© 2015 Journal compilation
http://biology.num.edu.mn
http://mjbs.100zero.org/
Volume 13(1-2), 2015

Mongolian Journal of Biological Sciences

ISSN 1684-3908 (print edition)
MJBS
ISSN 2225-4994 (online edition)

Original Article

Morphological Approach to Genetic Variability of the Asiatic Wild Ass (*Equus hemionus*) Using Non-metric Skull Characters*

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Abstract

Key words:
Asiatic wild ass, epigenetic variability, *Equus hemionus,* fluctuating asymmetry, non-metric skull characters, population genetics, Southern Mongolia,

Article information:
Received: 07 May 2015
Accepted: 19 Nov. 2015
Published: 26 Nov. 2015

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The Asiatic wild ass (*Equus hemionus*) is a globally endangered large mammal threatened by competition with livestock, poaching as well as habitat fragmentation and loss. Because of these environmental stresses it can be assumed that the species suffers from lower developmental stability and that barrier effects could cause genetic isolation. To gain more insight into the population genetics of the Asiatic wild ass, a series of 440 skulls found in two Gobi regions of Mongolia were examined. The epigenetic variability and epigenetic distance between the samples as well as their fluctuating asymmetry were studied by using 62 non-metric skull characters to test and evaluate possible genetic depletion, genetic isolation and the general influences during ontogenesis. The high epigenetic variability, $I_{ev} = 0.39$ did not differ between the two regions indicating no evidence of genetic depression. The very low, but significant epigenetic divergence of MMD = 0.05 between the Dzungarian Gobi and the Southern Gobi suggests restricted connectivity. The moderate degree of fluctuating asymmetry (FA = 0.11 – 0.15) found gives no signs of reduced developmental stability. Thus, our results suggest that Asiatic wild ass from Mongolia are from genetically viable populations.

Cite this paper as: Lkhagvasuren, D., Batsaikhan, N., Samiya, R., Schafberg, R., Stubbe, A., Stubbe, M. & Ansorge, H. 2015. Morphological approach to genetic variability of the Asiatic wild ass (*Equus hemionus*) using non-metric skull characters. *Mong. J. Biol. Sci.*, 13(1-2): 3-12.

Introduction

The Asiatic wild ass (*Equus hemionus hemionus* Pallas, 1775) is one of the most endangered large mammal species not only in Mongolia, but also in the world (Clark *et al.*, 2006; Moehlman *et al.*, 2008). It was once widespread throughout steppe and semi-desert habitats. Today it occurs only in the Dzungarian Gobi, Trans-Altai Gobi, Northern Gobi and Alashan Gobi in Mongolia

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*Results of the Mongolian-German Biological Expeditions since 1962, contribution No. 324

(Zevegmid & Dawaa, 1973; Reading *et al*., 2001; Feh *et al*., 2002; Clark *et al*., 2006, Kaczensky *et al*., 2011), and adjacent China (Chu, 2008; Bi, 2007). The Asiatic wild ass has lost about 50% of its distribution range in Mongolia during the last 70 years (Stubbe *et al*., 2012), and population size has decreased severely due to habitat fragmentation and loss, conflicts with livestock for pasture and water access, and poaching (Kaczensky *et al*., 2011; Stubbe *et al*., 2007). Therefore, many investigations on the species in different parts of Mongolia were conducted, including studies on social structure (Feh *et al*., 2001; Neumann-Denzau & Denzau, 2007), population characteristics (Reading *et al*., 2001; Lkhagvasuren, 2007; Tsendjav & Purevsuren, 2007; Ransom *et al*., 2012), habitat fragmentation and loss (Kaczensky *et al*., 2011; Ito *et al*., 2013), reproduction and mortality (Stubbe *et al*., 2007; Stubbe *et al*., 2012), age determination (Ansorge *et al*., 2007; Lkhagvasuren *et al*., 2013), as well as population structure and mortality (Lkhagvasuren *et al*., submitted). Nevertheless, for a more comprehensive evaluation of the population status of the Asiatic wild ass in Mongolia, there is still a need for more insight into the population genetics of the species. Of particular interest in this area is the degree of genetic variability and genetic divergence between potentially isolated subpopulations. The only study in this regard was done by Kaczensky *et al*. (2011) who examined the population genetics of the species by microsatellite DNA analysis identifying two subpopulations in the Dzungarian Gobi and in the joint Trans-Altai and Southeastern Gobi. The first is exactly one of our two survey areas, and the second includes our second region in large parts.

Besides molecular genetic techniques, morphological studies on the skeleton can be used to obtain information about genetic variability (Ansorge, 2001). Morphological investigations of non-metric skeletal characters also reflect the actual genetic background of phenotypic appearance. For this phenomenon the term "epigenetic" has been established at least since the middle of the last century (Berry & Berry, 1967; Ansorge, 2001). Non-metric characters are discontinuous variants in different parts of the skeleton. Their appearance results mainly from basic genetic control and they are independent of growth and direct external environmental influences qualifying them as morphological genetic markers (Bauchau, 1988). Therefore, they are suitable to assess genetic variability and divergence among populations (Sjøvold, 1977).

A large collection of Asiatic wild ass skulls from two regions of the Mongolian Gobi (Stubbe *et al*., 2005; Lkhagvasuren *et al*., submitted) offers the opportunity to utilize morphological investigations of non-metric skeletal characters for genetic studies. The aim of this study was to analyse the genetic variability and possible genetic distances between potentially isolated populations of the Asiatic wild ass based on epigenetic traits of the skulls. We expect a reduced genetic variability induced by possible genetic isolation and expressed by a lower variation of the morphological characters. A main objective of the study points at the question if there are isolated subpopulations as we predict because of several geographic barriers (e.g. mountain ranges, border fences). This represents the first report on the epigenetic use of non-metric skull characters for this species.

Materials and Methods

Sample materials and areas.

A total of 440 skulls of Asiatic wild ass were collected from carcasses in the Gobi region of Mongolia since 2001 by researchers of the National University of Mongolia (NUM) and Halle University (Stubbe *et al*., 2005; Stubbe *et al*., 2007; Lkhagvasuren *et al*., 2013). All of the skulls were found as dried carcasses; at least 41% were hunted illegally based on cut marks and bullet holes although the true number of poached animals was probably higher (Lkhagvasuren *et al*., submitted). In accordance with national legislation and international requirements, the skulls were shipped to Germany for technical processing and archival. They are kept in the collections of the Museum of Domesticated Animals "Julius Kühn" in Halle and the Senckenberg Museum of Natural History in Görlitz.

Most skulls were collected in the Southern Gobi (*n* = 379) between 2001 and 2004 (Stubbe *et al*., 2005). Furthermore fewer skulls are from the Dzungarian Gobi (*n* = 61) mainly collected in 2010 (Lkhagvasuren *et al*., 2013). The geographical location of the sample areas is shown in Fig. 1. The distance between the Dzungarian Gobi and the Southern Gobi is about 1,000 km with an additional barrier effect of the high mountain

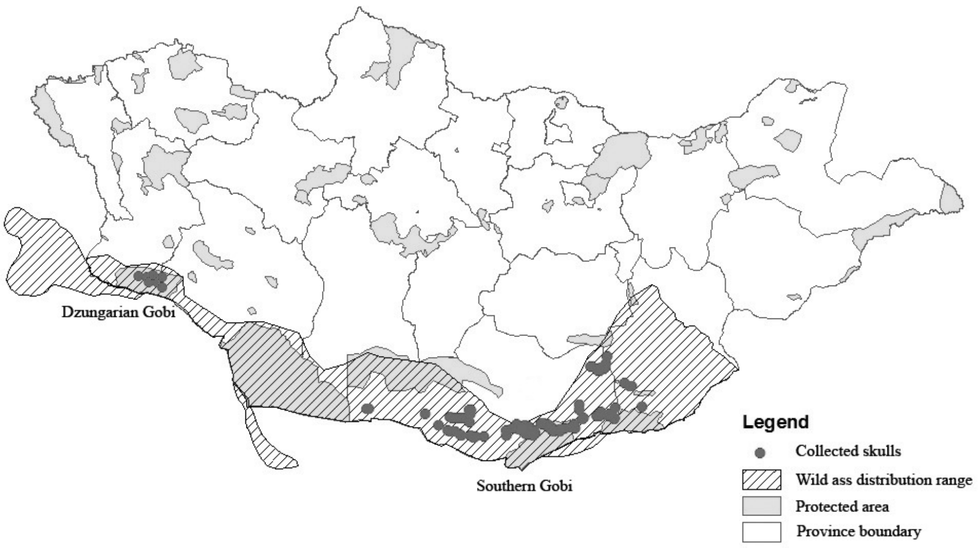

Figure 1. Distribution of the Asiatic wild ass in Mongolia and adjacent China (after Kaczensky *et al*., 2011; Chu, 2008; Bi, 2007), and the localities of the collected skulls.

ranges enhanced by the border fence between Mongolia and China (Kaczensky *et al*. 2011). Both Gobi regions are largely flat with scarce open water resources. The average annual precipitation is higher in the Southern Gobi (up to 200 mm) than in the Dzungarian Gobi (96 mm). The vegetation types vary from desert steppes with perennial grasses and shrub-dominated semi-deserts to true deserts (von Wehrden & Wesche, 2007). The extent of illegal hunting seems to be much higher in the Southern Gobi than in the Dzungarian Gobi (Wingard & Zahler, 2006; Stubbe *et al*., 2007).

Age and sex determination.

The age of the Asiatic wild asses was estimated by the eruption and replacement of the teeth and the annual lines in the tooth cementum. The sequence of tooth eruption and replacement shows the age up to five years (Ansorge *et al*., 2007; Lkhagvasuren *et al*., 2013). For older animals, longitudinal root sections of the first upper incisor produce incremental annual cementum lines allowing the exact age determination in years (Ansorge, 1995; Ansorge *et al*., 2007; Lkhagvasuren *et al*., 2013). After age determination all skulls were divided in five age groups to evaluate the potential dependence of non-metric character expression on normal growth.

To avoid including sex dependent non-metric characters, the sex of Asiatic wild asses was determined by the presence of canines in males, being mostly absent or rudimentary in females (Lkhagvasuren *et al*., 2013).

Epigenetic analysis by the use of non-metric characters.

The use of non-metric traits for population genetics is based on the high heritability of non-metric characters (Berry, 1978). Furthermore, the minor variants of non-metric skeletal characters are of lower importance for an organism than selectively more relevant traits. Therefore, such traits are exposed to a minimum of selective pressure, qualifying them as epigenetic markers reflecting the genetic circumstances of the relevant phenotype (Ansorge *et al*., 2009).

Nevertheless, no study on non-metric skull characters in any species of the Equidae has been carried out to date, whereas most carnivores or rodents have been considered (Ansorge *et al*., 2009, 2012). After first attempts (Ansorge *et al*., 2007), 62 non-metric characters were selected (Table 1, Fig. 2) based on their frequency and variability as well as on the results of earlier studies on ungulates (Markowski & Markowska, 1988; Markowski, 1993). They include 53 bilateral characters being registered separately on both sides of the skull.

The frequencies of the character expressions were checked for their homogeneity in both sexes and in the five age groups mentioned above. Sex- or age-dependent characters were excluded from further investigation after a χ^2-test at a significance level of $p < 0.05$ (Weber, 1980).

The characters occur with different frequencies resulting in single variability of every character from the numerical difference from a frequency

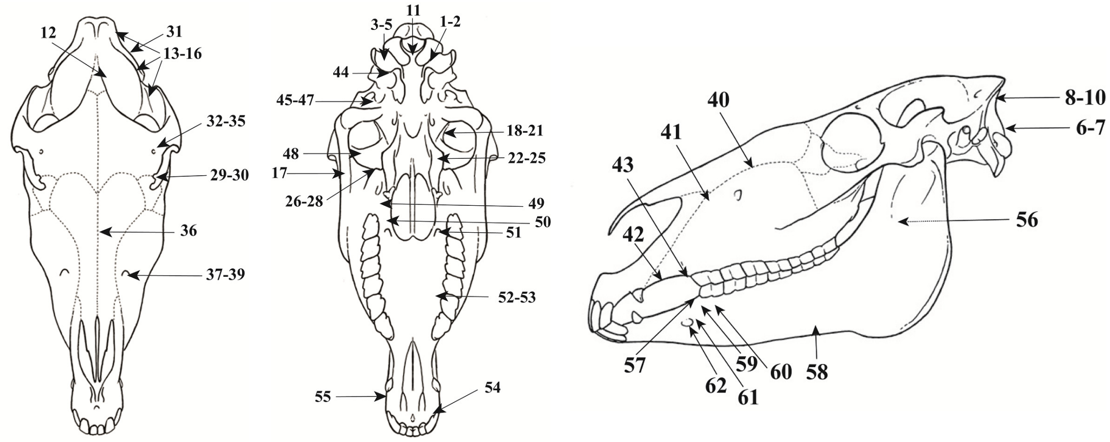

Figure 2. Position of 62 non-metric characters in the Asiatic wild ass skull.

Table 1. Non-metric characters in the Asiatic wild ass skull.

1	CF	Condylar foramen double
2	ACF	Accessory condylar foramen present
3	IHF	Internal hypoglossi foramen double
4	HF1	Accessory internal hypoglossi foramen 1 present
5	HF2	Accessory internal hypoglossi foramen 2 present
6	SFP	Supraoccipital foramen present
7	SFD	Supraoccipital foramen double
8	ESF	External supraoccipital foramen present
9	MFS	Medial supraoccipital foramen present
10	ISF	Intramedial supraoccipital foramen
11	IMF	Internal magnum foramen present
12	MPF	Major parietal foramen double
13	TWTF	Two temporal foramina present
14	THTF	Three temporal foramina present
15	FTF	Four temporal foramina present
16	MFTF	More than four temporal foramina present
17	ZPF	Zygomaticum posterior foramen present
18	EFD	Ethmoidal foramen double
19	AOC	Accessory optic channel present
20	AIOF	Accessory infra-orbital fissure present
21	SAF	Small alar foramen present
22	CAF	Caudal alar foramen present
23	PAOF	Posterior accessory oval foramen present
24	OIAOF	One interior accessory oval foramen present
25	TIAOF	Two interior accessory oval foramina present
26	APCF	Accessory palatine caudal foramen present
27	AMF	Accessory maxillary foramen present
28	ASF	Accessory sphenopalatine foramen present
29	ALF	Accessory lacrimal fossa present
30	LPF	Lacrimal process foramen double
31	TPF	Three parietal foramina present
32	OFF	One frontal foramen present
33	TWFF	Two frontal foramina present
34	THFF	Three frontal foramina present
35	MWFF	More than two frontal foramina present
36	NF	Nasal foramen present
37	AIIF	Accessory interior infraorbital foramen presen
38	ABIF	Accessory foramen beside infraorbital foramen present
39	IFBB	Infraorbital foramen with bone bridge
40	MFP4D	Maxillary foramen above P^4 double
41	MFP2	Maxillary foramen above P^2 present

42	MF	Maxillary foramen present
43	UFP	Upper first premolar present
44	SFD	Stylomastoid foramen divided
45	RF	Retroarticular foramen present
46	OARF	One accessory retroarticular foramen present
47	TARF	Two accessory retroarticular foramina present
48	IFD	Inferior foramen double
49	AMPF	Accessory minor palatine foramen present
50	PPFD	Posterior palatal foramen double
51	GPFBB	Greater palatine foramen with bone bridge
52	TWMF	Two maxillary foramina present
53	THMF	Three maxillary foramina present
54	IF	Incisive foramen present
55	CF	Canine foramen present
56	MFBB	Mandibular foramen with bone bridge
57	LFP	Lower first premolar present
58	VMF	Ventral mandibular foramen present
59	AMF	Accessory mental foramen present
60	PAMF	Posterior accessory mental foramen present
61	SAMF	Superior accessory mental foramen present
62	MFBB	Mental foramen with bone bridge

of 50%. The variability of the population was presented by the mean variability of all single characters of the sample. Thus, according to Smith (1981) the **degree of epigenetic variability**, I_{ev} for a population sample was calculated using the following equation:

$$I_{ev} = 1 - \frac{\sum_{i=1}^{n} \left|50\% - F_i\right|}{n\,50\%}$$

where, n = number of characters; F_i = frequency of the i^{th} character

The **degree of epigenetic distance** was calculated by the "Mean Measure of Divergence" (MMD) proposed and derived by Sjøvold (1977) from the Mahalanobis distances. The standard deviation S_{MMD} of the MMD indicated the statistical significance at the level of $p < 0.05$ to be MMD > 2 S_{MMD}.

$$MMD = \frac{1}{r}\sum_{i=1}^{r}\left[\left(\Theta_{1i} - \Theta_{2i}\right)^2\right] - v_i$$

$$S_{MMD} = \sqrt{\frac{2\sum_{i=1}^{r} v_i^2}{r^2}}$$

where, r = number of traits; n = sample size; p = frequency of traits

$\Theta = \arcsin(1 - 2p)$, $v_i = 1 / n_1 + 1 / n_2$

To assess the level of the MMD for populations connected without any barriers, the complete material from the Southern Gobi was divided into three subsamples: Southern Gobi West (*n* = 48), Southern Gobi Centre (*n* = 273) and Southern Gobi East (*n* = 48). These three and the Dzungarian Gobi populations were compared with each other by MMD calculations.

In addition, the **fluctuating asymmetry**, a non-directional difference in the right and left sides of bilateral non-metric characters, was used to measure developmental stability (Ansorge, 2001). The degree of fluctuating asymmetry (FA) is defined as the relation of the number of asymmetric occurrences of a single character to the sample size (Ansorge *et al*., 2012). The unscaled mean of all characters results in the degree of asymmetry of the population (Palmer & Strobeck, 1986). Student's *t*-test was used to test the significance of the mean differences in the fluctuating asymmetry of the subsamples ($p < 0.05$).

Results

Initial inspection of the chosen non-metric characters for homogeneity in age and sex found sex-specific expression in two characters (53 THMF, 58 VMF), and age-dependence in twelve characters (8 ESF, 17 ZPF, 30 LPF, 32 OFF, 33 TWFF, 37 AIIF, 40 MFP4D, 41 MFP2, 41 MFP2, 50 PPFD, 57 LFP, 58 VMF). For these 14 characters the χ^2-test adduced values from $\chi^2 = 4.08$ (df = 1, $p < 0.05$) to $\chi^2 = 104.0$ (df = 4. $p < 0.001$). Therefore, the traits were excluded from further analyses.

Epigenetic variability.

The epigenetic variability of the whole sample of all Asiatic wild asses pooled together was I_{ev} = 0.39. There was only a small difference between the geographic samples: Dzungarian Gobi I_{ev} = 0.389 and Southern Gobi I_{ev} = 0.387. This does not indicate any geographical trend.

Epigenetic population divergence.

The epigenetic distance between the Dzungarian Gobi population and the complete Southern Gobi population was quite low, but statistically significant (MMD = 0.05, S_{MMD} = 0.004) based on the remaining 48 characters. The theoretically imputed subsamples of the Southern Gobi showed no differences in two pair-wise comparisons, but also an epigenetic distance of MMD = 0.03 between the samples from Southern Gobi West and Southern Gobi Centre (Table 2). However, the cluster of the three Southern Gobi subsamples was slightly, but clearly separated from the Dzungarian Gobi.

Fluctuating asymmetry.

The degrees of fluctuating asymmetry were generally moderate. Asiatic wild asses from the Dzungarian Gobi showed the lowest level (FA = 0.11). The complete sample of the Southern Gobi population was a little more asymmetrical (FA = 0.15). However, this difference was statistically significant (t = 2.04, df = 1, p = 0.04). Interestingly, there was no significant difference in fluctuating asymmetry between the poached population part of the Southern Gobi sample and the sample part with unknown cause of death (t = 0.09, df = 1, p = 0.92).

Discussion

The relatively simple morphological tool of non-metric skeletal variants and the application of these characters for population genetics seem to be highly attractive to solve questions of genetic isolation, inbreeding or bottleneck effects, especially with regard to endangered species (e.g. Ansorge & Stubbe, 1995; Pertoldi *et al*., 2000; Ansorge *et al*., 2012; Ranyuk & Monakhov, 2011; Ranyuk & Ansorge, 2015). In fact, the study of the epigenetic variability and distances within and between the geographic samples of the Asiatic wild ass from Mongolia should help to assess whether habitat isolation and landscape fragmentation already affect the population. Although the assumption of high heritability of the non-metric characters was validated or experimentally confirmed only on humans and laboratory mice (Berry, 1968), these non-metric characters have become widely accepted as genetic markers (Bachau, 1988), and their frequency analysis is a well-established technique (Ansorge *et al*., 2012; Baker & Hoelzel, 2013; Tibbetts, 2013; Usukhjargal *et al*., 2013; Wiig & Bachmann, 2014; Ranyuk & Ansorge, 2015). It is therefore, reasonable to use them to clarify the questions of the genetic variability and possible genetic distances between populations of the Asiatic wild ass. On the other hand, because some variants in foramina seem to be less heritable, a potential environmental influence has to be accepted and faced with the simultaneous analysis of numerous traits (Sjøvold, 1977). Therefore, in this study on Asiatic wild ass in Mongolia the high number of 62 characters is the prerequisite for the epigenetic value of the chosen non-metric traits.

The analysis of these characters led to the surprising result of the highest degree of epigenetic variability (I_{ev} = 0.39) ever found not only in the scarce investigations on ungulates (Zima, 1989), but also in several small mammal populations including wood mouse (*Apodemus sylvaticus*), bank vole (*Clethrionomys glareolus*), common dormouse (*Muscardinus avellanarius*) and mole

Table 2. Mean measures of divergence (MMD) between the Asiatic wild ass samples (asterisks indicate statistical significance).

	DG	SGW	SGC	SGE
Dzungarian Gobi (DG)	0.0	0.064	0.054	0.024*
Southern Gobi West (SGW)		0.0	0.032*	0.0
Southern Gobi Centre (SGC)			0.0	0.0
Southern Gobi East (SGE)				0.0

(*Talpa europaea*) (Ansorge *et al*., 2012). Only some carnivore populations of polecat (*Mustela putorius*), pine marten (*Martes martes*), Eurasian otter (*Lutra lutra*) and badger (*Meles meles*) achieved similar high epigenetic variabilities (Ansorge *et al*., 2009). In ungulates, several populations of roe deer (*Capreolus capreolus*) from the territory of former Czechoslovakia showed an average variability of about 0.13 (Zima, 1989). For the population genetics of the Przewalski´s horse (*Equus przewalskii*) reintroduced in Mongolia, a preliminary report described a degree of epigenetic variability of 0.25 by the use of the same non-metric characters as in the present study (Usukhjargal *et al*., 2013).

The higher degree of epigenetic variability in Asiatic wild ass indicates the much higher population size and obviously no genetic depression by reproductive isolation or founder effects in the past. The almost identical epigenetic variability levels in the two geographic samples from the Dzungarian Gobi and the Southern Gobi point to the same conclusion. A DNA analysis of the Asiatic wild ass in Mongolia showed just as high a level of microsatellite diversity (Kaczensky *et al*., 2011). Based on a study on the evolutionary history of ass-like equids, the Asiatic wild ass also had the highest nucleotide and haplotype diversities compared to nine populations of three other wild ass species and subspecies (Rosenbom *et al*., 2015). This status of "apparent genetic health" is due to still widespread distribution of this subspecies although, the authors point out the loss of suitable habitats, declining populations, and increasing barriers in Mongolia and China (Rosenbom *et al*., 2015).

The epigenetic distances between the population samples varied very little, but they are statistically significant. However, the distance of MMD = 0.03 between two subsamples from the Southern Gobi that are connected without any geographical separation indicates the absence of actual differentiation. This result could be caused by, for example, few inhomogeneities of the sample material. On the other hand, the epigenetic distance between the populations from the Southern Gobi and the Dzungarian Gobi was three times higher, suggesting quite low, but distinct genetic differentiation of the populations. Recently, it has been reported that the Asiatic wild ass herds obviously do not migrate between both Gobi regions due to the barrier effects of the Altai Mountains, the border fences between Mongolia and China in the south and higher human and livestock density in the north (Kaczensky *et al*., 2011). However, the very low MMD of 0.05 does not indicate an effective reproductive isolation. Presumably, habitat fragmentation may not be truly significant for the roaming large ungulate species, such as the Asiatic wild ass. On the other hand, range constriction and barrier effects have been increasing only in the past few decades. In the time before the distribution area extended to the steppes in north of the mountain range ensuring the connectivity of the whole Mongolian wild ass population by unrestricted migration. This period of few generations may not be long enough for an effective epigenetic population differentiation. This is in contrast to the level of genomic diversity presented by a study on mitochondrial DNA of the Asiatic wild ass in Mongolia (Kaczensky *et al*., 2011). The authors described "one main genetic boundary" between the Dzungarian, Trans-Altai and southeastern Gobi, although they still expect genetic connection between them. A possible explanation could be the more integrative value of the non-metric characters at the epigenetic level, while the mitochondrial DNA shows higher effects induced by single population genetic occasions like bottlenecks (Ansorge, 2001).

Moreover, in conservation biology the detection of lower genetic diversity in endangered populations resulted from the analysis of non-metric traits (Parsons, 1990). The non-directional difference in the right and left sides of bilateral non-metric characters – fluctuating asymmetry – offers further opportunities as an epigenetic measure of stress in general (Parsons, 1990). It is caused by developmental noise during ontogenesis, e.g. environmental stress often related to anthropogenic influences like chemical or radioactive contamination, food shortage or parasitism (Ansorge, 2001; Tibbetts, 2013). Furthermore, fluctuating asymmetry can also be caused by genetic stress such as hybridisation or inbreeding (Parsons, 1990). Because of that the evaluation of the factors responsible for fluctuating asymmetry is often based on subjective assessment. Thus, the interpretation of fluctuating asymmetry should be treated very cautiously (Palmer, 1996; Gilligan *et al*., 2000; Baker & Hoelzel, 2013; Wiig & Bachmann, 2014).

The moderate degree of fluctuating asymmetry of both populations of the Asiatic wild ass corresponds well with data obtained from different roe deer populations in Poland (Markowski, 1993), whereas a study on roe deer from Germany showed asymmetry levels twice as high as in the wild asses (Zachos *et al*., 2007). In the recently reintroduced populations of the Przewalski´s horses in Mongolia, even a slightly lower degree of fluctuating asymmetry was observed (Usukhjargal *et al*., 2013). The more than 30% higher asymmetry of the Southern Gobi related to the Dzungarian Gobi population could result from more competition with human and livestock and higher hunting pressure in the former (Lkhagvasuren *et al*., submitted). A single outstanding factor may not be responsible for these differences. It should be emphasized that very little is known about diseases, parasites or predation of Asiatic wild asses. The quite low fluctuating asymmetry of the Asiatic wild asses in Mongolia gives no indication of reduced developmental stability and suggests a high degree of fitness in general.

However, the study suffers from the shortcoming of huge differences in sample sizes between the Southern Gobi and the Dzungarian Gobi. On the other hand the two geographic samples show almost identical epigenetic variability levels, very little epigenetic distances and nearly the same degree of fluctuating asymmetry. Thus, it is highly likely that the number of 61 skulls from the Dzungarian Gobi is quite sufficient for this epigenetic investigation. Nevertheless, in the future, additional materials from the Dzungarian Gobi should be collected as well as from the not already sampled areas of the Asiatic wild ass in Mongolia. As another prospect for further investigation, the use of the collected material for molecular analysis offers the chance to get more insight into the genetic nature of the Asiatic wild ass populations.

Acknowledgement

We are very grateful to Margit Hanelt (Görlitz), Diana Jeschke (Görlitz) and Roland Müller (Halle) for preparing and keeping all the wild ass skulls. Sincere thanks go to Bazartseren Boldgiv (NUM) for English revision. This research was partly supported by German Academic Exchange Service (DAAD), and the National University of Mongolia with a young researchers' grant.

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