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Novel exploration of the presence and morphological features *Spirometra* (Cestoda: Diphyllobothriidae) in Javan spitting cobra snakes (*Naja sputatrix)* in Sidoarjo, Indonesia

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Abstract. *Edila R, Effendi MH, Suwanti LT, Kwon H-K, Agumah NB. 2024. Novel exploration of the presence and morphological features* Spirometra *(Cestoda: Diphyllobothriidae) in Javan spitting cobra snakes (Naja sputatrix) in Sidoarjo, Indonesia. Biodiversitas 25: 3318-3324.* The study aimed to investigate the presence of spargana infection and provide a detailed description of the morphology, morphometry and histology in Javan spitting cobra snakes (*Naja sputatrix* Boie 1827) from Sidoarjo, Indonesia. Ethical approval was obtained and 51 living wild-caught *N. sputatrix* were collected from local sellers. All samples were euthanized and observed for the presence of plerocercoid. Identification of the plerocercoid as the larval infective stage was made using a carmine staining method drawn by using a camera lucida and routine histological study using hematoxylin-eosin staining. The positive rate of snakes in this study was 54.9%. A total of 165 spargana were collected which divided 82(49.6%) infecting muscles, 31 (16.7%) in viscera and 52 (31.5%) located in subcutaneous tissues. The plerocercoid length and width were 50-95 mm and 1.42-1.81 mm. Morphological analysis confirmed *Spirometra* characteristics and histological sections provided insights into spargana's structural complexity. This study represents the first detailed exploration of the morphological features, morphometry, and histology of *Spirometra* plerocercoids in Indonesia. The high prevalence of spargana in *N. sputatrix* underscores the importance of improving epidemiological databases, stringent wildlife trade regulations, and awareness programs to prevent sparganosis in Indonesia.

Keywords: Infectious disease, *Naja sputatrix*, neglected disease, sparganosis, *Spirometra*

INTRODUCTION

Sparganosis is a parasitic disease caused by the plerocercoid larva, commonly known as spargana, originating from tapeworms within the *Spirometra* genus (Cestoda: Diphyllobothriidae). This parasitic condition exhibits a notable prevalence across diverse vertebrate hosts, encompassing snakes, amphibians, and mammals, with documented occurrences in humans (Liu et al. 2015; Kudo et al. 2017). Subsequently, several Asian nations, including Korea, Thailand, and Japan, have also documented significant occurrences of sparganosis (Jongthawin et al. 2014; Hong et al. 2016; Kikuchi and Maruyama 2020). Plerocercoids can localize at various specific areas such as subcutaneous tissues, eyes, breast, spinal cord, and brain and undergo proliferation, causing significant tissue damage leading to serious pathological conditions such as paralysis, emaciation, and even death in chronic cases (Kim et al. 2018). However, sparganosis remains relatively obscure in Indonesia with limited reported cases.

This zoonotic disease, caused by *Spirometra* spp., follows a transmission pathway primarily associated with the ingestion of contaminated food or water (Hong et al. 2016). Within the Asian context, the intricate dynamics of *Spirometra* tapeworm transmission are characterized by the involvement of intermediate hosts, notably frogs and reptiles, among which snakes stand out as a particular emphasis, playing a pivotal role in the life cycle of the tapeworm. The close association between *Spirometra* and these intermediate hosts in the region significantly contributes to the propagation and prevalence of the parasite (Wang et al. 2011), encompassing diverse avenues for transmission to humans. This includes the ingestion of an infected copepod in natural water, underscoring the significance of waterborne transmission in certain regions, as well as the consumption of inadequately cooked amphibians, such as frogs or tadpoles, which emerges as a potential source of infection, emphasizing the zoonotic nature of *Spirometra*. Additionally, snakes, birds, and mammals, including rodents and pigs, serve as reservoirs for the tapeworm, posing a risk of transmission to humans through the consumption of undercooked or contaminated meat (Hong et al. 2016). Reptiles and amphibians, as intermediate hosts for *Spirometra* tapeworm, are already considered important sources for sparganosis transmission due to their zoonotic potential (Yudhana et al. 2021). This broad spectrum of transmission routes underscores the need for comprehensive public health measures to address and prevent *Spirometra*-induced infections, acknowledging the varied sources through which humans can come into contact with the parasite.

The elevated prevalence of sparganosis in Asia is potentially linked to regional dietary practices, particularly the widespread utilization of wild-caught snakes in culinary and traditional medicinal contexts (Liu et al. 2007). In Indonesia, some of the local restaurants serve wild-caught snakes in their dishes (Yudhana et al. 2020b). Furthermore, a significant number of individuals indulge in consuming raw snake meat, skin, and gall bladder, disregarding the substantial risk of parasite infection associated with such practices (Pranashinta et al. 2017). Cases of sparganosis in Indonesia have been reported in wild-caught animals consumed as food. Instances include Asian wild frogs (*Rana rugulosa* Wiegmann 1834) in Banyuwangi District, Javan spiting cobra (*Naja sputatrix* Boie 1827) oriental rat snakes (*Ptyas mucosa* Linnaeus 1758) in Sidoarjo District, and Asian Water Monitors (*Varanus salvator* Laurenti 1768*)* in East Java Province (Pranashinta et al. 2017; Yudhana et al. 2020a; Yudhana et al. 2021).

In light of the high prevalence of sparganosis and concerning snake consumption practices in Indonesia, our study aims to comprehensively investigate the occurrence of sparganosis in *N. sputatrix* snakes, commonly available in local restaurants. Our primary objectives include assessing the potential risks associated with human spargana infection through the consumption of wild-caught snakes. Additionally, this study provides a detailed description of the morphological characteristics of *Spirometra* obtained from *N. sputatri*x in Sidoarjo, Indonesia. Furthermore, the findings of this study may contribute to identifying sources of sparganosis infection, offering crucial implications for targeted sparganosis prevention programs, which is a neglected zoonotic disease in East Java Province, Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Vide No. 1.KEH.164.10.2023)

Study period and location

Naja sputatrix were collected from local sellers in Sidoarjo District, East Java, Indonesia between September and December 2023. Parasitological examinations were conducted at the Laboratory of Veterinary Parasitology, Division of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Snake samples identification

The Sidoarjo District located within the East Java Province, Indonesia at coordinates latitude -7.4478 and longitude 112.7183, constitutes the geographical focal point of this investigative study. A total of 51 snakes spanning various age cohorts, including hatchlings (0-40 cm), juveniles (41-80 cm), and adults (81-180 cm), were methodically stratified based on their length and subjected to scrutiny. It is pertinent to underscore that *N. sputatrix* selected for this study were explicitly categorized as wild-caught. This classification stems from the absence of captive breeding facilities dedicated to these snakes within the confines of the Sidoarjo District, accentuating the reliance on specimens sourced directly from their natural habitat for this research.

Parasitological examination

A total of 51 snakes were euthanized and subjected to necropsy as part of the research methodology. The examination for the presence of spargana in these snakes adhered to the procedure outlined by Ooi et al. (2000). After the snakes were skinned, their muscular, visceral, and subcutaneous tissues were carefully observed section by section. This observation was conducted visually, with plerocercoids being identified with the naked eye. In instances where spargana were detected, they were delicately extracted from their predilection sites using anatomical tweezers. Subsequently, the removed spargana were immersed in a physiological saline solution to facilitate debris removal. The enumeration of spargana obtained from the snakes was meticulously recorded to calculate both the prevalence rate and the intensity of infection.

Morphological observation

A total of thirty spargana, distributed among muscular, visceral, and subcutaneous tissues, with ten specimens in each category, underwent staining procedures utilizing a Semichon 's-acetocarmine solution for 30 minutes. The stabilized samples were then thoroughly washed and decolorized with 1% acidic alcohol and 1% alkaline alcohol solutions. The process continued with dehydration using varying concentrations of alcohol solution: 30% for 10 minutes, 50% for 15 minutes, 70% for 20 minutes, 90% for 30 minutes, and 95% for an additional 30 minutes. The resulting transparent parasite samples were immersed in xylol for 30 minutes and then sealed with Entellan® (Sigma-Aldrich, Singapore) (Yudhana et al. 2020c). The worms were observed under an Olympus CX-23 series microscope (Olympus Company, Tokyo, Japan). Images for specific identification were captured, and a lucida microscope (Nikon Eclipse e200) was employed for the drawing process. Therefore, 30 spargana were strategically selected and evenly distributed to gather precise measurements among various anatomical regions, including muscles, viscera, and subcutaneous tissues (10 in each category).

Histological preparations

The *Spirometra* spargana was fixed in a 10% formalin solution, then serially dehidrated in ethanol (70%, 80%, 90%, 96%, and absolute), and cleared with xylol. Subsequently, paraffin embedding was executed to generate a robust tissue block for further analysis. The resulting tissue blocks were delicately sliced to a thickness of 3 micrometers using a microtome, intricately mounted on an object glass, and subjected to staining employing the Hematoxylin and Eosin technique (Plumeriastuti et al. 2023). The sample slide was observed by a Nikon Eclipse Ci microscope.

RESULT AND DISCUSSION

The presence of *Spirometra* in *Naja sputatrix* snakes

This study confirmed spargana in *N. sputatrix* from Sidoarjo District and provided the detailed morphological description of plerocercoid *Spirometra*. The prevalence rate of Spargana was 54.9%. All snake samples were wild-caught from several sub-districts and consisted of hatchlings, juveniles, and adults. In total, 165 spargana worms were successfully collected. In each of the positive snakes, spargana were located in the muscles (Figure 1.A), viscera (Figure 1.B), and subcutaneous tissues (Figure 1.C) with intensity rates recorded at 49.6%, 18.7%, and 31.5%, respectively (Table 1). Grossly, spargana were identified to be flat, solid white with ribbon-like shape and pseudo segmentation (Figures 1.D and 1.E).

Owing to the elevated prevalence of sparganosis, the ingestion of reptiles poses a substantial risk for human sparganosis, given that both reptiles and humans serve as intermediate hosts for spargana, potentially facilitating the transmission of the disease (Liu et al. 2015). The primary mode of disease transmission occurs through the consumption of water contaminated with procercoid larvae, allowing for their migration to the intestine and subsequent infiltration into the muscle or subcutaneous tissues (Omar et al. 2023). The secondary mode of infection encompasses the consumption of raw or inadequately cooked frogs, snakes, fish, or poultry, all of which may harbor plerocercoid larvae. Conventionally, human infections typically ensue from the direct consumption of intermediate hosts, including the meat of reptiles or amphibians (Jeon et al. 2015; Edila et al. (2023). This complex web of potential transmission pathways underscores and heightens the importance of comprehensive preventive measures and heightened awareness regarding the consumption of reptiles in regions with a heightened sparganosis prevalence (Yudhana et al. 2021).

Table 1. Presence, intensity, and distribution of *Spirometra* infection in Javan spitting cobra snakes (*Naja sputatrix*) from Sidoarjo District, East Java Province, Indonesia

Age of snakes	Number of samples (N)	Percentage %	Intensity of Sparagna	No. of Spargana in tissues		
				Muscles	Viscera	Subcutaneous tissues
Hatchling (0-40 cm)	4	75	15	9	2	4
Juveniles (41-80 cm)	12	58.3	52	31	6	15
Adult (>80 cm)	35	51.4	98	42	23	33
Total	51	54.9	165	82	31	52

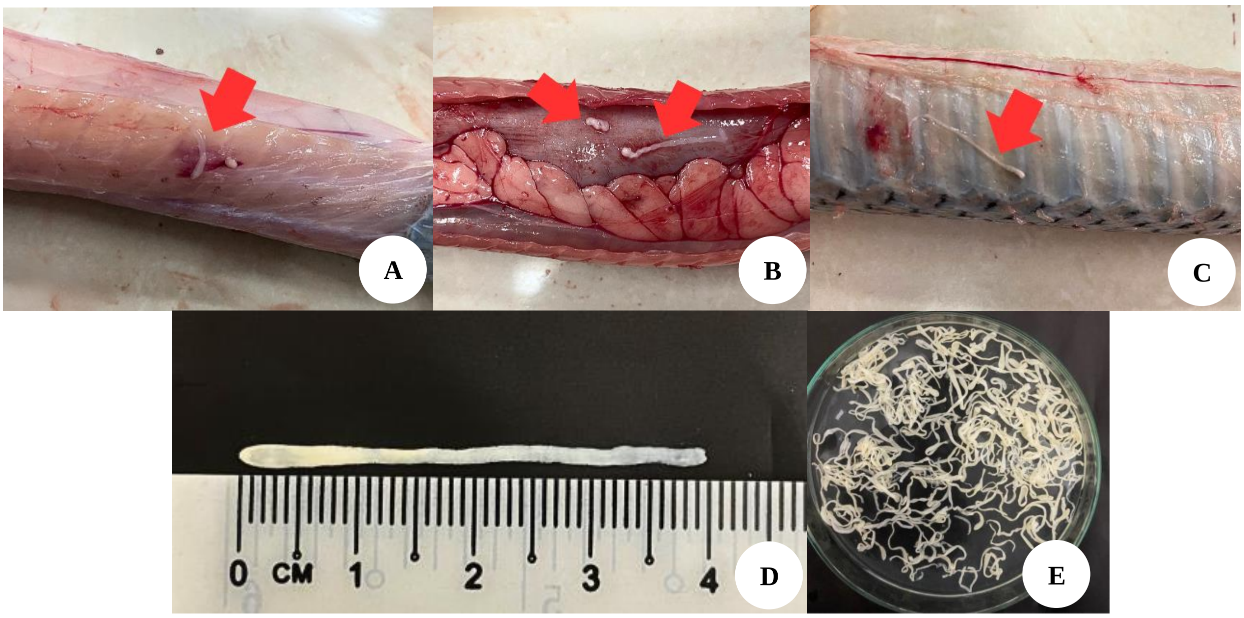

Figure 1. Spargana in Javan spitting cobra snakes (*Naja sputatrix*). A. Spargana in muscles (arrow); B. Viscera (arrows); C. Subcutaneous tissue (arrows); D, E. Macroscopic appearance of spargana

The *N. sputatrix* in Indonesia serve various purposes, including being kept as pets or used in culinary practices, driven by the belief that cobra components possess therapeutic qualities (Edila et al. 2023; 2024). Similar cases have been reported in Indonesia and China, primarily linked to the ingestion of raw snake meat, whole snake blood, and snake gallbladder (Wu et al. 2007). Moreover, improper cooking methods during the preparation of snake meat may elevate the risk of *Spirometra* transmission. The overlooked risk of parasitic diseases caused by *Spirometra* sp. in the utilization of *N. sputatrix* by the community categorizes this ailment as a neglected tropical disease, especially in Indonesia, where awareness is limited. This study highlights the function of snakes as transmitters for sparganosis transmission within the wildlife of Indonesia.

When humans are infected with plerocercoid (spargana), the larvae have the potential for visceral migration, settling in various tissues, and may be associated with diverse clinical indications. The infective spargana larvae can be identified in any region of the human body, although the prevailing pattern in clinical instances often involves their migration to subcutaneous tissue. Sparganosis typically presents as a subcutaneous nodule, clinically manifesting with inflammation or allergic symptoms (Laovachirasuwan et al. 2015). Conversely, past case reports in Korea delineate the clinical characteristics of human axillary sparganosis as a palpable mass resembling an enlarged lymph node devoid of inflammatory reactions such as fever or painful swelling (Kim et al. 2020). Sparganosis is not confined to East and South Asia but has been reported globally, including in Europe, America, Africa, and Australia (Yudhana et al. 2020b). In endemic areas such as China, Korea, and Thailand, the prevalence of sparganosis in definitive and intermediate hosts has been recorded up to 40% (Jongthawin et al. 2014; Hong et al. 2016; Edila et al. 2023). Previous studies in Indonesia have documented sparganosis in reptiles and amphibians, serving as intermediate hosts, including the oriental rat snake, white-lipped pit viper, and Asian wild frog (Liu et al. 2007; Pranashinta et al. 2017). Additionally, *N. sputatrix* in a different region, Banyuwangi District, showed a prevalence of 56.7% (Yudhana et al. 2020a).

Wild-caught *N. sputatrix* frequently prey on animals, potentially already harboring spargana, including frogs, snakes, or smaller lizards. It is imperative to heighten awareness and discourage culinary habits associated with the consumption of wild animal meat or products. A concerning trend is observed in many local restaurants, where reptiles like wild-caught reptiles or *N. sputatrix* are offered as culinary items, elevating the risk of sparganosis. Local governments should actively reinforce food safety inspections in restaurants that feature *N. sputatrix* meat as a main course. Additionally, collaborative educational initiatives are essential, urging restaurant owners to exclusively supply meat from farmed snakes or frozen sources to customers or ideally, to cease offering reptiles as culinary products altogether. These measures aim to mitigate the transmission of sparganosis from animals to humans.

Identification of plerocercoid *Spirometra* in cobra snakes

The specimens were confirmed as plerocercoids of *Spirometr*a tapeworm, commonly known as spargana based on the scolex has a depression which is the undeveloped bothria seen on the anterior aspect of the worm as described by Pranashinta et al. (2017) (Figures 2.A and 2.D.1). The cuticles appear with transverse striation on the scolex part, forming a distinct boundary between the clarity of the scolex and its body (Figures 2.A and 2.D.1). These proglottids exhibit a segmented appearance, resembling true tapeworm segments, although they lack true internal segmentation. The pseudo-segmentation is marked by external grooves that mimic the segmentation seen in mature tapeworms (Figures 2.B and 2.D.2). At the posterior end of spargana, there is a pointed or tapered structure with a gentle indentation in the middle, forming a distinct terminus opposite to the scolex (Figures 2.C and 2.D.3).

The spargana length is 40-75 (56.68) mm, while their body width is 1.8-2.2 (1.98) mm. The scolex region, situated in direct contact with the body, displayed a length of 4.14-5.4 mm (mean: 4.53 mm). The discernible cuticle on the spargana's scolex featured a width of 0.15-0.24 mm (mean: 0.18 mm). Notably, the bothria, crucial for identifying plerocercoid *Spirometra* in this study, exhibited dimensions of 0.03-0.06 (0.04) mm in length and 0.05-0.12 mm in width (mean: 0.08 mm). The reproduction system has not developed. The detailed breakdown of spargana measurements in each predilection presents in Table 2.

Table 2. Morphology and morphometry plerocercoid *Spirometra* sp. in *Naja sputatrix*

Characteristic	Measurement of spargana (mm)			Average (mm)
	Muscles (n=10)	Viscera (n=10)	Subcutaneous tissues (n=10)	
Spargana lenght	45-70 (56)	40-63 (53.2)	43-75 (58.4)	56.68
Spargana widht	1.8-2.1 (1.94)	1.98-2.12 (2.03)	1.8-2.2 (1.97)	1.98
Scolex lenght	4.14-5.16 (3.59)	4.28-5.27 (3.72)	4.18-5.46 (4.53)	4.53
Bothria length	0.03-0.06 (0.04)	0.03-0.05 (0.04)	0.03-0.06 (0.04)	0.04
Bothria width	0.06-0.12 (0.08)	0.06-0.12 (0.08)	0.05-0.1 (0.07)	0.08
Cuticule width	0.16-0.24 (0.18)	0.15-0.21 (0.18)	0.17-0.20 (0.19)	0.18

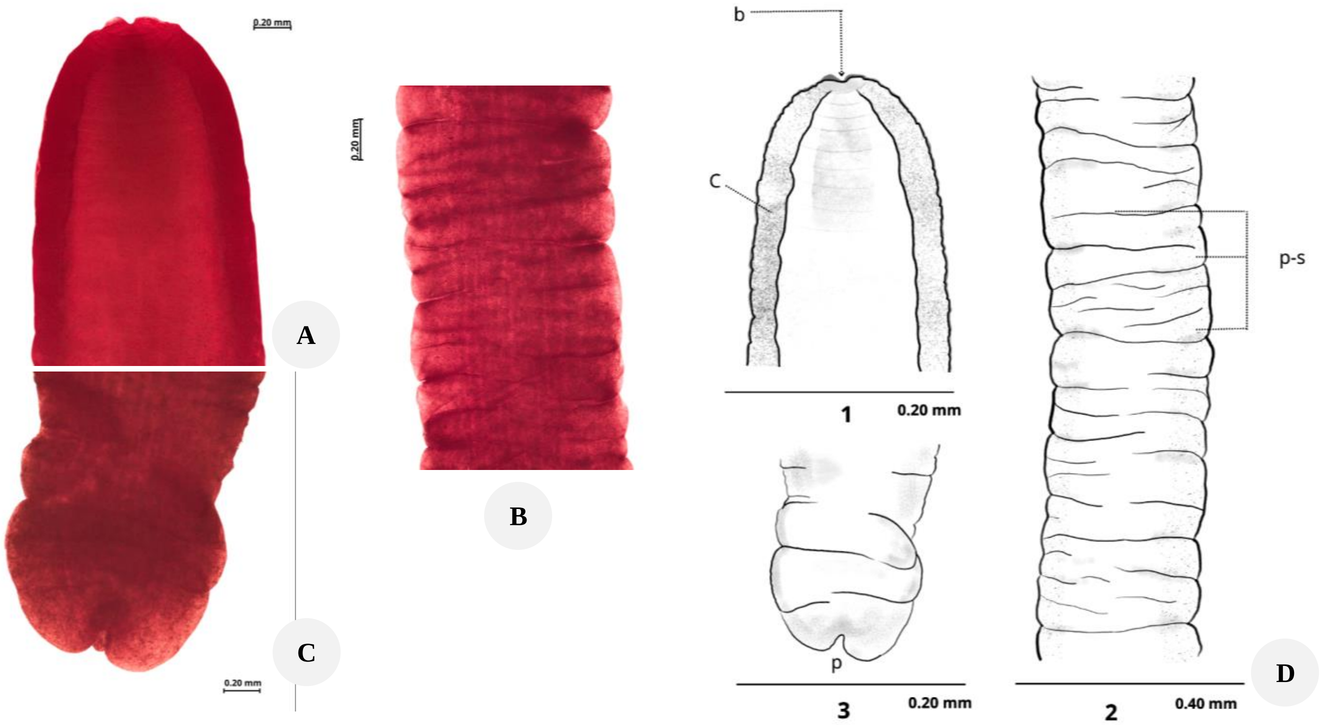

Figure 2. Photomicrograph of *Spirometra* in Javan spitting cobra snakes (*Naja sputatrix*). A. Anterior; B. Body part; and C. Posterior end of spargana with carmine stain (40x). D. Camera lucida drawing of spargana 1. Anterior; 2. Body and; 3. Posterior ilustrated. B. Bothria; c. Cuticule; p-s: Pseudo-segmentation; p: Posterior end

The plerocercoid stage of *Spirometra* sp. demonstrates remarkable longevity within intermediate and paratenic hosts, persisting until ingested by the definitive host. Throughout their maturation within the second intermediate hosts, these larvae undergo a gradual and deliberate growth process, manifesting in a steady increase in both length and width, as elucidated by Ambu et al. (2021). The spargana subjected to examination in this study reveal a distinctive morphology characterized by an anterior scolex (bothria) that assumes a club-shaped form. This scolex encompasses a broad middle part distinguished by ridges (body) and is devoid of sexual organs, culminating in a slender posterior part. This observed morphology closely aligns with the established description of the plerocercoid stage of *Spirometra* sp., illustrating the intricacies of their developmental processes within intermediate and paratenic hosts (Kavana et al. 2014; Xiao et al. 2015; Ambu et al. 2021).

Histology analysis of plerocercoid *Spirometra*

Microscopic analysis of histological sections, unveiled a spargana body wall characterized by varying thickness. This structure comprised an external layer with microvilli on the tegument, smooth muscle cells organized in a dual-layered structure, and a tegumental cell layer. Within the parenchyma, longitudinal muscle fiber bundles exhibited an irregular arrangement, accompanied by mesenchymal fibers, excretory channels, and calcareous corpuscles embedded in a loosely structured stroma (Figure 3). These intricate details provide a nuanced understanding of the morphological features of spargana in this study.

The external layer is characterized by microvilli on the tegument, indicating a surface adaptation and possibly for absorption or sensory functions (Barčák et al. 2019), suggesting a coordinated and efficient mechanism for movement or contraction (Morales et al. 2022). Additionally, the tegumental cell layer further contributes to the outer structure, potentially serving protective or interactive roles. The coexistence of mesenchymal fibers, excretory channels, and calcareous corpuscles embedded in a loosely structured stroma adds to the complexity of the observed tissue. The mesenchymal fibers could provide structural integrity and support to the internal organs, while excretory channels suggest a role in waste elimination. The calcareous corpuscles may contribute to mineral storage or other physiological processes (Vargas-Parada and Laclette 1999; Morales et al. 2022).

Generally, species identification of *Spirometra* tapeworm is based on the anatomical description of adult worms (Kavana et al. 2014). However, practical specimens commonly acquired are typically in the form of *Spirometra* larval stages. Differentiating morphological distinctions among plerocercoids within *Spirometra* tapeworms poses a challenge. Therefore, a molecular test has to be carried out on these larval forms to confirm the species of *Spirometra* sp. found in these wild-caught *N. sputatrix* in Sidoarjo, Indonesia.

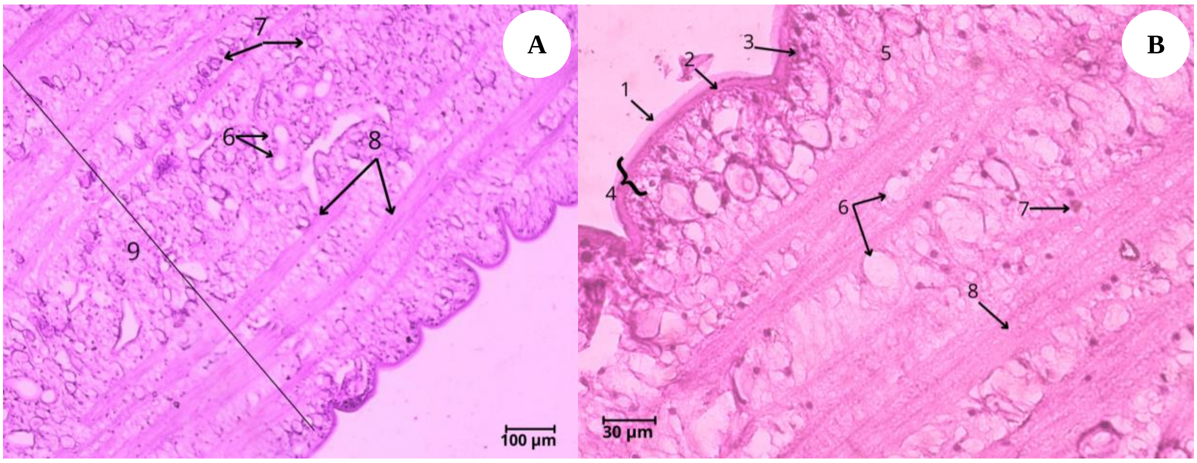

Figure 3. Microphotograph of histological section plerocercoid *Spirometra* sp. from *Naja sputatrix*. A. 100× and; B. 400×. 1. Tegument; 2. Longitudinal muscle fibers; 3. Row tegumentary cells; 4. Body wall; 5. Loose stroma; 6. Excretory channels; 7. Calcareous corpuscle; 8. Mesenchymal fiber and 9: Parenchyma

Based on the present study, we conclude that *N. sputatrix* have the potential for sparganosis transmission with a presence rate of 54.9%. The average width and length of spargana were 1-1.41 mm and 30-75 mm, respectively. The histological analysis provides a comprehensive understanding of spargana's structural composition, revealing a complex organizational structure and additional elements contributing to tissue complexity. Epidemiological databases focusing on precise diagnosis in reptiles and human hosts, must be enhanced with accurately identified spargana. The wildlife trade, especially in traditional merkets, should be regulated by government legislation to mitigate the potential risk associated with reptiles taken from the wild. Collaborative programs with local communities are essential to raise awareness about sparganosis as a neglected zoonotic disease with a significant impact in Indonesia.

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