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CYTOGENETIC EXAMINATION OF SOUTH AMERICAN TAPIRS, *TAPIRUS TERRESTRIS* (PERISSODACTYLA, TAPIRIDAE), FROM THE WROCLAW ZOOLOGICAL GARDEN

B. Kosowska[1], T. Strzała[1], M. Moska[1], R. Ratajszczak[2], T. Dobosz[3]

[1]Wroclaw University of Environmental and Life Sciences,
ul. Kożuchowska, 7, Wrocław, 51–631 Poland
[2]Wroclaw ZOO, ul. Wróblewskiego, 1–5, Wrocław, 51–618 Poland
[3]Wroclaw Medical University, ul. J. Mikulicza-Radeckiego, 4, Wrocław, 50–345 Poland
E-mail: barbarakosowska@poczta.fm (Barbara Kosowska).

Cytogenetic Examination of South American Tapirs, *Tapirus* terrestris (Perissodactyla, Tapiridae) from the Wroclaw Zoological Garden. Kosowska, B., Strzała, T., Moska, M., Ratajszczak, R., Dobosz, T. — Seven lowland tapirs (*Tapirus terrestris*) from Wroclaw ZOO (three females and four males), differing from each other with exterior and sexual behaviour were verified with cytogenetic analysis in order to check their taxonomic status. Cytogenetic analysis was done using two alternative methods of blood collection: 1) conventionally with venepuncture, and 2) with blood sucking bugs from the Reduviidae family. Lymphocytes capable of growing were obtained only with conventional method of blood sampling. Karyotypes and karyograms of all analyzed tapirs were created using classical cytogenetic methods of chromosomes staining. All possessed karyograms had diploid chromosome number equal 80 (2n = 80). Homologous chromosomes did not differ between each other with quantity, size, centromeres location, length of arms, G bands and all were classified as proper karyograms of *Tapirus terrestris* species representatives. The X chromosomes as well as the first pair of chromosomes (both metacentric), were the largest among all analyzed, respectively. All remaining 38 pairs of chromosomes were acrocentric with Y chromosome as the smallest one (in males' karyograms). Blood collected with blood sucking bugs proved to be unsuitable for cell culture. None of the seven established cultures was effective as lymphocytes obtained with this method did not show growth potential in prepared media. Thus, blood collected from the tapirs via *Dipetalogaster maxima* species did not show usefulness for cytogenetic studies due to the inability of cells to proliferation, even after a relatively short period of time elapsed since the blood sampling (1 to 2 hours).

K e y w o r d s: lowland tapir, cytogenetics, ZOO, taxonomic status.

Цитогенетическое исследование южноамериканских равнинных тапиров, *Tapirus terrestris* (Perissodactyla, Tapiridae), из Вроцлавского зоопарка. Косовска Б., Стшала Т., Моска М., Ратайщак Р., Добош Т. — Семь равнинных тапиров (*Tapirus terrestris*) из Вроцлавского зоопарка (три самки и четыре самца), отличающиеся друг от друга по экстерьеру и половому поведению, были использованы для цитогенетического анализа с целью проверки их таксономического статуса. Цитогенетический анализ проводили с использованием двух альтернативных методов забора крови: 1) традиционного с венепункции и 2) с помощью кровососущих насекомых из семейства Reduviidae. Лимфоциты, способные к росту, были получены только традиционным методом забора проб крови. Кариотипы и кариограммы всех анализируемых тапиров были созданы с использованием классических цитогенетических методов окрашивания хромосом. Все полученные кариограммы характеризуются диплоидным числом хромосом, равным 80 (2n = 80). Гомологичные хромосомы не различаются между собой по количеству, размеру, расположению центромер, длине плечей, G-полосам и все были классифицированы как таковые у представителей вида *Tapirus terrestris*. Среди всех проанализированных крупнейшими являются соответственно X-хромосомы, а также первая пара хромосом (обе метацентрические). Все остальные 38 пар хромосом — акроцентрические с наименьшей Y-хромосомой (в кариограммах самцов). Забор крови с использованием кровососущих насекомых оказался непригодным для культуры клеток. Ни одна из семи полученных культур не была эффективной, поскольку лимфоциты, полученные с помощью этого метода, не показали потенциал роста в готовых средах. Таким образом, кровь, собранная у тапиров с помощью *Dipetalogaster maxima*, оказалась непригодной для цитогенетических исследований в связи с неспособностью клеток к пролиферации, даже после относительно короткого периода времени, прошедшего с момента забора крови (от 1 до 2 ч).

К л ю ч е в ы е с л о в а: равнинный тапир, цитогенетика, зоопарк, таксономический статус.

Introduction

The population of South American tapirs (*Tapirus terrestris*) in the zoological gardens in Europe currently consists of about 300 individuals. All of them are included into the EEP breeding program (European Endangered Species Programme), the aim of which is to preserve genetically healthy population of tapirs that could be used in future reintroduction or reinforcement of local, limited in number natural populations. The family Tapiridae emerged in the Eocene on the area of North America (approx. 50 Ma). About 25 Ma, the animals included in the present genus *Tapirus* also lived in Asia, and about 5 Ma they colonized South America (Ashley, Norman, 1996). Only one species in Asia — Malayan tapir (*Tapirus indicus*), and four species in Central and South America — Baird's Tapir (*Tapirus bairdii*), mountain tapir (*Tapirus pinchaque*), South American Tapir (*Tapirus terrestris*) and *Tapirus kabomani* have survived to date (Schoch, 1989; Cozzuol et al., 2013). *Tapirus terrestris* (Linnaeus, 1758) is the most wide spread in South America among the five species of *Tapirus* genus (Bodmer et al., 1997). It is observed from northern Colombia to southern Brazil, northern Argentina and Paraguay (Hershkovitz, 1954; Eisenberg, 1989; Emmons, 1990). Only 4 of over 50 scientifically described forms and subspecies of South American tapir are currently accepted (Padilla, Dowler, 1994). These forms were described based on single individuals, which does not reflect the variation within the population and species. *Tapirus* genus has not obtained until now any critical taxonomic revision based on modern cytogenetic and genetic methods, as was in the case of other species. A considerable exterior polymorphism, manifested in differentiation of particular individuals' size and coloration, may be observed among South American tapirs kept in zoological gardens in Europe. Despite the significant differences, they all are classified as the same species. However, large natural range suggests the possibility of genetically diverse subpopulations occurrence, probably justify in their distinguishing in separate taxa.

The premise directly leading to this study undertaking was an unclear sexual behavior of tapirs observed in several zoological gardens in Europe, also in the Wroclaw Zoological Garden (WZG). The preference of only one male by the females, significantly differing with exterior both from females and the other males, was observed in the group of tapirs composed of several males and females. The differences concerned the coat color, body size and the type of construction. In addition, the ignored males showed no willingness to mating the females which were similar to them in terms of exterior. In this situation, only one from several males managed to pass its genes to the offspring, which significantly reduces genetic diversity in a small population. As presumed, the cause of this phenomenon could be non-described so far genetic differences, reflected in the sexual behavior of animals, or the in ability to fertilize. These issues were widely discussed at the annual meetings of the Advisory Group of Tapirs Breeding (TAG) of the European Association of Zoos and Aquaria–EAZA.

In case of this kind of doubts, the first step is usually performance of cytogenetic analysis, allowing preparing the karyotypes of the individuals. The results of examinations most often decide on the species affiliation, and also provide information whether obtained karyotypes contain differences, e.g., in the number and morphology of chromosomes. Cytogenetic studies on tapirs have a short history. Houck et al., (2000) were the first who demonstrated that karyotypes of 4 currently recognized species are characterized by a diverse diploid number of chromosomes, which in Malayan tapir (*Tapirus indicus*) is 52; in mountain tapir (*T. pinchaque*) is 76; in Baird's Tapir (*T. bairdii*) is 80, in lowland tapir (Brazilian, anta) (*T. terrestris*) is 80. Also the similarities and differences between chromosomes of 4 tapir species were studied based on band patterns (Houck, Ryder, 2006).

Specific evolutionary chromosomal transformation in karyotypes of the tapirs were described in comparative studies using advanced cytogenetic techniques, on the basis of which they can be attributed to the species (Trifonov et al., 2008). In order to perform classical cytogenetic test, fresh blood should be obtained from the examined individual in order to establish the culture of suitable cells, usually lymphocytes. Fresh blood collecting from wild animals with traditional method using a syringe is often combined with a stress reaction, which can affect the change in blood parameters and reduce lymphocytes survival in the culture (Capitanio et al., 1996; Reinhardt, Reinhardt, 2000).

An alternative method is blood obtaining through specially bred true bugs (Becker et al., 2006). In recent years, this method has been used in many different animal species, also in tapirs (Becker et al., 2006; Helversen et al., 2006; Stadler et al. 2007). In this study, in order to verify the taxonomic status of the groups of tapirs housed in the Wroclaw Zoological Garden, they were subjected to cytogenetic examinations using blood collected for analysis with two alternative methods — traditional and through true bugs.

Material and methods

The research material consisted of peripheral blood samples collected in 2009–2013 from all South American tapirs held during this period in the Wroclaw Zoological Garden during veterinary control of the health status.

1. Establishing of lymphocytes culture from blood collected from tapirs using the traditional method, directly with a syringe and needle. The veterinarian collected 1ml of peripheral blood directly with syringe from 7 South American tapirs (3 females and 4 males) during the control examinations over the research period. Blood samples collected to heparin tubes were transported to the Laboratory of Molecular Biology and Cytogenetics, Wroclaw University of Environmental and Life Sciences, and placed in culture flasks with 5 ml complete culture medium. Established cell cultures were incubated at 37 ° C for 72 h. Colchicine, which

is an inhibitor of mitotic divisions, was added at 70th hour of culturing. Finally, the cultures were exposed to hypotonic shock and then fixed three times with the mixture of acetic acid and methanol (1 : 3) and applied to microscope slides. After a week, the chromosomal preparations were stained with differentiating agent on the visualization of G bands, using trypsin digestion and Giemsa stain. Then, appropriate metaphase plates were selected, chromosomes were counted and their photographs were taken. The karyotype of each individual was prepared based on 10 normal metaphase plates containing the complete set of chromosomes. All the chromosomes were excised, and final karyograms of the tapirs were prepared based on the analysis carried out using polarizing microscope Nikon Eclipse 50iPOL. The data of 7 tapirs subjected to examination are presented below.

2. Establishing of lymphocytes culture from blood collected from tapirs using the alternative method — through true bugs. The true bugs of *Dipetalogaster maxima* (Reduviidae, Heteroptera) species, the only ones in Europe that can be bought from commercial breeding, were used to collect the blood from tapirs from the WZG (the same individuals from which blood was collected using the traditional method). The bugs were obtained from two cultures: 1) the Zoo in Berlin (Institute of Zoo and Wildlife Research Alfred-Kowalke-St., 17, 10315 Berlin, Germany, E-mail: thomsen@izw-berlin.de tel.: +49–30–5168701) and 2) the Zoo in Wuppertal (Zoo Wuppertal, Dipl. Biol. André Stadler, a.stadler@zoo-wuppertal.de). The bugs were sterile and intended for single use only. *D. maxima* develop in five larval stages (L1-5) until the imago (I). Each stage of insect's development has a characteristic size (e. g. L1 measures 0.4 cm, and imago 4 cm), therefore, the amount of blood possible to collect is precisely determined. In stage L2, it is possible to obtain maximum 0.3ml of blood, in stage L5 up to1.5 ml, and maximum 4 ml in imago stage (Voigt et al., 2006). Such amount of blood is sufficient to establish the culture of lymphocytes. Hungry bugs were applied to the skin of tapirs in the area of large vessels. When they ended meal after 6–20 minutes, they released themselves from the host. Then the blood from bug's abdomen was collected with syringe into the test tube and transported to the laboratory. Since the insect releases heparin into the blood, further blood preparation for analysis was not necessary (Voigt et al., 2006). The next step was to establish the culture of lymphocytes and further proceedings were consistent with the description in the first part of the methodology.

Results

1. The results obtained on the basis of blood taken by a veterinarian directly from tapirs using a syringe with needle.

Several normal metaphase plates were obtained from each individual using the traditional method of blood collection, and 10 from each set were selected for the evaluation. Following the use of visible G bands to the compilation of homologous chromosomes in pairs, all karyotypes from metaphase plates appeared to be similar in terms of shape, size and number of autosomal chromosomes (n = 78), but they differed in terms of number, size and shape of sex chromosomes. Three of them contained two X chromosomes and belonged to females, while four had XY chromosomes arrangement in the karyotype and belonged to males. The karyograms of 7 tapirs from the WZG were prepared based on the obtained karyotypes.

Figure 1 below presents sex emplary karyograms of tapir female and male. The description of chromosomes morphology was prepared based on the classification of Levanet et al. (1964). The vast majority of *T. terrestris* chromosomes presented small size. The largest chromosomes in the cell nucleus were X chromosome and chromosome of pair 1, both metacentric. The other 38 pairs were identified as acrocentric. The smallest chromosome in the image of male karyograms was acrocentric Y chromosome. The detailed analysis of karyotypes of all 7 tapirs did not show any morphological differences between them, both in the number and structure of chromosomes.

Finally, it was found evaluating the karyograms of examined tapirs, that all had diploid number of chromosomes equal to 80, the homologous chromosomes of the examined individuals did not differ in terms of features such as number, size, centromeres location, arm length, G band pattern, which indicates that all examined individuals had normal karyotypes, characteristic for *Tapirus terrestris* species.

2. The results of cytogenetic analysis based on blood of tapirs obtained through DM bugs.

Blood collected with syringe from abdomen of bugs that drank the blood of tapirs proved to be unsuitable for cell culturing, since neither one of seven established lymphocyte cultures was effective. Peripheral blood lymphocytes obtained using true bugs did not show any growth potential in the prepared conventional media. The presence of cell nuclei or normal metaphase

plates was not observed in the cultures after 72 hours. Even in case of an attempt of possibly the fastest blood collection from bug's abdomen immediately after it sucking out of tapirs, and fast blood delivery to the laboratory, there were no signs of lymphocytes growth potential and the attempts of culturing were unsuccessful. It can be concluded with a high probability, that enzymes quickly starting to digest the lymphocytes are present in the alimentary tract of the bugs. The confirmation of this assumption maybe the fact, that single, incomplete and very compact plates contained chromosomes with traces of banding (although trypsin was not used) during the culturing after the conventional staining (Giemsa stain). Thus, the blood collected from tapirs by bugs of *Dipetalogaster maxima* species did not show in practice the use fullness for cytogenetic studies, due to the lymphocytes inability to proliferate in the established cultures, even after a relatively short time from blood collection (1 to 2 hours).

Conclusions

The karyotypes and then karyograms of seven tapirs from the WZG were obtained in this study using conventional chromosomes staining techniques. All of them, had the same diploid chromosome number 2n = 80. Three individuals were normal females with a set of two X chromosomes, while four individuals were the males and, except X chromosome, contained in their cells also Y chromosome, which has rarely been presented in their search so far. The karyotypes of four male swith acrocentric Y chromosome distinctly the smallest in the male karyotype were obtained in this study. Comparative analysis of the obtained karyograms at the background of the images of chromosomes of South American and Baird's Tapir published by Houck et al. (2000) and Trifonov et al. (2008), allowed the statement that all of the examined tapirs, in terms of cytogenetic, with no doubts belong to *Tapirus terrestris* species. Two species of the South American tapirs *T. terrestris* and *T. bairdii* have the same diploid chromosome number 2n = 80. Houck et al. (2000) found the presence of at least 13 identical autosomes in karyotypes of Baird's and anta tapirs, and at least 15 in karyotypes of Baird's and mountain tapirs. In turn, Trifonov et al. (2008) compared inter alia the size of X chromosome and G bands pattern in Baird's, anta and mountain tapir (*T. pinchaque*), which proved to be similar in anta and Baird's tapir, while X chromosome of mountain tapir differ from other species in the size and are as of heterochromatin. Classical karyotyping does not allow however to show many details in the construction of chromosomes, which in conditions of high karyotypes similarity often prevents the interspecies differentiation of the individuals.

Karyograms of all species of tapirs are usually composed of very small chromosomes, where it is difficult to observe G bands. Moreover, two species of tapirs and — anta and Baird's, have the same diploid number of chromosomes 2n = 80, which suggests that both species can be mistaken in this aspect. However, the karyotypes anta and Baird's tapir can be already distinguished on the basis of classical cytogenetic examination, using only the shape of large chromosomes and position of centromeres in them. Karyograms of both species presented by Houck et al. (2000) and their detailed analysis demonstrate the differences in the shape of a few large chromosomes, caused by different position of centromeres, presence of shorter arms, and their size. Moreover, Houck et al. (2000), based on G banding in karyotype of Baird's tapir, demonstrated large changes in type of insertion/deletion in chromosome 2, which can be used in the differentiation. These differences can be clearly seen also in the study by Trifonov et al. (2008), which lists the chromosomes of both species together.

Especially significant differences concern large chromosomes: chromosome 1 of anta tapir is metacentric, while in Baird's tapir — submetacentric, more over chromosome 8 in anta tapir is acrocentric, while in Baird's tapir and — metacentric. These are important differences sufficient for the initial distinction between the representatives of both species, and even for possible recognition of hybrid. Although the situation of ex situ crossing of both species representatives now seems to be almost impossible, such a random event was described in the past in zoological garden in the USA (Tapir…, 1996). It is interesting, that

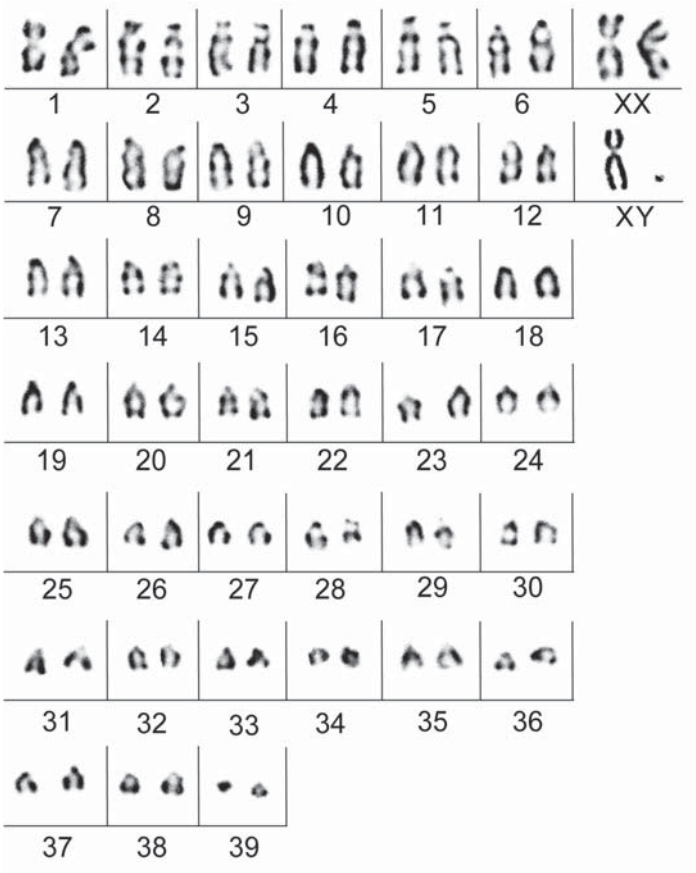

Fig. 1. Karyograme of the lowland tapir (*Tapirus terrestris*) from the Wroclaw Zoological Garden.

the resulting bastard was prolific and left two offsprings.

Due to the fact, that no karyotype differences were observed in this study in the examined individuals, and therefore all of them were qualified for *Tapirus terrestris* species, the observed among them exterior and behavioral differences could have originated from different subspecies affiliation. Among scientifically described over 50 forms and subspecies of anta tapir, only 4 are currently accepted (Padilla, Dowler, 1994). They are: *Tapirus terrestris terrestris* from Suriname, French Guiana, Brazil and Venezuela, *Tapirus terrestris aenigmaticus* from the south-eastern Colombia, Ecuador and Peru; *Tapirus terrestris colombianus* from northern Colombia, and Tapirus terrestris spegazzinii from southern Brazil, Bolivia, Paraguay and Argentina described by Ameghino (1909) as a new species. The distinction of four subspecies among *T. terrestris* was also confirmed by Groves (2006), based primarily on morphological characteristics such as coat color, or size of the individuals. However, these forms were described only on the basis of individual animals, and even just their skull, which certainly do not reflect the variation within population and species.

The researchers from South America report that cytogenetic studies of *T. terrestris* from eastern Colombia indicate a significant karyotype difference of this subspecies. Therefore, the actual existence of different karyotype patterns described in individuals of *T. terrestris* is taken into account. In the first of them, chromosome 1 and X chromosome are submetacentric and the others are acrocentric; that karyotype is characteristic for Central America tapirs (Houck et al., 2000; Hsu, Benirshke, 1975). In the second system described in anta tapirs from Colombia, the karyotype is composed of metacentric pair 1 chromosomes of and X chromosome, 6 submetacentric pairs and other acrocentric (Aguilera, Exposito, 2009.)

In the third karyotype pattern, which was reported in tapirs from Venezuela, chromosome 1and X are metacentric, and all others and — acrocentric (Aguilera et al., 2008).The authors believe that the assumed chromosomal differences described in karyotypes of anta tapirs from Colombia and Venezuela, deserve comprehensive, and concurrently detailed cytogenetic studies, and they also suggest to pay special attention to the samples from the area surrounding Lake Maracaibo in Colombia, since it is probably the habitat of *Tapirus terrestris colombianus* subspecies. At the background of the results presented by Aguilera et al. (2008), the results of this study are most similar to the pattern 3.

Many researchers emphasize that, although in the range of chromosomes number the karyotype of *T. terrestris* has the pattern 2n = 80, such features as fundamental number (FN) determining the number of arms in dual arm chromosomes, morphology of the first pair of autosomes and X chromosome morphology are the variable features. G banding of X chromosome obtained in the present study, as well as the position of its centromere, point to the metacentric chromosome, similar to its picture presented by Aguilera and Exposito (2009) for tapirs from Venezuela and Colombia, while Houck et al. (2000), examining anta tapirs from Panama, described their X chromosome as submetacentric one.

Some results of the research emphasize the existence of hypothetical differences in the patterns of anta tapir karyotypes. Except the diploid number, also fundamental number (FN) is sometimes provided, which is the number of chromosome arms. In Colombia, Sarria-Perea et al. (1999) reported the karyotypes, in which FN of autosomes was 92. The authors described chromosome 1 as a metacentric one, 6 autosomes as subtelocentric, and 32 consecutive autosomes as acrocentric ones. There are therefore some doubts about the presence of shorter arms in a few pairs of large autosomes. Houck et al. (2000) described acrocentric chromosomes with visible shorter arms in the samples from Panama.

In the karyogram presented in this study, sorter arms are also visible in four pairs of autosomal chromosomes (pairs 2–5), but they are so small that these chromosomes were not defined as two-armed submetacentric, but as one-armed acrocentric ones. Similarly, Aguilera and Exposito (2009), described the presence of short arms at least in five pairs of autosomal chromosomes, but also defined as acrocentric, single arm. It may be thus concluded that karyogram presented in this study and that described by Aguilera and Exposito (2009), are very similar. However, compiling in detail both presented karyograms, i. e., tapirs from current study descended from the founders from Brazil, and tapirs from Venezuela from the study of Aguilera and Exposito (2009), it is clear that the short arms of chromosomes of tapirs from Venezuela are visible only in the chromosomes of pair 2, in the other pairs they are virtually imperceptible compared to the karyogram obtained in this study, in which the short arms of chromosomes of pairs 3–5 are clearly visible and significantly longer. Thus, the differences are noticeable and have measurable character. It is essential that these discrepancies were explained, because on the one hand they may be merely subjective, resulting rather from nomenclature in evaluation of chromosomes morphology, on the other hand — a detailed comparison of particular pairs of chromosomes can reveal an existence of real differences between them. Evaluation of the existing diversity makes probable the existence of a subpopulation characterized by genetic differences within *T. terrestris* species.

Among fours species of tapir, *T. terrestrisis* a species the most common in South America, since it is observed from northern Colombia to southern Brazil, northern Argentina and Paraguay (Hershkovitz, 1954; Eisenberg, 1989; Emmons, 1990). It seems that such a large spread of anta tapirs on the continent was likely resulting from the differentiation of the population in geographically different areas, which may also be reflected in chromosomes diversity in their karyotypes.

In this study, in addition to karyotypes visualization and karyograms creation, an alternative method of blood collection for the search from tapirs using *D. maxima* bug was examined. Blood obtained this way appeared to be unsuitable for cell culturing, since lym-

phocytes did not exhibit the growth potential and culturing proved to be ineffective. The only researcher so far who successfully acquired the blood for cell culture (fibroblasts) from bug's abdomen and as a result received karyograms of two bats was Volleth (1985). She used commonly occurring in nature *Triatoma infestans* bug from Triatominae subfamily, of *Triatoma* genus (Reduviidae). The bug drank the blood of bats that were in a cage standing close to the researchers, the blood from the abdomen was collected immediately after the insect finished the meal, and was immediately delivered to the cytogenetic laboratory located nearby. The bug of other species from the family Reduviidae was used in the current study. It was the only bug species available in Europe in the commercial breeding — *Dipetalogaster maxima* of *Dipetalogaster* genus. This bug is less common in nature, but is more useful for the research, due to the large size of adult forms (L III and L IV), and primarily for that reason it is bred for the purpose of blood collecting from different animals, mainly in zoological gardens (Voigt, 2006). From time of the study by Volleth (1985), blood from many animal species, including the tapirs, has been successfully obtained using the bugs, and was then used for serological, biochemical and hormonal analysis (Helversen et al., 1986; Voigt, 2006; Stadler et al., 2007; Kruszewicz et al., 2009).

Considering inefficiency of this method in the present cytogenetic study, the time that elapsed since blood collection from a tapir by the bug, until blood recovery from insect's abdomen (bug's meal lasted from a few to several minutes), that was crucial for the lack of success, as well as the time at which the blood samples was transported to the laboratory, should be taken into account. Blood from the bug was collected with syringe from the back of the insect gut, which contains active digestive enzymes. These also include trypsin-like peptidases, since the result of their activity was observed indirectly — in incomplete and compacted metaphase plates of the lymphocytes, the bands similar to those caused by trypsin addition to cell culture were visible in the chromosomes. Alimentary tract enzymes of that bug are currently extensively examined due to the sudden increase in *D. maxima* importance in tropical medicine (Assumpção et al., 2011). This insect lives only in one region of Mexico and usually feeds on the blood of lizards. However, as a result of intense people inflow to the local suburban areas, the bug found additional hosts in humans and domestic animals. Due to a significant increase in related with it bites and infections, it has become a frequent subject of research in recent years (Salazar-Schettino et al., 2011).

In summary, the study demonstrated normal karyotypes of 7 tapirs, 3 females and 4 males, which after cytogenetic comparative analysis led to the conclusion, that all individuals belong to *Tapirus terrestris* species. The subspecies identification will be possible after comparative material obtaining, yet inaccessible in the literature. An alternative method of blood collection from tapirs through *D. maxima* bugs in order to obtain lymphocyte culture was considered unworkable.

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