Original Article

Determination of age and construction of population age structure of wild Asian elephants based on dung bolus circumference

Chalita Kongrit[1*] Chomcheun Siripunkaw[2]

Abstract

Estimating the age of wildlife is an important technique for the construction of a population age structure that could be useful for the prediction of population change. The age of wild elephants can be reliably estimated from the size of dung bolus circumference which correlates with elephant growth. This research aimed to determine a cut-off bolus circumference for the mature age class of wild Asian elephants at Salakphra Wildlife Sanctuary as inferred from the social behavior of male elephants. Males living within their natal groups were considered immature males, whereas solitary males were considered mature males. The largest bolus circumference of the immature males was used as a cut-off criterion for age class determination. Noninvasive molecular sexing was applied to determine the sex of elephant samples. From a total of 225 dung samples, 96% were successfully sex determined; 90 and 126 samples were identified as male and female, respectively. Among the male samples, 49 samples were from males living within their natal groups and 41 samples were from solitary males. The cut-off bolus circumference was determined to be 42.5 cm. The dung samples with bolus circumferences larger than the cut-off size were classified as belonging to mature elephants. The same criterion was applied to females as well. A population age structure of Salakphra elephants was created based on the bolus circumferences regardless of individual identification. The construction of population age structure based on dung sampling could be useful for a rapid population survey.

Keywords: age determination, Asian elephant, bolus circumference, population age structure

[1]*Animal Systematics and Molecular Ecology Laboratory, Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Ratchathewi, Bangkok 10400, Thailand*

[2]*Division of Biological and Natural Resources Science, Mahidol University, Kanchanaburi Campus, Kanchanaburi 71150, Thailand*

***Correspondence**: ch_kongrit@hotmail.com*

Introduction

Age estimation is an important technique for the construction of a population age structure that could be useful for predicting population change. The age of wild animals is difficult to determine unless a long-term studied population is used in which researchers can identify individuals and recognize members of the population easily. Even though the Asian elephant (*Elephas maximus*) is a large terrestrial mammal that can be researched, determination of elephant age requires skilled observation.

Age estimation of wild Asian elephants has been done using a photographic method by which elephants are photographed and their shoulder heights are compared with a reference height or photographs of other members of their social group (Sukumar, 1992; Arivazhagan and Sukumar, 2008). However, after elephants reach an asymptotic height at their age of maturity, comparison using other morphological features of captive elephants such as buccal depression and degree of ear folding is needed to determine age more accurately (Sukumar, 1992). Since wild Asian elephants live in forest habitats, age estimation by direct observation may not be practical. Dung bolus circumference is another feature that correlates with elephant age (Jachmann and Bell, 1984; Reilly, 2002; Morgan and Lee, 2003; Morrison et al., 2005). Measurement of bolus circumference or diameter does not require any expensive equipment. The technique produces reliable results and has been widely applied to the study of wild elephant populations (Jachmann and Bell, 1984; Reilly, 2002; Morgan and Lee, 2003; Nowak et al., 2009; Olivier et al., 2009). Most of the references for the use of bolus circumference for age estimation are derived from captive elephant studies. Interestingly, the bolus sizes of wild and captive elephants at similar ages are different (Jachmann and Bell, 1984; Morgan and Lee, 2003), and this may be partly due to the diet (Morgan and Lee, 2003). The amount of water uptake per day as well as their daily activities might also be factors that explain the difference. To create a criterion for age estimation of wild elephants, bolus sizes from wild elephants of known age are required. Growth curves based on the dung bolus diameter of previously aged African elephants (*Loxodonta africana*) have already been constructed (Morrison et al., 2005). Vidya et al. (2003) and Flagstad et al. (2012) determined the cut-off criteria for the adult age class of unknown age wild Asian elephants based on the distribution of dung bolus size of the studied population.

Wild elephants might be classified into four age classes: calf (0-1 year), juvenile (1-5 years), sub-adult (5-15 years) and adult (>15 years) (Sukumar, 1992), although discrimination between calf and juvenile, as well as sub-adult and adult, is difficult. Thus, classification of elephants into immature and mature age classes is usually sufficient for the prediction. Age at maturity could be inferred from the ability to reproduce offspring (Sukumar, 1992). Female elephants with their calves can be easily observed, and thus mature females can be determined and their age of maturity can be estimated from their shoulder heights by the photographic method. In contrast, the age of maturity in males is more difficult to determine. Social behavior in male elephants could be used to indirectly measure the age of maturity. Generally, the Asian elephant is a female philopatric species, whereby females of all ages stay with their natal group. Males, on the other hand, leave or are forced out of their natal group at maturity by adult female group members. Mature males live solitary lives when they are of mature age. Young adult males do sometimes form "bull groups" without any specific social bonds to gain advantages by foraging together in crop raids (Sukumar, 1992).

To study the population age structure of wild Asian elephants, a criterion to categorize immature and mature elephants is required. This study aimed to apply the social behavior of male elephants to the creation of a criterion for cut-off bolus circumference to help categorize immature and mature elephants. The cut-off circumference was then used to construct the population age structure of wild elephants at Salakphra Wildlife Sanctuary in Thailand. Unfortunately, a specific criterion for females could not be created because mature females in the elephant groups could not be identified. The same criterion was applied to both sexes. Sex determination of the Asian elephant by external morphology could possibly give erroneous results because males do not always have tusks and it is almost impossible to determine the sex of young elephants. A noninvasive genetic method using elephant dung samples and sex determination markers was used to determine the sex of elephant samples in this study.

Materials and Methods

Study area: This research was carried out in Salakphra Wildlife Sanctuary located in Kanchanaburi province, Thailand. The major forest types in the area are mixed deciduous forest and bamboo forest, including dry evergreen forest at higher elevations. These forest types serve as important habitat and food sources for wild elephants. Wild elephants have been observed frequently, especially in the forest edges nearby crop fields during the harvest period in the dry season, between November and February. A majority of wild elephants in Salakphra live in the middle and southern areas of the wildlife sanctuary where conflict between humans and elephants occurs regularly.

Sample and data collection: This research focused on elephants living in the middle and southern areas of the wildlife sanctuary. Sampling sites were locations where the elephants congregated in the dry season such as major waterholes, saltlicks, crop fields and resting areas. It is important to note that the elephant groups living in the northern area of Salakphra were not included in this study, and that the elephant groups did not mix. Each sampling site was visited at least twice during the sample collection period from December 2004 to February 2005. If a single fresh dung sample or a few dung samples at different defecation times were found at the sampling site and the dung size and roughness of fiber in the samples were similar, these samples were categorized as coming from solitary elephants. If several dung samples were found

in a particular area at the sampling site and the dung characteristics varied, these samples were categorized as coming from several elephants living within their natal group.

Approximately ten grams of the outer layer of each fresh dung sample was collected into a 25-ml polypropylene tube containing DET buffer (20% DMSO, 250 mM EDTA, 100 mM Tris, pH 7.5 and NaCl to saturation). The samples were kept at ambient temperatures at field sites for 7-10 days, and stored at 4°C after arriving at the laboratory at Mahidol University. Dung circumferences (cm) of three intact boli were measured using a measuring tape (Fig. 1). If dung boli were destroyed by insects or other animals, only the available intact boli were measured. Mean bolus circumference for each sample was calculated.

Figure 1 Measurement of a bolus circumference (cm) using a measuring tape

Molecular sex determination: Genomic DNA was extracted from the dung samples using a silica-based purification method (Boom et al., 1999). Regardless of individual identification, elephant sex was determined by co-amplification of the zinc finger protein (ZF) gene fragments present on X and Y chromosomes using the method outlined by Fernando and Melnick (2001). The re-designed primers, LaZFXYF: TGGGAAGCATTTCT CTCACAC (L. S. Eggert, unpublished data) and LaZFXYR: TCTTGCTATGGACTGCCAAA (Munshi-South et al., 2008), were used to amplify the ZFX and ZFY fragments located on the X and Y chromosomes, respectively. Polymerase chain reactions were carried out in 0.2-ml PCR tubes. The reactions consisted of 1X ImmoBuffer, 150 µM of each dNTPs, 1.3 mg of BSA, 3.0 pmol of forward and reverse primers, 2.0 mM $MgCl_2$, 0.8 unit of IMMOLASE™ DNA Polymerase and 4 µl of DNA extraction from dung sample. A negative control using purified type I water and positive controls using 20 ng of DNA extraction from blood samples of a male elephant and a female elephant were carried out along with the amplifications. The reactions were initially denatured at 95°C for 10 min., followed by 40 cycles of denaturation at 95°C for 1 min., annealing at 60°C for 1 min., extension at 72°C for 1 min., and a final extension at 72°C for 7 min. Five µl of PCR products were digested using 5 units of restriction enzyme BamHI at 42°C for 3 hours. The pre-digested and post-digested PCR products for each sample were run through 2.8% agarose gel side by side, and visualized by UV transillumination gel documentation. Genomic DNA of females produced one band of the ZFX fragment, whereas the DNA of males produced three bands, one band of the ZFX fragment and two bands of the digested ZFY fragments. Due to the noninvasive genetic approach, allelic dropout, in which only one of the two alleles is successfully amplified, could occur, leading to misinterpretation of the genotype or sex. Therefore, a "comparative multiple-tube approach" (Frantz et al., 2003) was applied by reamplifying the locus of interest and scoring the consensus results. An allele was accepted if it was produced at least twice. The molecular sexing process was repeated at least twice. Samples that yielded X and Y fragments twice were scored as male. For samples yielding only the X fragment twice, the molecular sexing process was repeated once more. If the result was consistent, with only X fragments being produced, they were scored as female.

Data analysis: The sex of the elephant samples was determined. The overall mean bolus circumferences of the male and female samples were compared. Frequency distributions of the bolus circumference of the female and male samples were constructed. The male samples were classified into two groups according to the characteristics of the dung samples observed at the sampling sites: males living with their natal groups and solitary males. Male elephants that came from an already formed bull group were considered mature solitary males. Frequency

distributions of the bolus circumference from the two male groups were constructed. A cut-off circumference between the immature and mature age classes was determined using the largest bolus size of males living with their natal groups as the cut-off point. Males with bolus sizes larger than the cut-off circumference were considered mature males. This criterion was applied instead of using the smallest bolus circumference of the solitary males because young adult males are sometimes forced to leave the natal group before they are fully mature. Since the sample collection was only carried out in a specific area of the wildlife sanctuary, a local population age structure that did not include the elephants from the northern part of the study site was constructed. The cut-off circumference criterion was applied to both male and female samples to construct a local population age structure of the elephants living in the sampling areas.

Results

A total of 225 dung samples were collected at the sampling sites. DNA extraction and molecular sexing were performed for each sample. The primer pair LaZFXYF and LaZFXYR co-amplified the ZFX and ZFY regions on the X and Y chromosomes, respectively, yielding the same PCR product size of 155 bp (base pair). The ZFY fragment contained BamHI restriction site, but the ZFX fragment did not. After the PCR products were digested with BamHI, the samples from males produced three product fragments, one ZFX fragment and two digested ZFY fragments; thus, three product bands were observed on the 2.8% agarose gel at 155, 93 and 62 bp. In contrast, female elephants do not contain a Y chromosome, so the samples from females yielded only one product of the undigested ZFX fragment after BamHI digestion at 155 bp (Fig. 2). An example of molecular sexing results on 2.8% agarose gel from the six dung samples, numbers 1-6, is presented in Figure 2. The pre-digested and post-digested PCR products of each sample were run on the agarose gel side by side. The samples numbered 2 and 6 yielded three products after the digestion, and they were categorized as males. The samples numbered 1, 3, 4 and 5 yielded one product after the digestion, and they were categorized as females.

Among the total sample collected, 216 samples were successfully amplified and categorized for sex. Ninety samples were categorized as males and 126 samples were categorized as females. The sex ratio for the overall samples was 1 male: 1.4 females. These samples were included in further analyses.

The ranges of the bolus circumferences of the female and male samples were 19.67-53.50 cm and 17.33-58.33 cm, respectively. The overall mean bolus circumference of the female samples was 38.43 ± 7.47 cm, and that of the male samples was 40.87 ± 8.97 cm. The overall mean and median circumferences of each sex were similar (Table 1). The overall mean circumferences of males and females in the population were significantly different from each other (t = -2.11, p = 0.036), in that the average bolus size of the males was larger than that of the females. The bolus circumferences of the male and female samples in the Salakphra population were normally distributed. The frequency distributions of bolus circumferences of male and female samples are presented in Figure 3.

The ranges of the bolus circumferences of the males living within their natal groups and those living independently from their natal groups were 17.33-42.50 cm and 35.00-58.33 cm, respectively (Table 2). Interestingly, a group of 13 dung samples collected from a sugar cane field at the same time were identified as males. This was an evidence of a bull group formation. The bolus circumferences of the male samples in the bull group ranged between 35.00 and 51.00 cm with the average bolus circumference of 45.15 ± 4.07 cm. Males in a bull group were considered as solitary adult males. Males living with the natal groups had the smallest mean bolus circumference among the three groups of males. The average bolus circumference of the males forming the bull group was smaller than that of the males living alone without forming a bull group (Table 2). The frequency distributions of bolus circumferences of the males living with their natal group and those living alone/solitary lives are shown in Figure 4.

Table 1 Minimum and maximum range (min.-max.), mean ± SD and median of bolus circumference (cm) of male and female samples collected at sampling sites. N = number of dung samples.

Sex	N	Min.-Max.	Mean ± SD	Median
Female	126	19.67-53.50	38.43 ± 7.47	39.33
Male	90	17.33-58.33	40.87 ± 8.97	40.92

Table 2 Minimum and maximum range (min.-max.), mean ± SD and median of bolus circumference (cm) of males living with their natal groups (Males_natal group), males forming a bull group (Males_bull group) and males living alone without forming a bull group (Males_independent solitary). N = number of dung samples.

Categories of males	N	Min.-Max.	Mean ± SD	Median
Males_natal group	49	17.33-42.50	34.37 ± 5.82	35.00
Males_bull group	13	35.00-51.00	45.15 ± 4.07	46.00
Males_independent solitary	28	39.50-58.33	50.25 ± 4.52	50.42

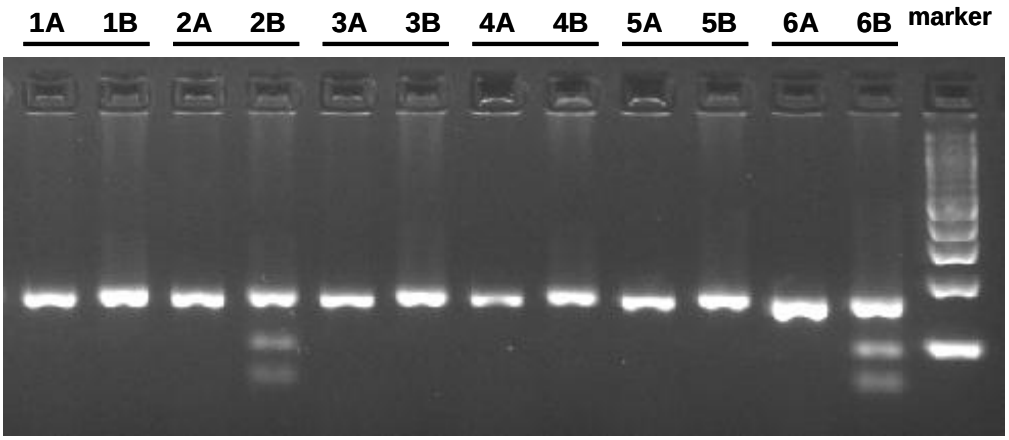

Figure 2 Agarose gel electrophoresis from molecular sexing method of the elephant samples numbered 1-6, and 100 base pair marker
Lanes 1A and 1B are the pre-digested and post-digested PCR products of sample no.1 (digestion yields 1 fragment, sample no.1 is female)
Lanes 2A and 2B are the pre-digested and post-digested PCR products of sample no.2 (digestion yields 3 fragments, sample no.2 is male)
Lanes 3A and 3B are the pre-digested and post-digested PCR products of sample no.3 (digestion yields 1 fragment, sample no.3 is female)
Lanes 4A and 4B are the pre-digested and post-digested PCR products of sample no.4 (digestion yields 1 fragment, sample no.4 is female)
Lanes 5A and 5B are the pre-digested and post-digested PCR products of sample no.5 (digestion yields 1 fragment, sample no.5 is female)
Lanes 6A and 6B are the pre-digested and post-digested PCR products of sample no.6 (digestion yields 3 fragments, sample no.6 is male)

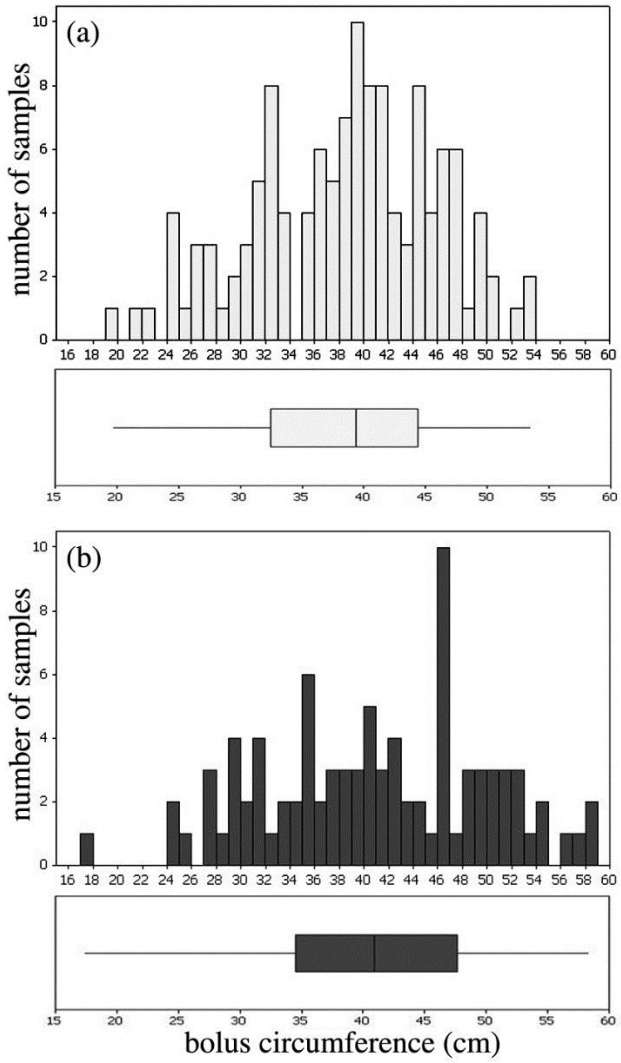

Figure 3 Frequency distribution and boxplot of the bolus circumference of (a) 126 samples of females and (b) 90 samples of males

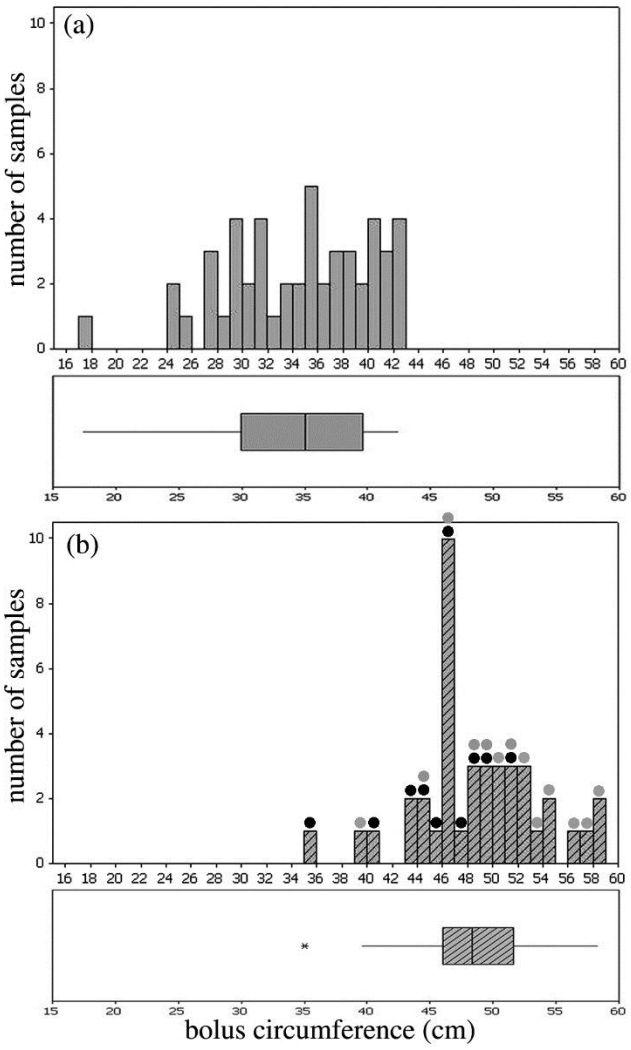

Figure 4 Frequency distribution and boxplot of the bolus circumference of (a) 49 samples of males living with their natal groups and (b) 41 samples of solitary males. The solitary male outlier was a bolus of 35.0 cm circumference. The dark circles mark the bolus circumference of males forming a bull group. The light circles mark the bolus circumference of males living solitary lives without forming a bull group.

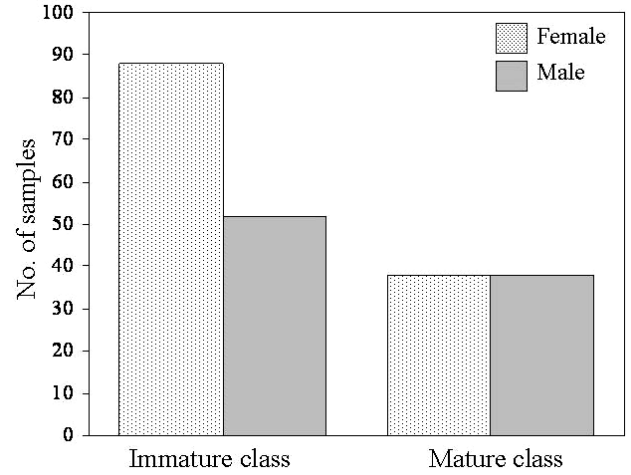

Figure 5 A local population age structure showing numbers of samples in the immature and mature age classes based on the cut-off circumference of 42.5 cm

The largest bolus circumference of the males living with their natal groups was 42.50 cm, and all males that left their natal group had bolus circumference larger than 42.50 cm. This value was used as the cut-off circumference for the construction of population age structure. The samples were classified into two age classes, immature and mature. A local population age structure based on bolus circumferences of Salakphra elephants regardless of individual identification is shown in Figure 5.

Discussion

The noninvasive genetic sampling yielded a sufficient amount of DNA for molecular sex determination with 96% amplification success. The re-amplification of the sex determination fragments produced consistent results. The re-designed primer pair yielded short fragments of PCR products that facilitated successful amplification. The molecular sexing method is reliable and a promising tool for

wildlife study, especially for endangered and elusive species that are rare or difficult to observe.

Although the overall mean bolus circumference of the males was significantly larger than that of the females, it cannot be concluded from this study alone that the body size of the males is larger than that of the females in the same age group because the age of the elephants was not known and the elephants were not individually identified. Although male and female elephants have slightly different growth rates, females reach asymptotic growth earlier than males making the female body size smaller than that of the males (Reilly, 2002; Morrison et al., 2005). Reilly (2002) found that there was no significant difference in dung bolus diameter between males and females of the Sumatran elephant.

The cut-off bolus circumference of 42.5 cm is similar to the criteria used by Vidya et al. (2003) of 37.68 cm for mature females and 43.96 cm for mature males in the studies of wild Asian elephant populations in Nagarahole and Mudumalai-Bandipur reserves in India. They defined the criteria based on the distribution of the bolus diameter of the elephants in the study areas. A similar criterion, 43.96 cm, was also used in the population study of the wild African elephants (Nowak et al., 2009). In the current study, it was noticed that there were three solitary males with bolus circumferences smaller than 42.5 cm (Fig. 4). Two of them formed a bull group. The smallest bolus circumference of the solitary male was 35.00 cm. It is possible that these males matured and left the natal group early. It was also possible that these males were forced by the female members to leave the natal group before they became fully mature. The results indicate that none of the males with bolus circumferences larger than 42.5 cm were living within their natal group. In addition, the results from this study revealed that the young adult males formed a temporary bull group with other adult males, whereas the old males with bolus circumferences larger than 51.0 cm all lived solitary lives (Fig. 4).

Regardless of individual identification and the fact that the same cut-off criterion was applied to both males and females, the local population age structure revealed that the adult sex ratio was not different from 1 male: 1 female. This corresponds with the conservation-related fact that there is no direct poaching of a particular sex in this population. In the areas where intense poaching occurs, molecular sexing techniques could reveal a biased sex ratio toward one particular sex (Vidya et al., 2003). The population age structure based on dung samples regardless of individual identification should be interpreted with caution because it does not represent the actual numbers of elephants in the studied populations. The dung sampling technique might underrepresent the elephants in the younger age class, including calves and juveniles, because of the low defecation rate (Jachmann and Bell, 1984; Olivier et al., 2009).

Applying only molecular sexing to the population does not yield as robust a result as using microsatellite genotyping, but this method is simple and does not require a high budget. If poaching for a particular sex is not an issue in the area, it is not necessary to carry out molecular sexing because there is a significant financial cost for DNA extraction from dung samples. A population study based on the dung sampling technique could be useful in a rapid survey of a population to identify age structures and trends for population change. The rapid survey could be carried out periodically. However, in a rapid survey, the survey should be done in a short period of time to prevent re-sampling of the same elephant individuals. Moreover, if possible, the survey should be carried out during the dry season to ensure that dung boli stay intact and to prevent moisture from causing the boli to deteriorate.

The cut-off criterion obtained from this study will be used in further studies of the elephant population in the Salakphra Wildlife Sanctuary. The study of microsatellite genotyping and sequencing of mitochondrial fragments of this elephant population to identify elephant individuals, and to evaluate genetic diversity and examine gene flow within the population should be further investigated.

Acknowledgements

We are grateful for the financial support provided by Mahidol University and the Royal Golden Jubilee program to C. Kongrit and the Thailand Commission of Higher Education scholarship to C. Siripunkaw. We thank the Department of National Parks, Wildlife and Plant Conservation for permission to work in Salakphra Wildlife Sanctuary. We are deeply thankful to W. Y. Brockelman for discussion about this study, V. Akkarapatumwong for laboratory support and discussion about the study, J. Plotnik for the valuable comments on the manuscript and to the Salakphra wildlife officers for field support and assistance with the development of our field sampling techniques.

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บทคัดย่อ

การระบุอายุและการสร้างโครงสร้างอายุประชากรของช้างป่าจากขนาดเส้นรอบวงกองมูลช้าง

ชลิตา คงฤทธิ์[1*] ชมชื่น ศิริผันแก้ว[2]

การประเมินอายุของสัตว์ป่านับว่าเป็นวิธีการที่สำคัญวิธีการหนึ่งในการสร้างโครงสร้างอายุประชากร ซึ่งจะเป็นประโยชน์ในการคาดคะเนการเปลี่ยนแปลงขนาดประชากร อายุของช้างป่าสามารถประมาณได้จากขนาดเส้นรอบวงกองมูล ซึ่งมีความสัมพันธ์กับการเจริญเติบโตของช้าง งานวิจัยนี้มีเป้าหมายในการกำหนดเกณฑ์ขนาดเส้นรอบวงกองมูล ซึ่งจะใช้ในการจำแนกช้างออกเป็นช้างในชั้นอายุก่อนเต็มวัยและช้างในชั้นอายุเต็มวัย โดยศึกษาประชากรช้างป่าในเขตรักษาพันธุ์สัตว์ป่าสลักพระ โดยใช้พฤติกรรมทางสังคมของช้างเพศผู้ในการกำหนดเกณฑ์การจำแนกชั้นอายุ ช้างเพศผู้ที่อาศัยอยู่กับฝูงครอบครัวเป็นช้างที่ยังไม่เจริญเต็มวัย ส่วนช้างเพศผู้ที่อาศัยอยู่ตัวเดียวเป็นช้างที่เจริญเต็มวัยแล้ว และใช้ขนาดเส้นรอบวงกองมูลที่ใหญ่ที่สุดของช้างเพศผู้ที่ยังอาศัยอยู่กับฝูงเป็นเกณฑ์การระบุชั้นอายุของช้าง การระบุเพศช้างป่าทำโดยใช้วิธีการระบุเพศทางอณูชีววิทยาจากตัวอย่างมูลช้างป่า จากตัวอย่างมูลช้างทั้งหมด 225 ตัวอย่าง พบว่าร้อยละ 96 ของตัวอย่างสามารถระบุเพศช้างได้ โดยระบุได้ว่าเป็นตัวอย่างที่มาจากช้างเพศผู้ 90 ตัวอย่าง และจากช้างเพศเมีย 126 ตัวอย่าง และพบว่า ตัวอย่างจากช้างเพศผู้จำนวน 49 ตัวอย่างมาจากช้างเพศผู้ที่อาศัยอยู่กับฝูงครอบครัว และจำนวน 41 ตัวอย่างมาจากช้างเพศผู้ที่อาศัยอยู่ตัวเดียว เกณฑ์ในการกำหนดชั้นอายุจากเส้นรอบวงกองมูลมีค่าเท่ากับ 42.5 เซนติเมตร ดังนั้น ตัวอย่างมูลช้างที่มีขนาดเส้นรอบวงกองมูลมากกว่านี้ถูกจำแนกเป็นช้างเต็มวัย โดยใช้เกณฑ์เดียวกันทั้งในช้างเพศผู้และเพศเมีย ผู้วิจัยใช้เกณฑ์นี้ในการสร้างโครงสร้างอายุประชากรของช้างป่าสลักพระจากตัวอย่างกองมูลช้าง โดยไม่มีการระบุตัวช้าง การสร้างโครงสร้างอายุประชากรด้วยวิธีการสำรวจกองมูลนี้จะเป็นประโยชน์ในการสำรวจประชากรสัตว์ป่าอย่างรวดเร็ว

คำสำคัญ: การระบุอายุ ช้างเอเชีย เส้นรอบวงกองมูล โครงสร้างอายุประชากร

[1]ห้องปฏิบัติการซิสเตมเมติกซ์ของสัตว์และนิเวศวิทยาโมเลกุล, ภาควิชีววิทยา, คณะวิทยาศาสตร์, มหาวิทยาลัยมหิดล, ถ.พระราม 6, เขตราชเทวี, กทม. 10400 ประเทศไทย

[2]สาขาวิชาวิทยาศาสตร์ชีวภาพและทรัพยากรธรรมชาติ, มหาวิทยาลัยมหิดล วิทยาเขตกาญจนบุรี, กาญจนบุรี 71150 ประเทศไทย

****ผู้รับผิดชอบบทความ** E-mail: ch_kongrit@hotmail.com*