produced 50 000 sampled trees. After each analysis the log-likelihood scores were plotted against generation time to assure whether the -lnL values achieved stationarity. Subsequently, first 20% of trees (10 000) was discarded as a burn-in. A 50% majority rule consensus tree was produced from the posterior distribution of trees, and posterior probabilities were calculated as the percentage of sampled trees recovering any particular clade (Huelsenbeck and Ronquist 2001). Yet another method, maximum parsimony (MP), producing ultrametric trees was used for a dataset containing only the genus *Galago* to show more clearly differentiation among lineages. All crown groups in an ultrametric tree are equidistant from the root and usually provide easily readible pattern. MP analysis was performed by PAUPup 1.0.3.1 (Calendini and Martin 2005) as a graphic platform of PAUP* 4.0b10 (Swofford 2002) under the heuristic search with 100 random stepwise addition and tree-bisection-reconnection (TBR) branch swapping. To measure between-species genetic differences, uncorrected *p*-distances were calculated in MEGA 5 (Tamura et al. 2011). *P*-distances are summarized in Tab. 5 and Tab. 6.

To construct the haplotype network I had to avoid the problem with missing data (i.e. nonsequenced animals) within the dataset, for these can highly influence the topology of the network and thus all deduced interpretations (Joly et al. 2007). The haplotype network was therefore based only on the first targeted region of the cyt *b* (307bp, primers L14841 and H15149) for which all animals have been successfully sequenced. Only the

four species of the genus *Galago* were included into this analysis. Haplotype network was constructed in Network 4.6.1.0 using the median joining algoritm (Bandelt et al. 1999).

4. RESULTS

As already mentioned, the GTR + G was selected as the most appropriate model of sequence evolution. Base frequency as calculated in jModelTest was as follows: f(A) = 0.3200, f(C) = 0.3308, f(G) = 0.1033, f(T) = 0.2460; gamma shape parameter = 0.2510. The final alignment containing all available representatives of family Galagonidae reached the length of 680 bp, 255 positions were variable and 175 of them parsimony informative (*Lemur catta* as an outgroup exluded). When only species belonging to the genus *Galago* were assessed, the number of variable positions was 92 with 42 of them being parsimony informative.

ML analysis resulted in a topology with log-likelihood = -3552.90147 which was comparable to the one recovered from MrBayes (mean lnL = -3608.15). Both trees resulted in the same topology. All studied genera formed monophyletic clusteres except of the genus *Galagoides* which was polyphyletic with *G. demidoff* standing completely apart from the two other species (*G. granti, G. zanzibaricus*). All species of the genus *Galago* formed very well supported (100/1; bootstrap support values are always in the order ML/BI from no on) monophyletic clade. Within this monophylum, the GenBank sequence of *G. matschiei* (AF271409) formed a sister lineage to all remaining specimens, the latter clade being well supported (87/1). This clade consists of three well defined clusters, however, their mutual relations remained unresolved. 1) The first included animals from the Zoos in Zájezd, Plzeň and Amersfoort. Except of one animal from Zoo Plzeň differing in two nucleotide position, all haplotypes were identical. Because of those two mutations the within-group *p*-distance for this lineage exceeded zero value (= 0.003*). 2) The second group was formed by animals from the Zoos Prague and Poznan. Unfortunately, I did not succeed in sequencing of the second targeted region of cyt *b* in any of Prague´s animals, therefore the comparison is based only on the first targeted region. These two Zoos share the same haplotype and it is very unlikely that the nucleotide sequence from the hind part of the cyt *b* of Prague´s animals would differ when the first region is identical. 3) The third cluster consisted of the GenBank data of *G. gallarum* and *G. senegalensis* and the GenBank as well as our *G. moholi* sequences, however this group did not recieve a noticeable ML bootstrap support (-/98).

* genetic distance is proportion of variable nucleotides versus the number of conserved sites expressed as a proportion of 1, i.e. for instance a *p*-distance eguals 0.01 means 1% genetic divergence

G. gallarum from Somalia and *G. senegalensis* of an unknown original source form a well supported clade (80/0.99) sister to *G. moholi* (97/1).

Animals from the Zoo Amneville and Copenhagen shared identical haplotype differing in 4 mutational steps from those from Frankfurt. Average between-species genetic distances within the Zoo samples of *G. moholi* are equal to 0.012. Surprisingly, the GenBank sequences of *G. senegalensis* do not cluster together with the Zoo samples and are more closely related to *G. gallarum* instead. Average *p*-distance of the GenBank *senegalensis* from those from the Zoo Prague and Poznan is 0.028, from those from the Zoo Plzeň, Zájezd and Amersfoort is 0.042. Genetic distance between both Zoo groups of *senegalensis* equals 0.023. The between-lineage comparison of *senegalensis* clades is summarized in Table 5. A full list of uncorrected genetic distances within the genus *Galago* is shown in Table 6. The final phylogenetic tree is shown in Fig. 7.

Tab. 5. Average genetic distances between the three lineages of *G. senegalensis*.

G. senegalensis	**1**	**2**
1. GenBank		
2. Ghana + Togo	0.028	
3. Guinea	0.042	0.023

Tab. 6. Uncorrected pairwise distances comparing between-lineage divergence. Major clades recoverd in our analysis are separated by lines, only one representative for each Zoo depicted.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	AF271409__matschiei														
2	AF212970_gallarum	0.063													
3	AF271410_moholi	0.063	0.04												
4	AF212971_moholi	0.059	0.036	0.004											
5	AY441470_moholi	0.063	0.032	0.016	0.012										
6	Amneville_moholi	0.063	0.04	0.008	0.004	0.008									
7	Copenhagen_moholi	0.063	0.04	0.008	0.004	0.008	0								
8	Frankfurt_moholi	0.063	0.032	0.024	0.02	0.008	0.016	0.016							
9	AY44147_senegalensis	0.067	0.012	0.051	0.047	0.043	0.051	0.051	0.043						
10	AF212969_senegalensis	0.063	0.008	0.047	0.043	0.04	0.047	0.047	0.04	0.004					
11	Praha_senegalensis	0.055	0.024	0.055	0.051	0.04	0.047	0.047	0.04	0.028	0.024				
12	Poznan_senegalensis	0.055	0.024	0.055	0.051	0.04	0.047	0.047	0.04	0.028	0.024	0			
13	Zajezd_senegalensis	0.059	0.036	0.059	0.055	0.043	0.051	0.051	0.043	0.04	0.036	0.02	0.02		
14	Plzen_senegalensis	0.067	0.043	0.067	0.063	0.051	0.059	0.059	0.051	0.047	0.043	0.028	0.028	0.008	
15	Amersfoort_senegalensis	0.059	0.036	0.059	0.055	0.043	0.051	0.051	0.043	0.04	0.036	0.02	0.02	0	0.008

The result of the MP analysis (Fig. 8) highlights the above mentioned differentiation of *G. moholi* on one side and the two independent lineages of *G. senegalensis* on the other. In contrast with both previously described computational methods, MP analysis clusters both *senegalensis* groups together.

Median joining haplotype network (Fig. 9) shows clear haplotype structure concordant with those obtained from all tree-constructing methods. None of the monophyletic groups (i.e. *moholi*, *matschiei*, *gallarum*, *senegalensis* from GenBank, *senegalensis* from Guinea and *senegalensis* from Ghana and Togo) shared haplotype with any other. *G. moholi* formed quite structured cluster of haplotypes what may be caused by many various sources of tissues or sequences and thus many various possible places of geographic origin of the samples. On the other hand, *G. senegalensis* from Ghana + Togo and from Guinea formed more star-like pattern with one central haplotype with only several detached individuals. Rather than by limited variation within these lineages this may be caused by very limited sampling localities representing each clade.

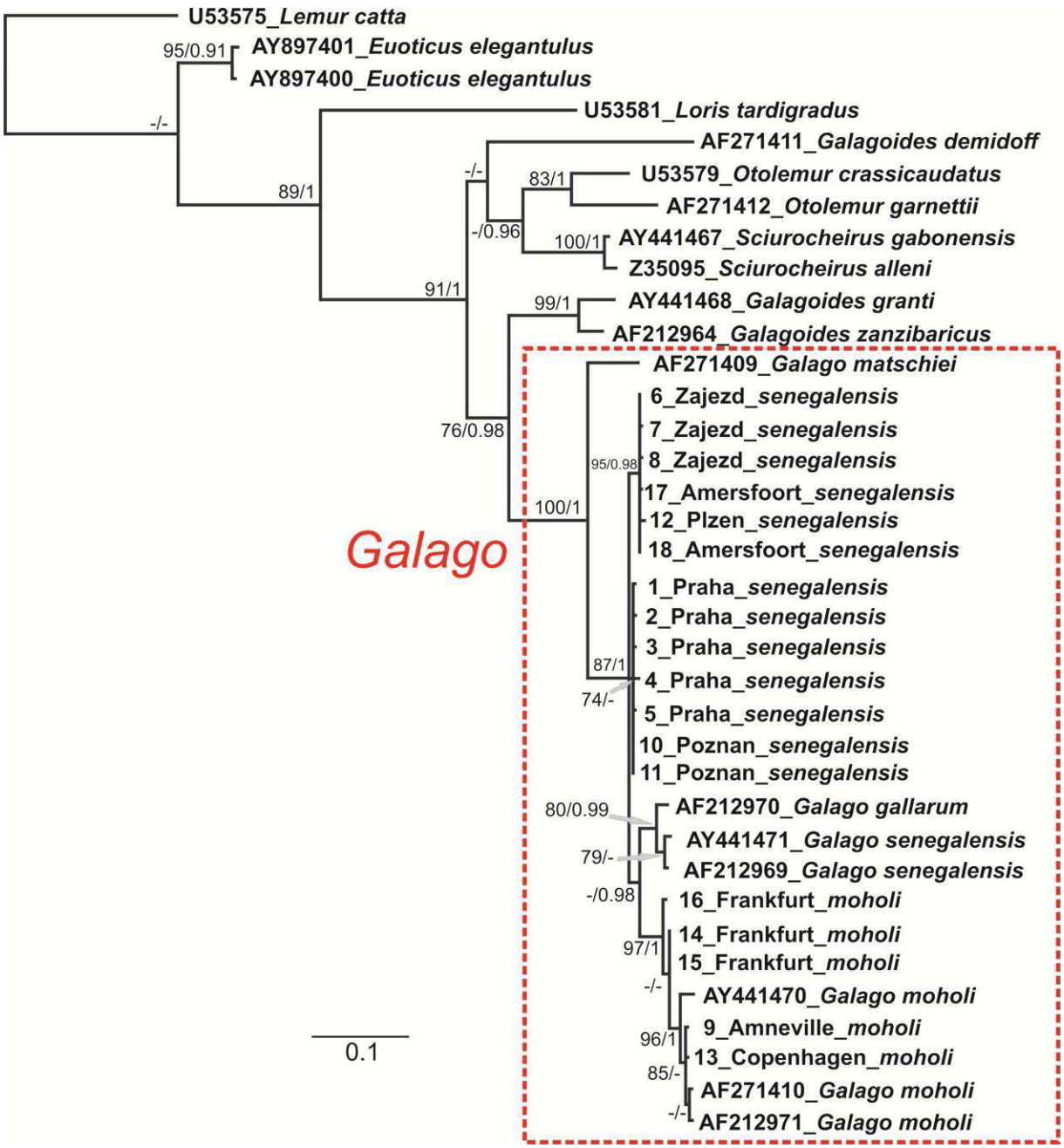

Fig. 7. Phylogeny of the family Galagonidae based on data available in GenBank and our sequences of animals from European Zoos. Both ML and BI trees resulted in the same topology, only the BI tree is presented. Numbers by nodes are bootstrap values of the ML analysis followed by bayseian posterior probabilities. Values below 70% (ML) and 0.9 (BI) not shown.

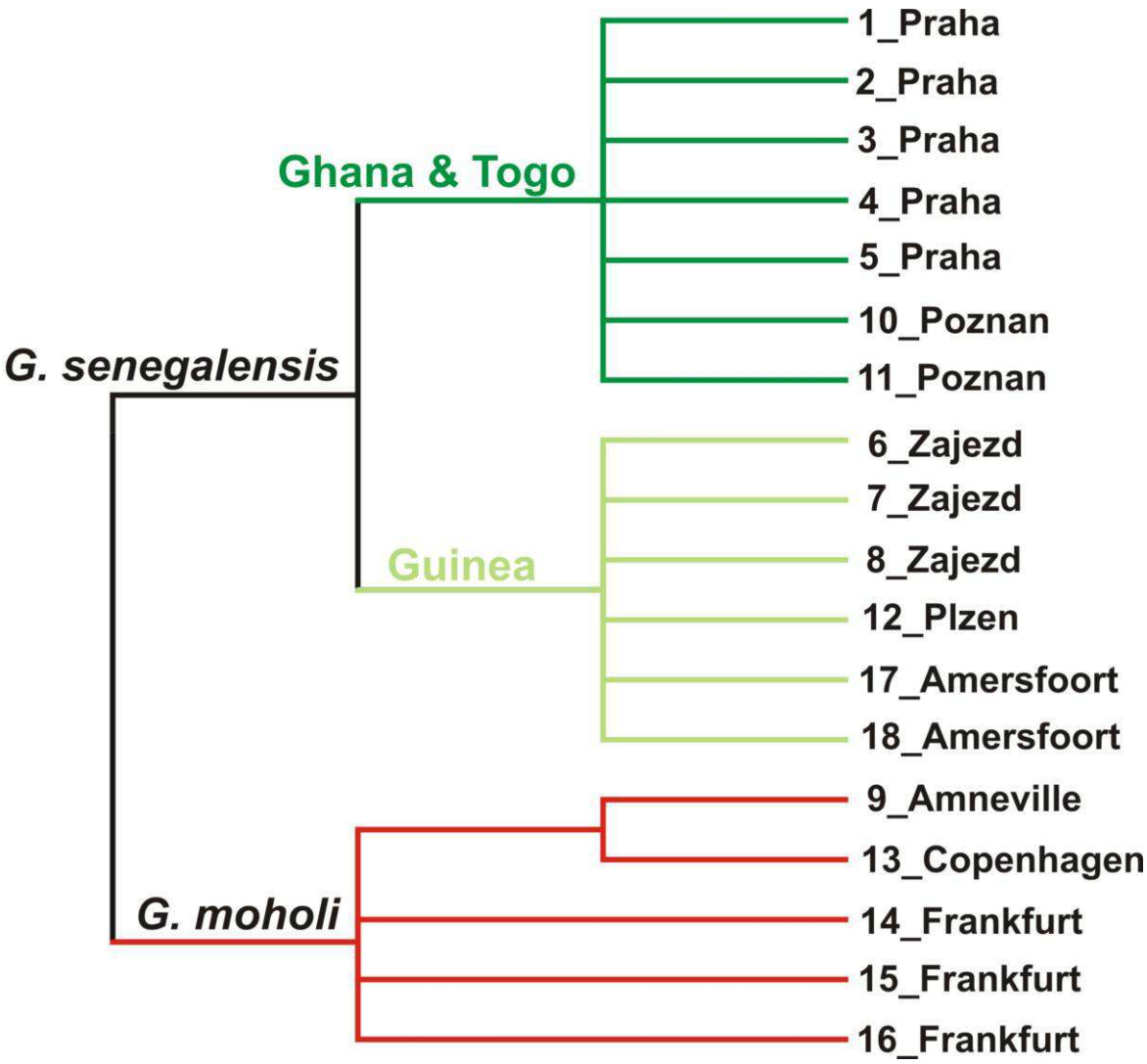

Fig. 8. Strict consensus maximum parsimony phylogenetic tree of galagos from European Zoos. Colours of individual lineages correspond with colours in the haplotype network.

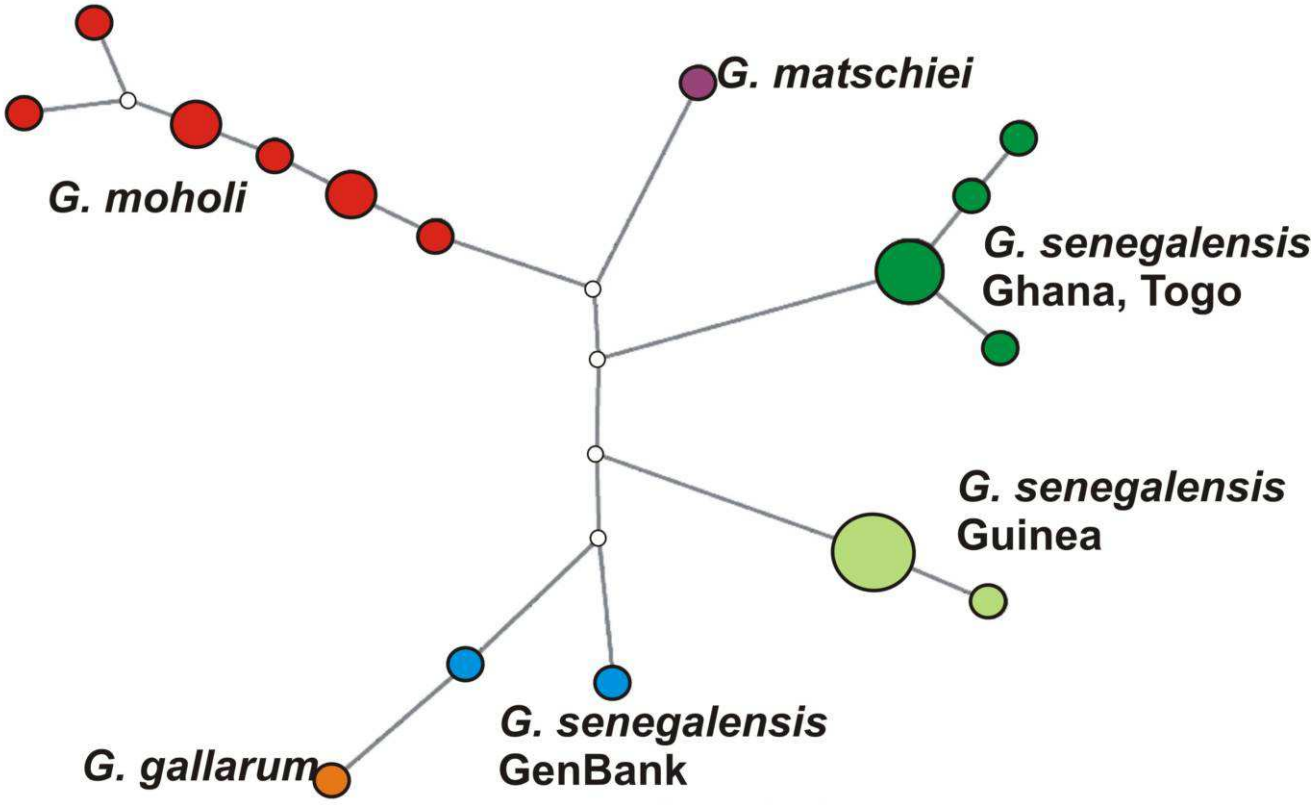

Fig. 9. Median joining haplotype network of the genus *Galago*. Colours of individual lineages correspond with colours in the MP tree.

5. DISCUSSION

This work relied on a material collected in a form of buccal swabs in European Zoos. As one of the most non-invasive methods of collecting tissue for genetic analysis (behind faeces sampling), buccal swabs are widely used in works with endangered species or in forensics (Rudbeck and Dissing 1998). However, extracting DNA from swabs claims higher sterility and the yields are usually much lower when compared with DNA extraction from regular tissue pieces (muscles, liver, spleen etc.). When workig with the primate DNA, the risk of contamination by human is higher than in other taxa, especially in amplifications with universal vertebrate PCR primers. For our purposes of sequencing mtDNA which is usually easier to amplify than nuclear DNA (higher amount in cells, absence of heterozygotes) the buccal swab were a sufficient cell source. So even if DNA extracted directly from tissue resulted in higher yields and easier amplifications, the advantage of buccal swabs as a non-invasive collecting method is still overwhelming and can hardly be replaced by for instance ear clips in galagos.

The results of the genetic analyses of the combined dataset of our samples of galagos from various Zoos and the GenBank sequences are in some degree concordant with already published works (DelPero et al. 2000; Stiner and Turmelle 2003; Roos et al. 2004; Masters et al. 2007; Chatterjee et al. 2009). As already reported by Stiner and Turmelle (2003), the genus *Euoticus* formed a basal lineage sister to all other galagids, although with a rather low bootstrap support. The phylogeny based on seven mtDNA genes supporting sister relationship between *Euoticus* and *Galago* provided by Chatterjee et al. (2009) seems more robust and probable. All other analyzed genera formed monophyletic clades in our results, the only exception was the genus *Galagoides*. Whereas *Galagoides zanzibaricus* and *G. granti* form a monophylum, *G. demidoff* stays completely apart of these two. This morphologically problematic group aptly called „*a wastebasket taxon of plesiomorphic species*“ by DelPero et al. (2000) consists of more independent genera with retained ancestral characters impeding more accurate morphological distinction.

To my big luck, all species recognized nowadays as members of the genus *Galago* were available in the GenBank. In both ML and BI analyses the genus *Galago* was clearly monophyletic with supports leaving no doubts about its shared common ancestor. Inner

relationships within *Galago* differ in almost every publication: all authors who had *gallarum* at their disposal (DelPero et al. 2000; Stiner and Turmelle 2003; Chatterjee et al. 2009) considered *gallarum* sister to *senegalensis*, these two than sister to *moholi*. In other works where *gallarum* sample was missing (Roos et al. 2004; Masters et al. 2007), *senegalensis* was recognized as a closest relative to *moholi*. Unlike the others, Stiner and Turmelle (2003) were not able to resolve mutual relations within *Galago*, although they were the first who analyzed all four species. There is no doubt that comparing publications where some species were not included does not really make much sense, therefore a comprehensive study on more independent genes and all species was needed. Chatterjee´s et al. (2009) results based on seven mtDNA and three nDNA genes can be taken as the most reliable, however, *matschiei* was not included in the analyses.

Since my dataset is a compilation of all these published data a certain degree of overlap in the results is not surprising. The sequence of *G. matschiei* from the central African Burundi is the most distinct from the others and represents a basal branch. All animals determined as *G. moholi* cluster together with the GenBank *moholi* sequences confirming thus its monophyly. Unfortunately the place of origin of neither the GenBank nor the Zoo *moholi* is known. According to the degree of variability (none halpotype shared) among these sequences all animals were apparently imported from different populations. Average intraspecific differentiation within *moholi* clade equals 1.2% which does not exceed standard degree of cyt *b* variability in one species (Johns and Avise 1998).

The GenBank sequences for *gallarum* and *senegalensis* cluster together. Although the sequence of *gallarum* and one of *senegalensis* (AF212969) are from one publication and could indicate contamination, the second sample of *senegalensis* (AY441471) from different source and clustering with the first *senegalensis* implies a contamination was not likely. The topology is especially remarkable when our Zoo samples are taken into account. Even though they were morphologically determined as *G. senegalensis* by many independent Zoo curators they do not form a cluster closely related with the GenBank *senegalensis*. The easiest explanation of this topology would be misidentification of one *G. senegalensis* with *G. gallarum* which would be then deposited in GenBank under the latter name. Confusion of these two species seems rather unlikely since they can be easily recognized on the basis of different face mask coloration (Butynski and de Jong 2004;

de Jong and Butynski 2010 and the gallery therein). But even in the case of misidentification, *senegalensis* would evince quite high degree of intraspecific genetic variability and, moreover, would not be a monophyletic species. When the within-species variability of *moholi* is compared with that one of *senegalensis*, then we can still clearly see *moholi* forming a well defined and isolated lineage whereas *senegalensis* falls apart into three independent clusters corresponding exactly with the geographic origin sites. When we assume individual *moholi* samples have different geographic origin as well, the distinction within *senegalensis* is even more obvious.

The degree of genetic variability within *G. senegalensis* spanning from 2.3 to 4.2% is in agreement with mtDNA differentiation in other primates. Melnick et al. (1993) reported a difference of 3.75% between the rhesus (*Macaca mulatta*) and the Japanese macaque (*M. fuscata*). In even closer related primates, lemurs, Pastorini et al. (2003) in their multilocus study brought an evidence for a subspecific distinction between *Eulemur fulvus fulvus* and *E. f. rufus* reaching 2.33 – 2.41%. For another subspecies of the same lemur (*E. f. rufus* and *E. f. collaris*), Yoder et al. (1996) shows a distinction of 4.2% in the cyt *b* gene. Johns and Avise (1998) summarized previous studies on cyt *b* genetic distances for pairs of sister species across vertebrate groups, most mammalian sister species diverge in 4 – 7%.

By combining all these information it is apparent that individual groups of *G. senegalensis* are distinct on at least subspecific level. However, the most distinct lineage are the GenBank animals with an unknown origin (meaning geographic location of the original wild populations) so they cannot be on the basis of geographic distribution certainly ascribed to any recognized subspecies. Animals from the Zoos in Zájezd, Plzeň and Amersfoort originally imported from Guinea are the closest to the type locality of *G. senegalensis* in Senegal and as such could belong to the nominotypic subspecies. On the other hand, according to available data (Kingdon 1997), the nominotypic subspecies should span from Senegal eastwards to Sudan and west Uganda thus covering also Ghana and Togo, the original locality of galagos kept today in the Zoo Prague and Poznan. This cannot be resolved until material from the type locality in Senegal is analyzed. But as Kingdon (1997) also notices the boundaries between these populations (i.e. subspecies) are still uncertain, so both Zoo lineages can belong to some already described subspecies. This would cause a massive expansion of the range of distribution of one of the east African

subspecies (*braccatus*, *dunni*, *sotikae*) on one hand and dramatic shrinkage of the range of the true *senegalensis* on the other. In the case of the GenBank data for *senegalensis* we can only guess where they are from. Final resolution of this problem would be to sequence animals from type localities of all subspecies which would clearly delimit what lineages bear which subspecific name.

Thus, with the current knowledge, the galagos kept in the Zoos Prague and Poznan should be held separately from the animals kept in Zájezd, Plzeň and Amersfoort. Despite their morphological determination as *G. senegalensis* they evince substantial genetic differences testifying long-term independent evolution of each clade. We can easily overlook speciation in its beginnings. Although it might not cause problems like viability decrease or health problems in potential hybrids, keeping both lineages together would result in creating a „Zoo lineage“, an entirely new animals we might not encounter anywhere in the wild with. This has already happened in gibbons (Geissmann 1984; Mootnick 2006).

Unlike *G. senegalensis*, *G. moholi* do not exhibit such a degree of variability, if there are any geographically correlated distinctions we are not able to detect them for the time being. So the restrictions suggesting separated breeds of *senegalensis* do not apply to *moholi*, at least for animals from the studied Zoos.

All members of the family Galagonidae exhibit certain conservativeness in their morphological characters as can be seen in the unstable taxonomy, in the case of *Galagoides* paraphyly or in the subtle morphological differences between *Galago senegalensis* and *G. moholi*. The inability to notice some phenotypic difference (if there is any) between our two Zoo *senegalensis* lineages is then not surprising, moreover when none curator has a comparative material for both of them at disposal. Lacking detailed morphological data this molecular evidence is now our only lead how to assign possible new imports of galagos from the west Africa. In a case of new imports, the animals should be genotyped before they will be intermixed with some already existing breeding group with known affinities.

Next task should be to find out whether there are any morphological, behavioral or bioacustic differences between the two Zoo lineages. Taking into account problematic determination of individual species of the genus *Galago* this may be a bit of a challenge. Morphology has been thoroughly studied into the slightest details such as hair

ultrastructure (Anderson 2001), but usually in studies comparing different species, intraspecific variability was not taken into consideration or was minimal (Anderson 1999, 2000, 2001). Contradictory results were reported in works on intraspecific variability in vocalization. Whereas Anderson et al. (2000) did not reveal any variation accross the range of *Galago moholi*, Ambrose (2003) found four distinct types of vocalization in *Sciurocheirus alleni* on relatively small area covering Gabon, Cameroon and Bioko Island. Possible variability in vocalization of *Galago senegalensi*s remains to be discovered. From genetic point of view, nuclear genes should be analyzed to reveal whether galagos differ only on the level of mtDNA with high mutational pace or if there are also differences in more conservative nuclear genes. In a case of finding variability in nuclears then the question of existence of independent subspecies becomes really interesting.

6. CONCLUSIONS

- Galagos from the Zoos in Frankfurt, Amneville and Copenhagen were genetically determined as *G. moholi*, galagos from all the other analyzed European Zoos as *G. senegalensis*. These results are in concordance with the morphological determination. Therefore morphological characters used to distinct these two species are reliable.
- *G. moholi* exhibit only a minor intraspecific variability in sequences for the cyt *b* gene. Average within-species differences reach 1.2% what falls into the standard intraspecific variance inside one species in primates.
- Individuals of *G. moholi* from the Zoos we have analyzed can be bred together.
- There are three lineages that *G. senegalensis* clusters into:
  1) animals from the Zoos in Prague and Poznan whose ancestors have been imported from Ghana and Togo
  2) animals from the Zoos in Zájezd, Plzeň and Amersfoort originally from Guinea
  3) the GenBank data
- Average genetic distance between individual *senegalensis* lineages spans from 2.3 - 4.2%. This range is comparable with distinctions found in other primates on subspecific or even specific level.
- Individuals of *G. senegalensis* from Prague and Poznan can be bred together, as well as can be animals from Zájezd, Plzeň and Amersfoort. Nevertheless, these two groups should remain in mutual isolation.
- New potential imports from Africa should be genotyped first to find out what group they belong to, only after that animals could be intermixed with already existing breeds.

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