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Research Article

COMPARATIVE ANALYSIS OF PROBING AND CONTRAST RADIOGRAPHY FOR GENDER DETERMINATION IN RETICULATED PYTHONS (*Malayopython reticulatus*)

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ARTICLE HIGLIGHTS

- Accurate gender identification supports effective breeding programs and ensures snakes' welfare.
- This study emphasizes that probing is more reliable and efficient than contrast radiography for gender determination in reticulated pythons.
- The probing method proved to have 100% accuracy in determining the gender of reticulated pythons, outperforming contrast radiography, which only achieved 75% accuracy.

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ABSTRACT

Gender determination is necessary in reticulated pythons, due to lack of significant gender differences. This study aimed to compare probing and contrast radiography methods for gender determination of reticulated python in terms of efficiency and accuracy. A total of 6 reticulated pythons with body lengths, tail lengths, and body diameters of 128-167 cm, 5.4-9.6 cm, and 1.4-3.4 cm, respectively, were examined twice using these methods. The results showed that probing had 100% accuracy, with probe lengths ranging from 1.37-3.60 cm and 0.3-0.76 cm for male and female snakes, respectively. Contrast radiography had 75% accuracy, with the hemipenis appearing as a white silhouette, while female snakes appeared colorless. The accuracy rates signified the effectiveness of probing over contrast radiography for reliable gender determination in reticulated pythons. The selection of appropriate methods for gender determination is important to ensure accuracy and efficiency, which is crucial for breeding programs and the welfare of snakes.

Keywords: contrast radiography, probing method, reproductive health, reticulated pythons, snake gender determination

INTRODUCTION

Determining the gender of snake is vital for captive snake owners and breeders. Accurate gender determination is crucial to avoid unintended breeding and effective management of snake populations. Some owners may decide to rear several snakes in the same terrarium and wish to avoid breeding activities. Various methods are used to determine gender of snake, such as molecular techniques and morphological characteristics. Previous studies reported molecular techniques, such as quantitative PCR (Rovatsos *et al.* 2015). Laopichienpong *et al.* (2017) presented a novel molecular method of using gametologous genes for gender identification in caenophidian snake. However, a comprehensive comparison of probing and contrast radiography methods specifically for reticulated pythons has not been extensively documented. Knowledge of snake gender can also help in diagnosing diseases affecting reproductive organs (Gnudi *et al.* 2009). Differentiating between male and female snakes is challenging due to the relatively simple morphology, signifying the importance of gender determination (Bonnet *et al.* 1998).

The most common method used for snake gender determination is manual probing, which is conducted specifically to identify hemipenes (Vincent *et al.* 2006). This can be achieved by inserting a smooth, blunt, and thin probe into the cloaca to deviate caudally. In male snake, the probe passes through the hemipenes and reaches more deeply into the tail compared to that in female snake (Stahl 2002). The depth of the male hemipenis and the female homologue varied between species. An alternative to the probing method is popping which is more traumatic, suitable for small snakes, and not recommended for larger snakes. Popping includes the eversion of the hemipenes outside the cloaca by rolling a finger from the tip of the tail toward the vent (Divers & Mader 2005). Ultrasonography and contrast radiography are methods used to determine gender and evaluate breeding problems. Contrast radiography was performed by inserting a radiopaque medium into the cloaca to fill cavities, such as inverted hemipenes (Vetere *et al.* 2022). Ultrasound was conducted using a 3-9 MHz transcutaneous convex probe and underwater scans for gender determination have also been conducted (Mathew *et al.* 2015).

In regard to reproduction, snakes are either oviparous (egg-laying) or viviparous (giving birth). Examples of viviparous snakes with a placenta, include boa constrictors and green anacondas. In contrast, most vipers do not have placental connections and lay eggs that hatch before parturition (previously defined as ovoviviparous).

Pythons are common snakes kept as pets among reptile keepers and all the species are oviparous (Booth & Schuett 2016; Raharjo *et al.* 2008). *Malayopython reticulatus*, formerly known as Python reticulatus, is the longest nonvenomous snake in South and Southeast Asia, reaching a length of up to 10 m (Somaweera 2017). It is also a native to Indonesia, where many captive breeding programs are being held nationwide. *Malayopython reticulatus* is still being heavily exploited because of the high demand for its skin for fashion commodities or captured alive to be sold as a pet (Murray-Dickson *et al.* 2017).

As reticulated pythons are being heavily exploited, the development of local breeders and breeding programs establishment are essential. An important aspect of a snake breeding program is an accurate gender determination. Efficient and minimally stressful gender determination methods is needed for breeders to achieve the highest success rate. Improper methods can not only slow down the breeding program rate, but also raise welfare concerns for many animals. This study aimed to compare probing and contrast radiography methods, for gender determination of reticulated pythons in terms of efficiency and accuracy.

MATERIALS AND METHODS

Materials and Tools

Materials

Reticulated pythons used in this study were selected based on availability and the willingness of owners to participate. These pythons varied in body length from 1.2 m to 1.7 m, representing a diverse range of sizes to ensure the applicability of the methods across different snake dimensions. The animals used were prepared without knowing their gender. Each snake was first subjected to manual probing, followed by contrast radiography to determine the gender. This procedure was conducted 2 times at an interval of 7 days to allow sufficient time for the contrast agent to clear from the lumens of the reproductive organs (cloaca and hemipenes). The interval was calculated from day 1 (the day of the first procedure) to day 7 (the day of the second procedure). Supporting materials used were Iopamidol (Iopamiro 300 mg/mL), KY jelly lubricant, and 70% alcohol.

Tools

Tools used in this study were a 0.07-inch diameter snake probe, digital X-ray machine, 24 G polyethylene catheter, cloth, and snake hook. This experiment was designed not to harm the experimental snakes and followed the established protocol for handling and care. All procedures were approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences of IPB University.

Procedures

Manual Probing

Snake was handled manually and restrained without sedation or anesthesia. During data collection, a single probe was used to handle one snake. The probe was lubed with KY jelly lubricant, placed within the vent, and directed laterally and caudally. Furthermore, the probe was gently advanced into the base of the tail toward the tip. Rotating the probe while gently advancing helps minimize trauma and allows the instrument to pass deeply into the hemipenis or shallowly into the female blind diverticulum. Both the right and left hemipenes and diverticulae were probed. Subsequently, a radiograph of the tail region was obtained, while the probe was still inserted. Snake was positioned on the radiography table under manual restraint. The X-ray machine was set to 40 kVp and 5.00 mAs with FFD 40 inch. Finally, the depth of the probe inserted was measured from the cloacal region up to the end of the instrument and recorded (Fig. 1a).

Contrast Radiography

Plain radiography of the tail region was performed before administering the contrast medium. Subsequently, Iopamidol was injected into the cloaca using a 24 G polyethylene catheter, positioned caudally without a catheter needle. During the injection of contrast medium, the snake was restrained manually and no pressure was applied to the tail area (Fig. 1b). Injection was stopped once the medium began to leak from the vent. A radiopaque needle or probe was placed perpendicular to the snake body at the cloacal vent opening, as a radiographic marker. The snake was positioned on the radiography table under manual restraint. Furthermore, digital radiography of the dorsoventral projection of the tail region was performed. Radiography machine was set to 40 kVp and 5.00 mAs with FFD 40 inch.

Data Analysis

The data obtained from manual probing were analyzed by determining the radiography image of the probe inserted into the cloaca and the length was measured. For contrast radiography, data from the X-ray machine were analyzed by observing the presence of a radiopaque hemipenis. The length of contrast of the hemipenis was also measured and recorded. Radiographs were examined using ImageJ and ORCA® (OR Technology Cloud Archiving). The length of the inserted probe and positive contrast were measured and recorded. The means were then calculated for a better comparison and determination of gender. Both tests were examined to determine the accuracy and consistency of the methods. For data analysis, a dependent T-test was initially considered, but was not used due to the small sample size and the descriptive nature of the study. Future studies with larger samples should incorporate more efficient statistical analyses to validate these results.

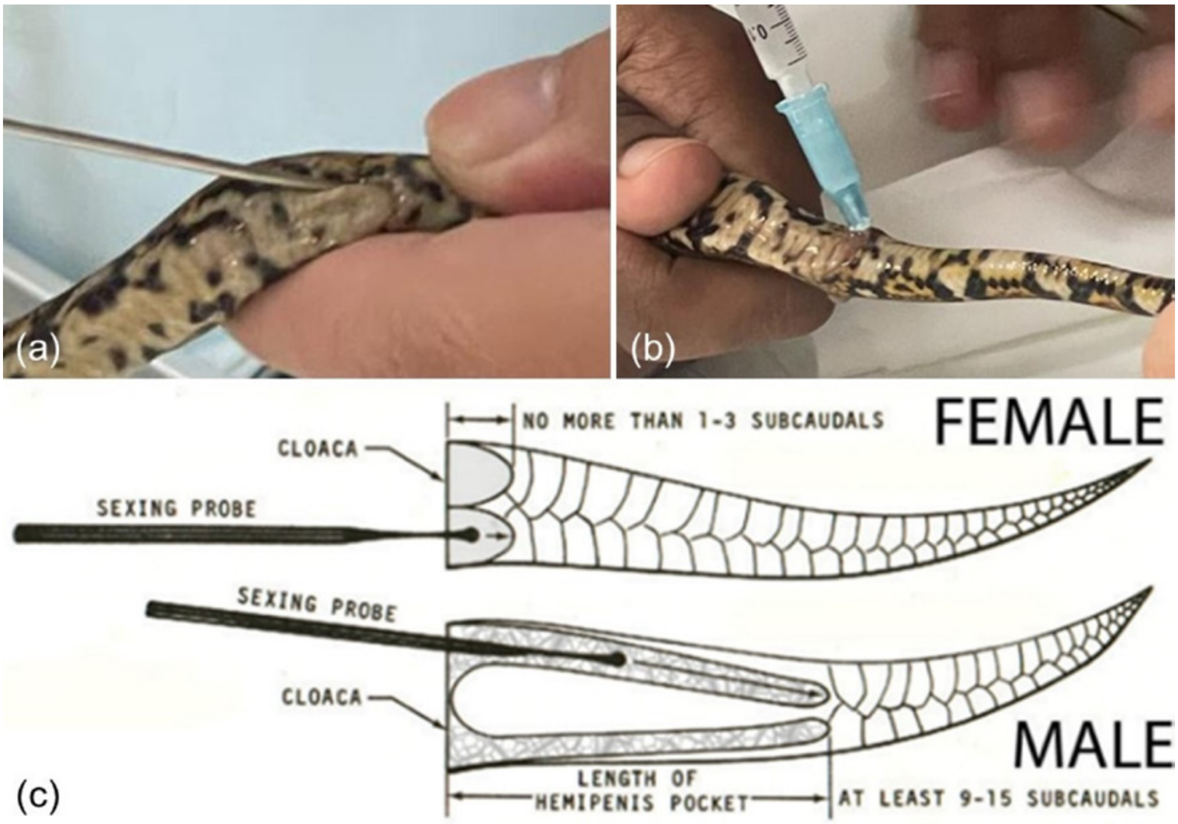

Figure 1 Probing and contrast medium administration on a snake

Notes: (a) Inserting the probe in the cauda-ventral opening of the cloaca
(b) Administering contrast medium (Iopamidol) at the cauda-ventral opening of the cloaca
(c) probing technique applied in snakes (Laszlo 1975)

RESULTS AND DISCUSSION

Results of measurement of body length, tail length, and body diameter of reticulated pythons used in this study are presented in Table 1. The body length ranged from 128.00 cm to 167.00 cm, with an average of 139.05 cm and a standard deviation of 14.88 cm, emphasizing significant variation. Meanwhile, the tail length ranged from 5.40 cm to 9.60 cm, with an average of 6.93 cm and a standard deviation of 1.50 cm, showing higher consistency. The body diameter ranged from 1.40 cm to 3.40 cm, with an average and a standard deviation of 2.28 cm and 0.75 cm, respectively, signifying moderate variation. These data provided a general overview of the physical size of reticulated pythons, which was helpful for further studies or comparisons with other snake species.

A total of 36 radiographs were taken throughout the study. Each snake had two plain radiographs, i.e., two with an inserted probe and two with administered contrast medium. The results showed differences in probe insertion depth and contrast visualization between male and female snakes. Snakes 3, 2, and 5 were chosen to be the observed representatives due to the superior quality of radiographs for interpretation (Fig. 2). Snakes 3 and 5 represented the male and female snakes, respectively.

Male snake was identified by the presence of positive contrast of the hemipenes and the probe insertion was deeper. Meanwhile, female snake was determined by the absence of positive contrast and the probe insertion was relatively shallower. Snake 2 was not identified by gender based on contrast radiography images, because the images were controversial and inconsistent, requiring further explanation. Radiopaque needle tips and probes were perpendicular to the body of the snake to signify the cloacal vent. Radiograph was taken in the tail region, making the caudal and cloacal vertebra radiopaque. Contrast will be radiopaque when the probe is inserted, while muscles will mostly be radiolucent.

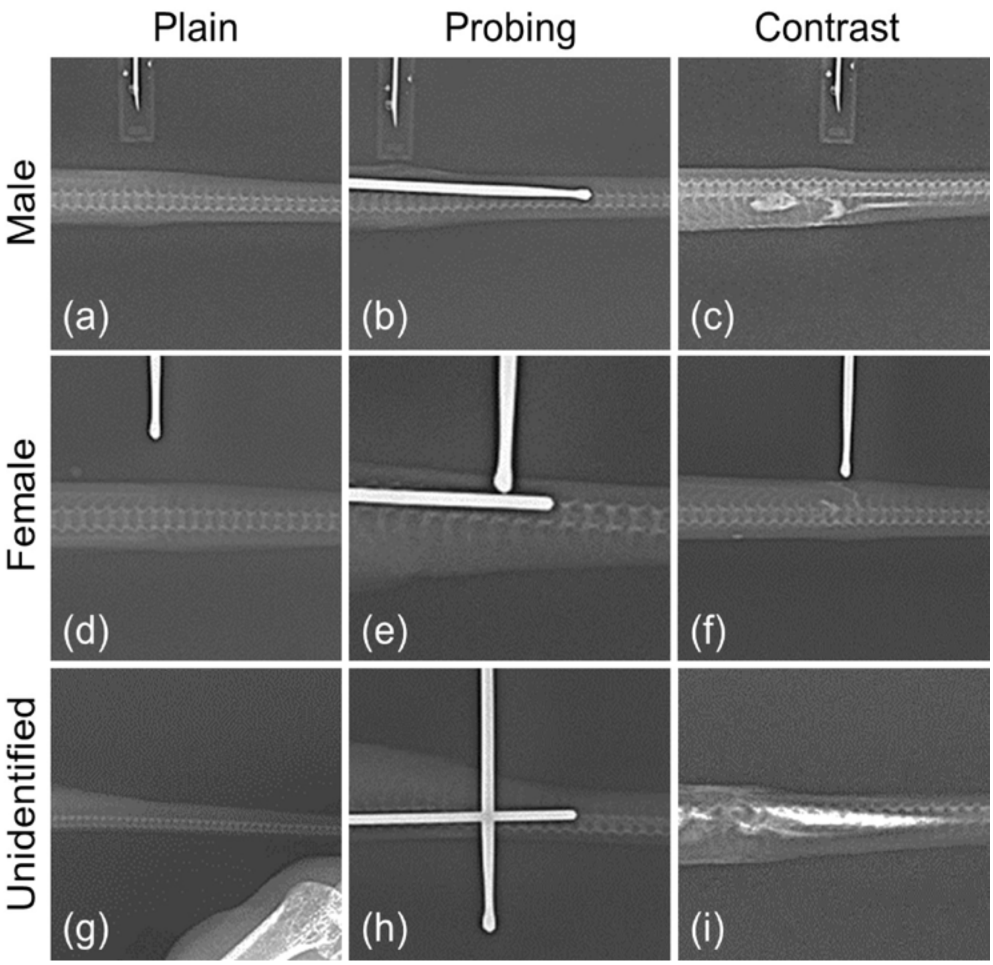

Figure 2 Radiographs of plain, probing, and contrast radiography during gender determination of reticulated pythons

Table 1 Measurement of body length, tail length, and body diameter of reticulated pythons used in this study

Measured parameter	Snake number						Mean ± standard deviation
	1	2	3	4	5	6	
Body length (cm)	133.20	128.00	131.50	167.00	130.00	144.60	139.05±14.88
Tail length (cm)	7.50	5.40	5.90	9.60	6.30	6.90	6.93±1.50
Body diameter (cm)	2.30	1.40	2.50	3.40	1.50	2.60	2.28±0.75

Table 2 Length of hemipenis and accuracy of reticulated pythons gender determination by using probing and contrast radiography

Length of hemipenis (cm)	Snake number					
	1	2	3	4	5	6
Probing I	2.46 (True Male)	0.76 (True Female)	2.31 (True Male)	3.60 (True Male)	0.75 (True Female)	2.14 (True Male)
Probing II	2.19 (True Male)	0.50 (True Female)	2.32 (True Male)	2.64 (True Male)	0.36 (True Female)	1.37 (True Male)
Probing mean±Standard deviation (cm)	**2.33±0.19**	**0.63±0.18**	**2.32±0.01**	**3.12±0.68**	**0.56±0.28**	**1.76±0.54**
Probing Accuracy (%)	**100**					
Contrast I	1.59 (True Male)	1.64 (False Male)	n.a. (False Female)	1.13 (True Male)	n.a. (True Female)	n.a. (False Female)
Contrast II	1.14 (True Male)	n.a. (True Female)	1.82 (True Male)	1.93 (True Male)	n.a. (True Female)	1.93 (True Male)
Contrast mean±Standard deviation (cm)	**1.37±0.32**	**1.64±n.a.**	**1.82±**	**1.45±0.57**	**n.a.±n.a.**	**1.93±n.a.**
Contrast Accuracy (%)	**75**					

Note: n.a. = contrast silhouette did not appear on the radiogram.

This study showed no abnormalities or foreign bodies in the tail region of the snakes before any procedures was performed (Fig. 2a, d, and g). The probe presented in Figure 2b, was inserted much deeper than that in Figures 2e and h. The probe usually goes deeper into the tail of a male snake. A clear structure of one of the hemipenes of a male snake was seen in Figure 2c. Meanwhile, lack of contrast and shallow probe insertion was shown in Figure 2f, because female snake has no hemipenis. A positive contrast in the tail area and a very short probe insertion signified a high probability of gender being female (Fig. 2i). Therefore, radiography results could not confirm the gender of the snake.

All 6 snakes were subjected to double probing and contrast radiography (Table 2). During the probing procedure, a male snake tended to have a deeper cavity, allowing for probe insertion at a depth of approximately 1-3 cm. On the contrary, the probe could only be inserted at less than 0.8 cm for a female snake. For contrast radiography, a male snake was identified by observing the presence of hemipenes in a radiopaque state. On the other hand, female snake showed an absence of positive contrast in the results of contrast radiography.

This study indicated that the accuracy of probing method was relatively higher than the accuracy of contrast radiography method, as false-positive outcomes were observed in contrast radiography on Snake 2. Multiple probing results corresponded, showing high consistency. Meanwhile, the results of contrast radiography were inconsistent, as some male and female snakes showed false-negative and false-positive outcomes, respectively. Actual male and female snakes signify true positives and true negatives, respectively. Contrast radiography accurately showed the structure of the hemipenis, thereby providing a better picture of the reproductive organs of male snakes.

Length of the inserted probes were longer than the length of positive contrast (Tables 1&2). The discrepancy suggested limitations in the administration of contrast medium, because the contrast medium may not all have been fully delivered into the hemipenis cavity. A potential reason was that the small size of hemipenis cavity prevented contrast medium from fully entering the cavity.

Determining the age of a young snake is challenging, but can be facilitated by several methods. Size criteria based on snout-vent length (SVL) are commonly used to categorize pythons into different age groups. For instance, a study on water snakes in Lake Erie classified the snakes with under 25 cm SVL as young-of-the-year, signifying an age of less than one year (King 1992). Similarly, in a study of Javanese keelback water snakes, hatchlings were identified to be less than 59 cm long, juveniles between 60-79 cm, and adults over 79 cm (Yudhana 2024). Investigation on red-sided garter snakes showed that female fecundity increases with body length, in correlation with age, due to the indeterminate growth of snakes (Rollings *et al.* 2017). This suggested that body length is an indicator of age, particularly concerning reproductive capacity. Collectively, the methods signify that body length and weight can be effectively used to infer the age and condition of snakes.

Contrast radiography presents multiple false-positive and false-negative results (Table 2). During evaluation, some radiographs of male snakes showed the absence of positive contrast on the hemipenis, leading to false negative results. There was only one false positive, which was on snake 2. Contrast observed was relatively thicker than the other true-positive male results. By comparing the outcomes of probing and contrast, it was discovered that the probes were inserted at a very shallow depth, while a contrast showed negative results. After analysis, Snake 2 was confirmed to be a male snake. The observed positive contrast results were also proven to be false positives.

In terms of accuracy, probing yields consistent and accurate results. Meanwhile, some radiographs were accurate, with one false positive and two false negative results, leading to an accuracy of only 75% (Table 2). The radiograph method remains an excellent option in gender determination, but requires more tools, such as X-ray machines and contrast media, which makes it costly and time-consuming.

Based on contrast radiography results of (Gnudi *et al.* 2009), the overall accuracy was approximately 81.5%, and the presence of a single hemipenis was frequently reported. The results of this study were also similar, as only one hemipenis was observed. Among the two hemipenis sheaths, only one could be filled with contrast medium, because of the proximity of the catheter tip to the opening. The feces most likely contributed to the uneven distribution of contrast medium within the cloaca.

In terms of gender, the male and female snakes, are with and without hemipenis sheath, respectively, causing a significant difference in the inserted probe or contrast medium. During probing, the probe instrument is usually inserted at a maximum depth of 3 subcaudals into the tail of a female snake. Meanwhile, the probe easily enters a pocket or one on either side of the tail of a male snake and penetrates between the eighth and sixteenth subcaudals (Laszlo 1975). A male snake has paired hemipenes, which lie in sacs caudal to the cloaca in the ventral part of the tail (Vasaruchapong 2014). Typically, the reproductive tract of a female snake consists of 2 ovaries and 2 oviducts. The right ovary of a female snake is relatively larger and located anteriorly than the left ovary. The urogenital papillae allow the oviducts, which travel from the ovaries to the urodeum and into the body (Di Girolamo & Selleri 2017).

All snakes used in this study were not adults, as the lengths were between 1.2-1.7 m (Table 3). The hemipenes of the experimental snakes were relatively smaller and shorter. Python is categorized as adult when the male python reaches approximately 2.5 -3 m in body length (Mukherjee *et al.* 2012). The mean length of the hemipenes of adult reticulated pythons was 6.36±0.46 cm (Enriquez *et al.* 2011).

The implemented probing method showed that the inserted probe was between 0.56-0.63 cm deep for female snake and 1.75-2.62 cm deep for male snake, respectively. The differences between these lengths were significant. There is a possibility of the probe being further inserted. This is because the probe instrument is rather thick for the cavity and the snake is smaller in size. Organic solids, such as feces, may accumulate in the hemipenis sheath, reducing the depth to which the probe can penetrate and producing false results (Kane *et al.* 2022).

Despite probing method being generally considered reliable for determining the gender of snakes, this method has some limitations, such as misidentification, particularly in cases of inexperienced probing or ambiguous results (Mayer *et al.* 2023). Therefore, gender determination can be further confirmed by comparing the outcomes of contrast radiography.

Some disadvantages of probing method include injury and distress to snakes when not performed with extreme care and precision (Katz *et al.* 2020). Delicate tissues of the cloaca can be easily damaged, leading to pain, infection, or other complications. The process can also be stressful and may negatively affect the well-being of the snake. Probing method provides information solely about the gender of snakes without giving insight into other reproductive health conditions or abnormalities. The method does not offer a comprehensive evaluation of the overall reproductive health of the animals.

Contrast radiography is a useful diagnostic tool for evaluating hemipenis. By using contrast medium, such as radiopaque dyes or gases, reproductive structures were identified, and the anatomy and functionality of the reproductive organs were assessed. Contrast radiography of hemipenes can provide information regarding abnormalities, blockages, or structural issues within these organs. The contrast radiography method facilitates the diagnosis of conditions such as hemipenile prolapse, abscesses, tumors, or strictures within hemipenes (Rivera 2008). The use of contrast medium in snake radiography allows for enhanced visualization and helps identify specific reproductive conditions or disorders that are not easily detectable with regular radiography.

Depending on the size and anatomy of snake, accessing and positioning reproductive organs for contrast radiography can be challenging. Furthermore, contrast radiography signifies intestinal obstruction, colon obstruction, radiolucent foreign bodies, and masses (Banzato *et al.* 2013). Another challenging aspect is the interpretation of contrast radiography of the reproductive organs. Differentiating typical structures from abnormalities can be challenging, primarily when subtle changes or variations occur. It is important to acknowledge that misinterpretation of radiography results can lead to inaccurate diagnoses or unnecessary interventions.

CONCLUSION

In conclusion, probing method showed higher accuracy, however the method is potential to cause distress in the observed snakes. Despite the lower accuracy of contrast radiography, the method provided valuable insights into the reproductive anatomy of giant snakes. The combination of both methods with molecular techniques offered a comprehensive approach to gender determination in reticulated pythons. Limitations experienced in this study were (1) the sample size of snakes is relatively small, which may limit the generalizability of the results; (2) the samples in the study were relatively young, which affected the accuracy of the methods when applied to older or larger snakes; (3) the use of contrast radiography requires more specialized equipment and expertise, which might not be readily available in all settings. Recommendations for further study were: (1) breeders prioritize probing for routine gender determination and consider contrast radiography for more detailed reproductive health assessments and (2) study with larger, more diverse samples and standardized handling techniques is needed to validate these results and explore other potential methods for snake gender determination.

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