

Characterisation and Regeneration of *Diopatra claparedii* Nervous System

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Diopatra claparedii has the potential to serve as an animal model for Neurodegenerative Disease (ND) studies due to its complete nervous system and regenerative ability. However, the characteristics of the nervous system in intact *D. claparedii* and during the regeneration process are still unknown. In this study, the intact samples were fixed with Hematoxylin and Eosin (H&E) staining for characterisation. Amputated samples were let to regenerate for 60 days with a 20-day interval before histology assessment. The major findings suggested that the intact *D. claparedii*'s nervous system is built by an anterior dorsal brain and ventral nerve cord, which is segmentally ganglionated. Histology also revealed that the nervous system of regenerated samples on day 60 differs morphologically in terms of brain size. The presence of the brain and ventral nerve cord validates that *D. claparedii*'s nervous system resembles the nervous system organisation in humans. Thus, it is potentially suitable for further investigation of neurodegenerative studies.

Keywords: *Diopatra claparedii*; neurodegenerative diseases; polychaete; regenerative study; hematoxylin; eosin staining

I. INTRODUCTION

Neurodegenerative-related diseases are becoming a threat globally. The statistics suggest an increment pattern and an increase in life expectancy (Procaccini *et al.*, 2016). The diseases have affected older people and emerged among young people (Gong *et al.*, 2016). They are defined as slow but progressive neuronal dysfunction, which causes the progressive losses of neurons in the Central Nervous System (CNS). The loss of neurons leads to ataxia, which is functional loss or sensory dysfunction, or dementia (Solanki *et al.*, 2016). Many approaches and studies are being conducted to find a cure for these diseases since human Neurodegenerative Diseases (ND) are among the hardest to study (Sandoe & Eggan, 2013).

One of the challenges is to find an appropriate study model prior to conducting clinical studies on humans. Current models are being developed using both vertebrates and invertebrates. The ultimate goal is to establish a model that can imitate the human nervous system. Note that it has been reported that organisms from the phylum Annelida have a similar nervous system to humans and may have the capability to be the animal model (Ilmie *et al.*, 2019). Polychaetes or Polychaeta are a class of marine worms from phylum Annelida. In Malaysia, a total of 64 polychaetes from 31 families have been identified, and many marine species, including polychaetes, have not yet been discovered (Idris & Arshad, 2013). One of the species identified is *Diopatra claparedii* from the family Onuphidae. Its tube is submerged

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in the sediment and easily can be recognised during the medium-low tide (Idris & Arshad, 2013).

The nervous system of class Polychaeta has a lot more to discover. In polychaetes, a ground nervous system pattern is suggested and described based on developmental and regeneration studies (Orrhage & Mu, 2005; Nazri *et al.*, 2021; Helm *et al.*, 2022). *Diopatra claparedii*, which can be discovered from Malaysia shores, has the ability to perform both anterior and posterior regeneration (Nazri *et al.*, 2021). Anterior regeneration of *D. claparedii* includes the regeneration of the nervous system. The polychaete may have potential as an ND study model (Nazri *et al.*, 2019). However, the complete nervous system of *D. claparedii* is still unknown. Some invertebrates and vertebrates have been used as models to study ND. However, despite many advantages, there are also disadvantages, such as the difficulties in measuring complex behaviours and less possibility of representing the ND (Nazri *et al.*, 2019). Polychaetes can be used as an animal model for ND studies, which might provide better results than other models in terms of better coverage measurement of the nerve tissues and complexity of the nerve actions. Yet, the nervous system of most of the polychaete classes is less studied. Although many studies have been conducted on various regeneration capabilities, detailed morphological characteristics of regeneration abilities, specifically in the nervous system, are scarce (Müller *et al.*, 2003). Due to the capability of *D. claparedii* to regenerate both anterior and posterior, we were interested in exploring its nervous system and how different it is during regeneration. Hence, this study aimed to investigate the morphological characteristics of the intact and regenerated nervous system of *D. claparedii*.

II. MATERIALS AND METHODS

A. Sampling

Tubes containing *D. claparedii* were collected using a shovel by digging deep into sediment from the intertidal mudflat area of Pantai Morib, Banting, Selangor. Subsequently, the presence of the polychaetes was checked inside the tube. Empty tubes were placed back into the sediment. The collected tubes with polychaetes inside were transferred into an ice chest covered with a wet towel to maintain the

humidity. A total of 20 polychaetes were collected with their tubes. Sediment was also collected at the same spots where the samples were collected. Consequently, the sediment underwent a debris separation and drying process. Other organisms, such as small crabs, foreign polychaetes or seashells, were separated so that they would not interfere with the polychaetes during the laboratory conditions. The sampling was done on 22nd July 2017, from 9 am to 2 pm during the low tide. Parameter readings such as temperature, pH and salinity were obtained in the sampling area to mimic their habitat in laboratory conditions (Nazri *et al.*, 2021).

B. Laboratory Conditions

The samples were removed from their tubes and rinsed with artificial seawater to remove the debris and other living organisms attached to the tubes. Correspondingly, tanks were prepared with sediment from Pantai Morib and artificial seawater with a ratio of 1:3 (Pires *et al.*, 2012; Hussain *et al.*, 2018; Che Soh *et al.*, 2020; Nazri *et al.*, 2021) with water conditioner (Tensiongon, Mydilib). Then, the tubes were placed into tanks filled with 6 kg of sediment and 18 L of artificial seawater with a salinity of 28±1. The acclimatisation process took place for ten days. Samples were fed commercial fish food every 2~3 days. Note that the excess foods were cleaned occasionally and never allowed to accumulate to avoid fungal growth and contamination (Pires *et al.*, 2012).

C. Characterisation *D. claparedii*'s Nervous System

Characterisation was done by dissecting the targeted regions of the samples before processing them in a tissue processor. However, the tissues were damaged badly during the processing process prior to histology. Therefore, we did not dissect, instead taking the entire anterior region to process. The anterior regions were fixed using 10% formalin in artificial seawater and stored in formalin for 24 hours before processing. They were cut into two parts, 0.2 cm and 0.3 cm from the end part, as shown in Figure 1. Before processing the samples, the whole samples were cut transversely and sagittally. Subsequently, the processed samples were proceeded with histological analysis.

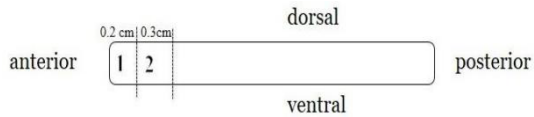


Figure 1. The plan of cuts of anterior heads. Part 1 was 0.2 cm from the anterior, and Part 2 was 0.3 cm from Part 1.

For the suspected ventral nerve cord, the middle part with a 2 cm length from the anterior end was cut before processing. The samples dissected for the brain and ventral nerve cord were processed in a tissue processor. The processed samples were vacuumed in the vacuum oven at 80°C degrees for 2 hours. Then, the samples were embedded, sectioned horizontally and fixed on a glass slide. The sections were stained by Hematoxylin and Eosin (H&E) staining, covered with a coverslip and viewed under a compound microscope (Ilmie *et al.*, 2015).

D. Regeneration Experiment

The regeneration experiment was conducted after ten days of the acclimatisation process. The samples were immobilised using a 4% magnesium chloride solution in artificial seawater prior to the amputation. Six samples of *D. clapedii* were amputated at the 10th chaetiger to observe the anterior nervous system. The posterior ends of the samples were left on the sediment inside the seawater to let the samples recover from the anaesthetic state. Consequently, the recovered samples were inserted back into their tubes and placed into their tanks (Pires *et al.*, 2012). The samples were observed for 60 days, with a 20-day interval for each polychaete. Starting with the control, the regeneration experiment started on day 20, day 40 and lastly, day 60. Two samples from the six samples represented each day and were stained separately for histology to view the process of nervous system regeneration. After each interval, the samples were fixed in 4% formalin for 24 hours. Then, tissues were processed, embedded, sectioned and fixed on a glass slide (Ilmie *et al.*, 2015).

E. Histology

The specimens of *D. clapedii* underwent a tissue preparation process prior to H&E staining. The tissues were dehydrated using 50~100% ethanol in a tissue processor

(Leica TP1020, Illinois, USA). Correspondingly, the specimens were cleared using xylene and embedded in paraffin. Then, the specimens were sectioned to 5µm of thickness using a microtome (HistoCore BIOCUT, Leica, Illinois, USA). The sections were transferred to 40°C of the water bath for the fishing process. Finally, the sections were fished using glass slides and allowed to dry on a hot plate (Ilmie *et al.*, 2015). The staining process was started by soaking the slides in xylene for 2 minutes each. Then, the slides were soaked in absolute alcohol, 90% and 80% alcohol, for 2 minutes each. The process was continued by rinsing the slides in tap water and soaking them in hematoxylin for 20 minutes. The slides were rinsed again in running tap water. Consequently, the slides were dipped into acid alcohol for 3 seconds and again were rinsed in running tap water for 5 minutes. Next, the slides were dipped into ammonia water for 10 seconds and rinsed again for 2 minutes. Next was the eosin, where the slides were soaked again for 2 minutes. The process was continued by soaking the slides again in absolute alcohol, 90% and 80%, for 2 minutes each. The last step was to soak the slides into xylene twice for 2 minutes each. Then, the slides were covered with a coverslip and were viewed under a light compound microscope (Ilmie *et al.*, 2015).

III. RESULTS

A. Parameter Reading from Sampling Site

The average temperature at the site was 30.9, the average pH was 7.83, and the average salinity was 26.67. These parameter readings were applied to set up the tanks for laboratory conditions.

B. Characterisation of Brain

Characterisation of the nervous system was done by identifying the brain and ventral nerve cord of *D. clapedii* by the histological method. The suspected brain was identified by the presence of some neuronal cells, as illustrated in Figure 2. The brain has a symmetrical morphology with two lobes, one left and one right. There are innervations of nerves called palp nerves pointing towards the uppermost anterior. Note that nuchal nerves are observable from this method to complete the anterior nervous system organisation (Figure 2).

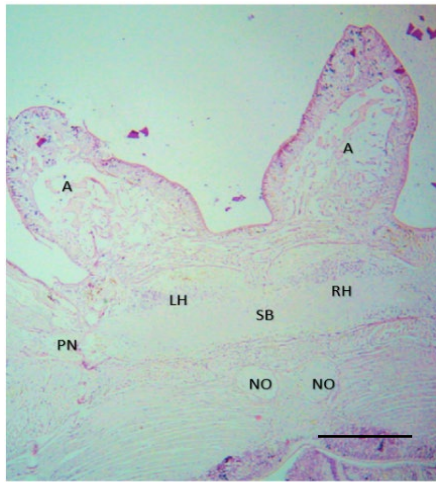


Figure 2. Micrograph of the anterior end of *D. claparedii* stained by H&E. The Suspected Brain (SB) displaying Left Hemisphere (LH) and Right Hemisphere (RH), Palp Nerve (PN) and Nuchal Organs (NO) below the Antennae (A) at a magnification of 10x. Scale bar= 200µm.

The same method identified the suspected ventral nerve cord (Figure 3). Our finding reveals that the segmented body of *D. claparedii* also contains segmental nerves. Every segment consists of subesophageal ganglia at both left and right.

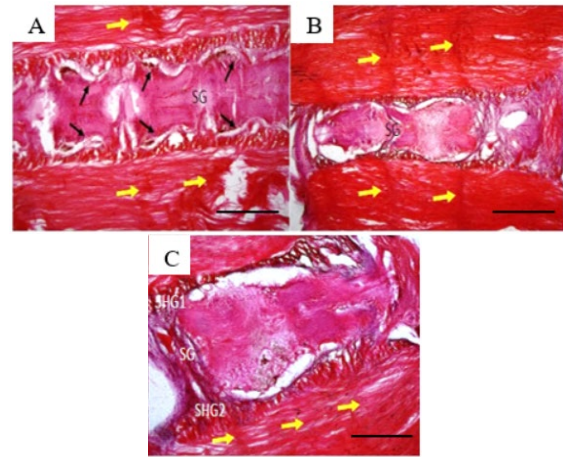


Figure 3. Micrograph of the ventral part of *D. claparedii*: (A) The presence of ventral nerve cord (black arrows) at each segment (yellow arrows show septa) at a magnification of 5x, (B) The presence of suspected Subesophageal Ganglion (SG) at the segments (yellow arrows), and (C) The presence of a pair of Suspected Hemiganglia (SHG1, SHG2). Scale bar=200µm.

C. Regeneration Experiment

During the regeneration experiment, the number of regenerated segments of the amputated *D. claparedii* was observed. Table 1 provides the percentage of regeneration after amputation at the 10th segment for every 20 days interval.

Table 1. Percentage of regeneration of amputated polychaetes every 20 days.

Worm	Day	No. of segments amputated (anterior)	No. of regenerated segment after amputation	Percentage of Regeneration (%)
1	0	10	0	0
2	20	10	6	60
3	40	10	0	0
4	60	10	24	240

After every 20 days interval, each polychaete, as listed in Table 1, has been observed for physical features resulting from the regeneration phase with failure of regeneration in

worm 3. Table 2 below represents the physical description of *D. claparedii* after every 20 days interval exhibiting the gradual growth change upon completion.

Table 2. Physical description of *D. claparedii* after every 20 days of interval.

Worm	Day	Physical
1	0	No regenerated segments
2	20	Segments regeneration
		Incomplete head formation
		Incomplete jaw formation
		Incomplete antenna formation
		Incomplete parapodia formation
3	40	No regeneration was observed except for a small protrusion at the amputation site
		White in colour
4	60	Segments regeneration of both anterior and posterior
		Complete head formation
		Complete antennae formation
		Complete parapodia formation
		Light green colour for the new segments

D. Characterisation of Regenerated D. claparedii 60 Days After Amputation

The characterisation of the nervous system of regenerated *D. claparedii* was also conducted to compare the difference with the intact *D. claparedii*. The brain of the regenerated polychaete was identified through histological staining, as portrayed in Figures 4 and 5. The brain at 60 days after amputation is observed as a lot smaller compared to that of an intact brain. Other parts of the nerves are also observed as not fully developed.

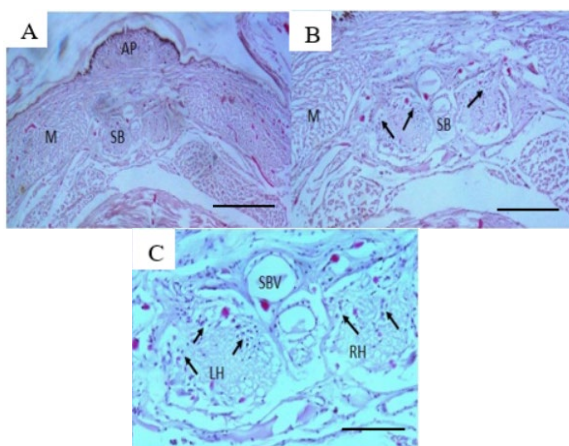


Figure 4. Micrograph of regenerated brain in horizontal view at 60 days after amputation of worm 4: (A) Position of Suspected Brain (SB) at the Anterior (AP) accompanied by a pair of Muscles (M) at a magnification of 5x, (B) SB with the presence of neurons (arrows) at a magnification of 10x, and

(C) SB with the presence of both left and right hemisphere, neurons (arrows) and suspected blood vessels at a magnification of 20x. Scale bars=200µm.

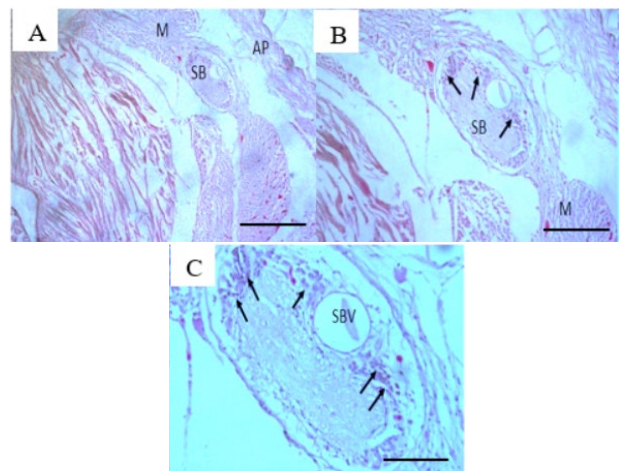


Figure 5. Micrograph of the regenerated brain in horizontal view at 60 days after amputation of worm 5. (A) Position of the Suspected Brain (SB) at the Anterior (AP) accompanied by a pair of Muscles (M) at a magnification of 5x. (B) suspected brain with the presence of neurons (arrows) at a magnification of 10x. (C) SB with the presence of neurons (arrows) and suspected blood vessels at a magnification of 20x. Scale bars=200µm.

IV. DISCUSSIONS

Prior to conducting this research, it was essential to create a suitable condition for the samples to live in a laboratory because we could not ascertain the occurrence of regeneration. Thus, the laboratory condition was regularly checked and adjusted according to the parameter readings taken from the sampling area. The same parameter readings were applied to set up the tanks for the regeneration experiment. This step was done and caused less stress to the amputated polychaetes, which was least likely to affect the survivability rate. Certain unfavourable conditions such as different age, nutritional status and environmental condition potentially impairing the regeneration process (Bely, 2010) as we seen in worm 3. Thus, before the regeneration experiment, the samples were put in a laboratory condition called acclimatisation. During this period, some samples were discovered to be dead within three weeks of the acclimatisation procedure. Following with the death of samples, we discovered that fungal growth was the cause. Moreover, the leftover foods were immediately removed as we noticed they might cause a fungal growth inside the tank. This is supported by a previous study (Pires *et al.*, 2012). Precaution steps have been taken, such as using a water conditioner to avoid contamination and reduce sediment turning black (rotten) and smelly during the experiment. Hence, we managed to increase the survivability in our experiments.

The prostomial dissection at the ventral side was quite tricky to perform and easily damaged the targeted regions. Therefore, dissection by sagittal cutting was processed. However, this also resulted in the break of paraffin-embedded tissues during sectioning. This method was considered unsuitable for characterisation due to the delicate dissected and exposed tissues of *D. claparedii*. The second method was then applied, which was processing the whole sample without dissecting.

We have performed H&E staining to identify brain region in the anterior part of the samples. Even though H&E stain is regard as principal tissue stains, the structure of brain can be visualised clearly as shown in Figure 2. The suspected brain was located below the antennae, separating the left and right hemispheres at the magnification 10x along with palp nerve and nuchal organs. Palp nerves are connected to the

dorsolateral and ventral-lateral parts of the brain (Beckers *et al.*, 2019). Meanwhile, the brain could be observed when the sample was cut transversely and sectioned horizontally. Furthermore, the brain only can be viewed in part 1, as demonstrated in Figure 1. The result explains the position of the suspected brain in *D. claparedii*, which is 0.2 cm away from the anterior part. Figure 6 illustrates the position of the brain *D. claparedii*.

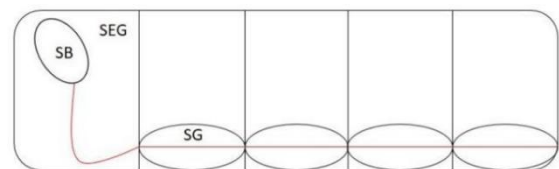


Figure 6. Representative diagram of anterior part of *D. claparedii*. The position of the Suspected Brain (SB), ventral nerve cord (red line) and Suspected Ganglia (SG) with the presence of segments from a lateral view.

The suspected ventral nerve cord was viewed after staining the tissues, as portrayed in Figure 3. The suspected ventral nerve cord is presented clearly along the middle of the abdomen. As displayed in Figure 2, the ventral nerve cord is accompanied by suspected ganglia at each segment. Each segmental ganglion is paired by suspected hemiganglia at each end. Figure 7 demonstrates the overview of the suspected ventral nerve cord and the position of the suspected ganglia in *D. claparedii*.

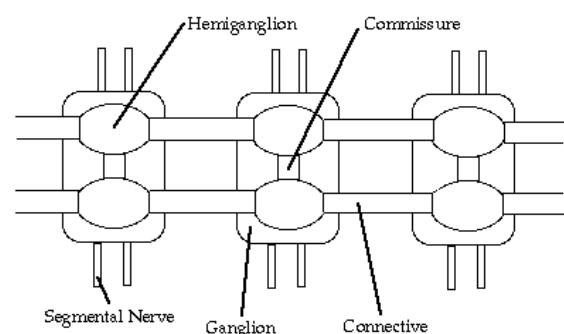


Figure 7. Overview of the suspected ventral nerve cord (red line), Suspected Ganglia (SG) and Suspected Hemiganglia (SHG) with the presence of segments.

This was supported by the presence of a ventral nerve cord in phylum Annelida, as exhibited in Figure 8. According to Zattara and Bely (2011), in annelids, the ventral nerve cord is

innervated by a ganglion at each segment consisting of a pair of hemiganglia, as displayed in Figure 8 below.

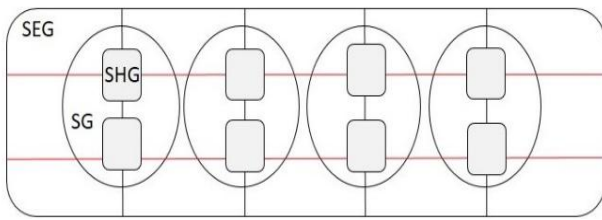


Figure 8. The ventral nerve cord in Annelids.

According to Ohtaki and Shioda (2015), the spinal cord is a part of the CNS, while the ganglia are included in peripheral nervous system (PNS). Like humans, *D. claparedii* has the presence of a ventral nerve cord representing the spinal cord, which consists of ganglia.

The experiments in the present study were conducted for 60 days considering that *D. claparedii* can completely regenerate its head at around 60 days after amputation, as stated by Pires *et al.* (2012) and Nazri *et al.* (2021). From our observation, even though the size of the regenerated anterior is similar to its original body, our results suggest that the nervous system is not fully developed. Our results could be strengthened by more advanced techniques, such as antibody staining, to prove the stages of nerve regeneration. Weidhase *et al.* (2014) reported that their samples, *Cirratulus cf. cirratus*, were still growing at the final state of regeneration in the study and full regeneration remains uncertain.

The presence study revealed the existence of a complex nervous system in *D. claparedii* by at least having a CNS that consists of the brain and nerve cord (Brodal, 2004). This is in accordance with that general overview of Annelid's nervous system. Beckers *et al.* (2019) reported that the anterior CNS consists of a dorsal prostomial brain with several commissures and connectives connecting to the ventral nerve cord. Other than that, *D. claparedii* has also been reported to possess those characteristics based on the study conducted by Nazri *et al.* (2021).

Our investigation supports that polychaetes from the genus *Diopatra* have a complete and advanced nervous system that

leads to their special ability to regenerate the nervous system (Pires *et al.*, 2012; Pires *et al.*, 2016; Nazri *et al.*, 2021). This understanding might be beneficial to be applied in medicine as an animal model. An established animal model such as *Caenorhabditis elegans* or roundworm has long been regarded as a subject to model human diseases that include NDs (Baumeister, 2002). Furthermore, *C. elegans* has rather simple cellular structures and genes in every part of its body. With only 1 mm long (Gonzalez-Moragas *et al.*, 2015), *C. elegans* is easy to manage and culture. However, due to its small size, more information tends to be ignored when comparing humans. Thus, we proposed that a larger animal with a complete nervous system structure that consists of major tissues such as the brain and nerve cord is worth considering to become a disease model. The fact that *D. claparedii* could anteriorly regenerate is another added point to consider this species. Therefore, molecular studies and more advanced approaches are needed to explore the potential that *D. claparedii* could offer.

V. CONCLUSION

In conclusion, the results reported that the suspected brain of *D. claparedii* is more like the human brain. This is due to the presence of neurons and the orientation of the brain, which is more like the human brain. The results also reported that the ventral nerve cord of *D. claparedii* was also identified in the intact sample of both ganglia and hemiganglia. This ventral nerve cord resembles that of the spinal cord in humans. Moreover, this research has primarily proved that *D. claparedii* has the potential to be an animal model for ND's research.

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