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Research Article

Concentration and Distribution of Oligochaeta Worms in the Waters of Kejapanan, Pasuruan, Indonesia Polluted by Mercury Waste using DNA Barcode

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Abstract

Physiological monitoring of mercury waste contamination can be carried out using the biota around the waters. This study aims to identify concentration of Hg and the types of worms in the waters of Kejapanan, Pasuruan, East Java with a molecular approach. Target gene amplification was carried out using the mitochondrial genome COI barcode primer. Analysis of molecular identification was performed with DNA analysis and phylogenetic, similarity, DNA sequence variation, genetic distance, and the BOLD System. The concentration Hg was analyzed using AAS and the distribution of mercury in the worms was analyzed using SEM Edax Mapping. The results showed that the pollutant source area (St2 sample) has the highest concentration of mercury compared to other locations. The results of molecular identification indicate the formation of two clusters. The amplified samples produced DNA bands according to the target (600-700 bp), and the process was continued with morphological-based-key identification. The results showed that they consist of the family Nadidae with two species, namely *Limnodrilus hoffmeisteri* and *Branchiura sowerbyi*. A DNA length of 709 bp as well as nucleotide composition. BLAST results showed that species *L. hoffmeisteri* and B. sowerbyi had similarity indexes of 99% and 86%, respectively. Based on the research results, it was found that there was an accumulation of mercury exposure in worms in polluted areas. For this reason, the results of this study can provide a novelty that worms can be used as biomonitoring of water pollution using the barcode data.

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1. Introduction

The waters in Kejapanan Village, Pasuruan, East Java have been polluted due to household, industrial, and agricultural wastes (Adam *et al*., 2018). Furthermore, several types of metals have been identified as contaminators of these waters including mercury at a dose of 0,015 ppm (Widiastuti *et al*., 2019), lead at dose 0,035 ppm, and cadmium at dose 0,02 ppm (Adam *et al.,* 2019a). Mercury is one of the most toxic heavy metals (Boening, 2000) and is highly bioaccumulative (Shi *et al.,* 2010). Its bioaccumulation is very influential on aquatic biota and humans (EPA, 1989, 2016), morphological changes (Adam *et al*., 2019b), death (Clarkson and Magos, 2006), biochemical disturbance (Barregard *et al.,* 2010), and physiology (Adam *et al.,* 2022).

Various types of biotas live in these waters including Oligochaeta worms, fish, plants, gastropods, mollusks and several types of plankton. These invertebrates play an important role in the waters and are tolerant to chemical contamination (Rodriguez and Reynoldson, 2011). The role of oligochaeta in waters is as a disperser of organic material and to increase soil aeration. Apart from that, oligochaeta can also be used as animal feed and aquaculture (Kosman and Subowo, 2010). The morphologically identified species in Kejapanan include *Tubifex tubifex, Limnodrilus hoffmeisteri*, and *Branchiura sowerbyi*. However, thorough certainty is still needed to avoid errors in their identification. DNA barcode technique is one of the methods used to identify species quickly and accurately, based on the nucleotide base sequence of a standardized marker gene (Hebert *et al.,* 2003). It can also be carried out by matching the fragment sequence of the selected gene to a reference containing the DNA sequence generated from the identified specimen (Wibowo *et al.,* 2018; Anggorowati *et al.,* 2019). The technique is often used to distinguish organisms as well as to identify hard-to-recognize specimens (Hebert *et al.,* 2003). DNA-based analysis was carried out to re-examine species with a wide distribution and are large in numbers. DNA barcoding is a powerful tool for identifying organisms (Elsaied *et al.,* 2022), thereby making it possible to identify morphologically unidentified specimens (Ali *et al.,* 2020). The use of mitochondrial DNA in the systematic analysis is not compatible with morphological evaluation because the characters often indicate cryptic species phenomena. Result study (Zhou *et al.,* 2021) explained that DNA barcodes from oligochaeta with COI are able to identify and explain the role of worms as indicators for monitoring waters.

The cytochrome oxidase subunit 1 (COI) gene is one of the encoding genes in the mtDNA genome. Several parts of COI are often conserved and then used as DNA barcodes (Ahmed *et al.,* 2019). A short COI fragment can be used as an accurate marker in identifying a wide variety of animals to the species level (DeSalle and Goldstein, 2019) as well as to reconstruct phylogenetics at the species level (Becker *et al.,* 2021). One of the advantages of the gene is having universal primers that can cover a large 5' part of the animal phylum. It also has a greater range of phylogenetic signals compared to other mitochondrial genes. Based on its superiority in identifying species, it is necessary to carry out a rapid assessment through molecular identification with DNA barcoding. No statement has been found that worms can be used as biomonitoring for mercury contamination. For this reason, the results of this study can provide a novelty that worms can be used as biomonitoring of water pollution using the barcode data. Therefore, this study aims to concentration of Hg and identify the types of worms in the waters of Kejapanan, Pasuruan, East Java using molecular identification.

2. Materials and Methods

2.1 Materials

The materials used in this study are water samples, sediment samples, and worm samples as test animals, as well as other materials used in laboratory testing 70% alcohol; sterile distilled water; NaCl; Tris-HCL; EDTA; proteinase K; sodium dodecyl sulfate; concentrated H2SO4; concentrated HNO3; KMnO4, 3% glutaraldehyde buffer; osmium tetroxide buffer; Phosphate Buffered Saline (PBS) solution; distilled water; ethanol/acetone, ZR Tissue & Insect DNA MiniPrepTM kit (Zymo Research, Irvine, CA, USA), My TaqTMRed Mix (Bioline, BIO-25044, Thomas Scientific, USA).

Meanwhile, the tools used in the study include those used for sample collection and measurement in the field, such as scoop; sample container Dark bottle; light bottle shovel; sample container. Water quality parameters including temperature, pH, and total dissolved solids (TDS) were measured onsite with a multiparameter water quality meter (Hanna HI98194, Hanna Instruments, Romania). For ammonia and dissolved oxygen (DO), water samples were measured with Salifert test kits for ammonia (NH3) and dissolved oxygen (DO) (Salifert BV, Netherlands); as well as the tools used in laboratory testing electric heater; centrifuge; beakers; watch glass; Atomic Absorption Spectrophotometer (Shimadzu AA-7000 Model, Japan); BLAST (Basic Local Alignment Search Tool); Hitachi TM3000 Benchtop Scanning Electron Microscope (SEM), Taihuru Nukurangi, Japan.

2.1.1 Ethical approval

The animals used in this study originated from local area, not conduct any testing or treatment, the ethical clearance is not applicable in this study.

2.1.2 Preparation Sample

Worm samples were collected from sediments in the waters of Kejapanan, Pasuruan, East Java with the coordinates LS 07°838' and BT 112°28' shown in Figure 1. Worms are taken at a predetermined sampling for 4 (four) station (St). St1 samples were taken at the pollutant source; St2 samples were taken right at the pollutant source; St3 and St4 samples were taken at the location after the pollutant source. The worms are taken directly (3-5 individuals/St) with the sediment and separated from the adhering dirt. Then the worms are washed in running water until clean and stored in a container. They were then preserved in 60% absolute ethanol and immediately stored at -20°C until the extraction process.

2.2 Methods

2.2.1 Mercury Concentration (Hg)

Oligochaeta worms that have been cleaned, then measured the concentration of the metal, namely mercury (Hg). Measurement of metal concentrations in each sample (water, sediment and Oligochaeta worms) used the Atom Absorption Spectrophotometer (AAS) method based on (BSN, 2006). The procedure for determining the concentration of Hg refers to (EPA, 1994).

2.2.2 DNA Extraction and Isolation

The alcohol washing process was carried out before DNA isolation by immersing the samples in sterile distilled water, followed by homogenization in STE buffer containing 1M NaCl, 10mM Tris-HCL, and 0.1mM EDTA with a pH of 8. They were then crushed and lysed using 0.125 mg/ml proteinase K and 1% sodium dodecyl sulfate. Genomic DNA was isolated with the ZR Tissue & Insect DNA MiniPrepTM kit

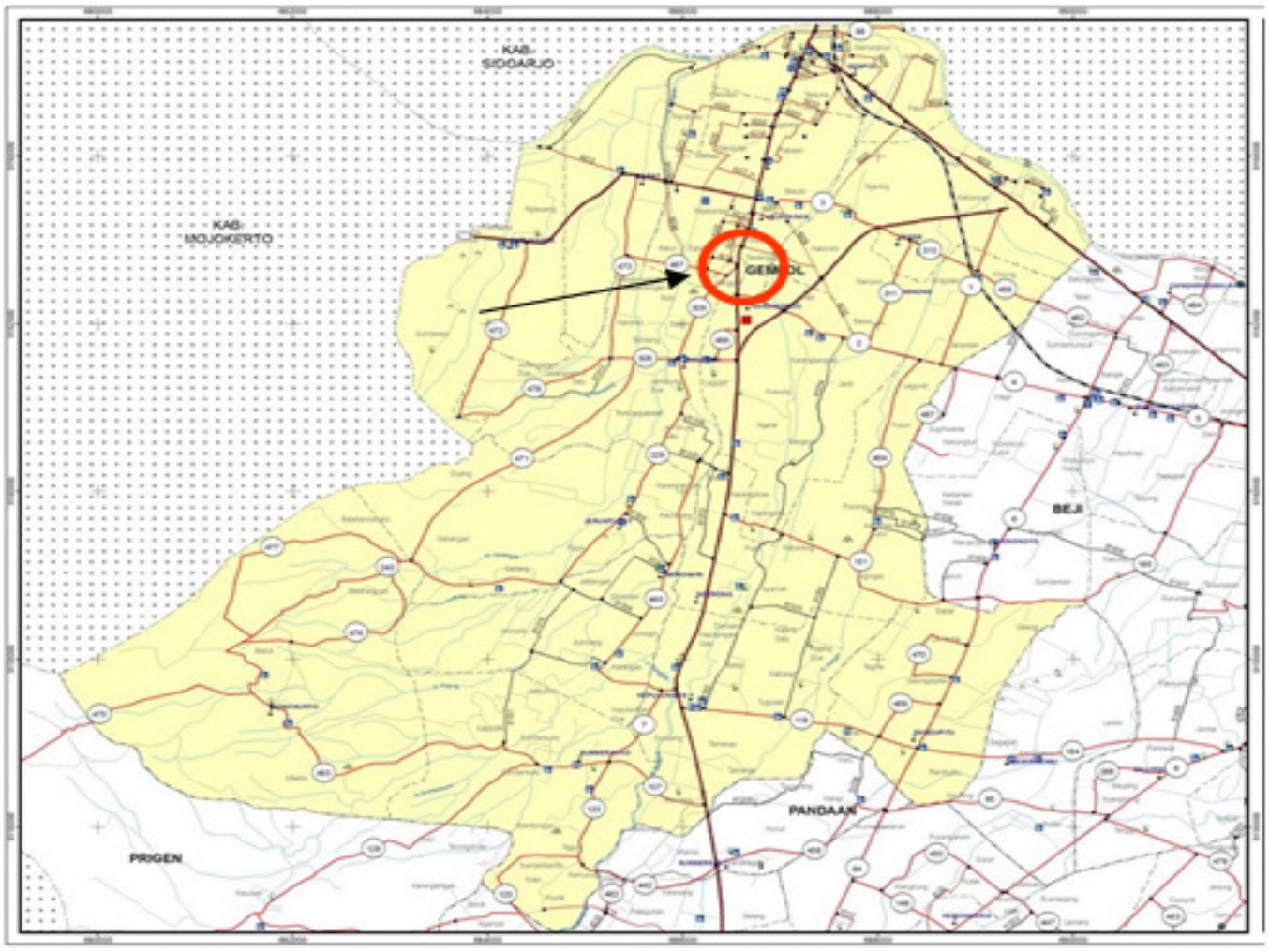


Figure 1. Research Maps Kejapanan, Pasuruan, East Java.

protocol. Subsequently, pure DNA was collected and stored at -70˚C.

2.2.3 DNA Fragment Amplification and Visualization

The COI gene segment of the mitochondrial genome was amplified using universal DNA barcode primers, namely LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994). The success of the PCR was observed with the 6% polyacrylamide gel electrophoresis (PAGE) method, which was carried out at a voltage of 200 V for 50 minutes, followed by silver sensitive staining (Byun *et al.*, 2009). DNA extraction was performed based on the ZR tissue & Insect DNA MiniPrepTM kit procedure. PCR amplification using My TaqTMRed Mix (Bioline, BIO-25044) was carried out with the following temperature settings: pre-denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 10 seconds, 35 cycles of annealing at 52°C for 30 seconds, and extension at 72°C for 45 seconds and the temperature was held at 4°C for 1 cycle.

2.2.4 DNA Analysis and Phylogenetic

The mold used for PCR sequencing is an amplicon with single-band quality using the same big dye terminator and primer method as the initial amplification. The results of the nucleotide sequencing were edited manually based on the chromatogram and then used as input in the search for gene similarities using BLAST (Basic Local Alignment Search Tool). This was carried out to compare the DNA sequence database contained in the BOLD (Barcode of Life database), which can be accessed at the NCBI GenBank (www.blast.ncbi.nlm.nih.gov). The most similar GenBank sequences are characterized by the same Max and Total Scores, Query Coverage close to 100%, E-value close to 0, and I-dent close to 100% in each database. Nucleotide sequences of all samples and homologous BLAST results were aligned using Clustal W version 2.0 contained in the MEGA program version 06.6 (Tamura *et al.*, 2011) to determine the level of homology in the analyzed DNA base sequence. The order submitted to NCBI was retrieved from the database in FASTA format. Databases with high similarity, namely a similarity threshold of 99% and threshold length of 90% using BLASTclust of files were placed in one group.

2.2.5 Scanning Electron Microscopy (SEM)

The preparations were made using the modified method (Devos *et al.*, 1998). After dissection, they were thinly cut to a thickness of 2–3 mm, the paraffin was removed from the sections, followed by immediate immersion in 2% glutaraldehyde, placed in 0.05 M osmium tetraoxide (pH: 7.4; fixative osmotic pressure: 310 mosmol l-1) for 90 min. Subsequently, each of the samples was placed in buffer 0.15 osmium tetraoxide under room temperature for 4 × 10 min. They were then left to dry for 5 min and placed in 30, 50, 70, 90, and 100% acetone solution for 5 min each, followed by drying at the critical point (critical point dryer, Balzers CPD 030). The sample was mounted on a silver paint stub by maintaining the primary lamella parallel to the stub, and it was gold-coated with the sputtering method (Balzers). Two worm samples were then examined under a scanning electron microscope, namely Philips XL 20 for each experimental condition. At least four non-contiguous areas were randomly selected from each fish for morphometric analysis.

Source: Data in Table 1 is obtained from the mean ± stdev in each sample (n = 3)

3. Results and Discussion

3.1 Result

3.1.1 Concentration of Hg

Table 1 shows that the St2 sample has the highest concentration of mercury compared to other locations, both in water (0.0363 ± 0.0050 mg/L), sediment (0.2683 ± 0.0055 mg/L), and in Oligochaeta worms (0.2543±0.0065 mg/L). The lowest concentration of mercury in the St4 sample. The concentration of mercury in the sediments and on Oligochaeta is higher than that in the water.

St2 samples are samples taken right at the pollutant source. St1 samples were taken before the pollutant source which had a high concentration of mercury followed by St3 and St4 samples taken at the location after the pollutant source. These results explain that the closer the location is to the pollutant source, the higher the pollutant concentration.

3.1.2 DNA Amplification and visualization

The COI gene that was successfully amplified had a length of approximately 700 bp using a 3000 bp DNA ladder as a comparison, as shown in Figure 1 (A). Cloning was then carried out to obtain clearer results. The cloned COI gene that was successfully amplified had a length, as shown in Figure 1 (B). The process was then continued with colony PCR (8 colo-

nies) using electrophoresis with 1% TBE agarose. The COI gene that was successfully amplified by PCR has a length as shown in Figure 2 (C).

3.2 Discussion

Referring to (Goverment Regulation, 2001),

Table 1. Concentration of mercury (Hg) in water, sediment and worms.

Sample Stations	Hg water (mg/L)	Hg sediment (mg/L)	Hg worms (mg/L)
St1	0,0357±0,0050	0,2417±0,0031	0,1697±0,0074
St2	0,0363±0,0050	0,2683±0,0055	0,2543±0,0065
St3	0,0280±0,0020	0,1640±0,0001	0,0930±0,0056
St4	0,0277±0,0015	0,1413±0,0067	0,0917±0,0031

Source: Data in Table 1 is obtained from the mean ± stdev in each sample (n = 3)

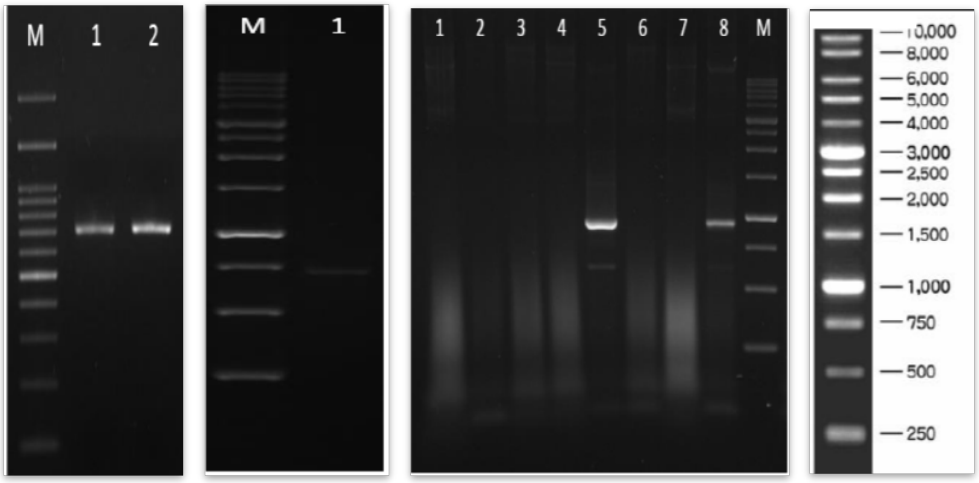


Figure 2. Electropheogram of COI gene (A) amplification of worm specimens; (B) Results of Agarose Gel Analysis on sample-1; (C) Colony PCR (8 colonies) on Sample-1 (3µL PCR Products were assessed by electrophoresis with 1% TBE agarose); M DNA ladder 1 Kb.

3.1.3 DNA Analysis and phylogeny

The BLAST results show the similarity level of *Limnodrilus hoffmeisteri* species with the same Max score and total score of 1179, as well as 92% query cover, E value of 0.0, and 99% Ident 99%. The similarity level of the species *Branchiura sowerbyi* was indicated by the same Max score and total score of 777, as well as 95% query cover, E value of 0.0, and 86% Ident, as shown in Table 3.

The Limnodrilus sample was ingroup with KU 668562.1 Branchiura, while the sample was outgroup with the GenBank reference species as shown in Figure 3.

3.1.4 Mercury concentration and distribution in worms

Based on the results of the SEM-EDX test, the worm samples contained several elements as shown in Figure 4. The majority of them are organic except Al, Si and Ti. Aluminium (Al), Silicon (Si), and Titanium (Ti) are metals that are used by organisms.

the maximum recommended concentration of mercury is 0.001 mg/ up to 0.002 mg/L. has been contaminated with mercury. The International Association of Dragging Companies/Central Dreging Association (IACD/CEDA) divides the quality standards for mercury concentrations in sediments into 5 levels, namely the target level (0.3 ppm); limit level (0.5 ppm); test level (1.6 ppm); intervention level (10 ppm) and hazard level (15 ppm), while the results showed the concentration of mercury in the study locations ranged from 0.1413 ± 0.0067 mg/L to 0.2683 ± 0.0055 mg/L. This shows that the mercury in the research location is still below the target level so it is not too dangerous for the environment. As is the result of a study that has been carried out (Adam *et al.*, 2018) explains that sampling areas or stations close to pollutant sources are exposed to a higher level than areas far from pollutant sources.

The results of mercury measurements showed that the highest concentration was found in sediments. The amount of heavy metals contained in sediments indicates the level of contamination in water bodies. In

general, the content of heavy metals in sediments is higher than in water because heavy metals have the property of easily binding and settling on the bottom of the water and uniting with sediments (Zhang *et al.*, 2022). Heavy metals that enter the waters will experience precipitation, dilution and dispersion, then be absorbed by organisms that live in these waters (Peng *et al.*, 2018). Metal deposition occurs because the specific gravity of metals is higher than the specific gravity of water so the heavy metal content in the sediment becomes higher than in the water (Zeng *et al.*, 2022).

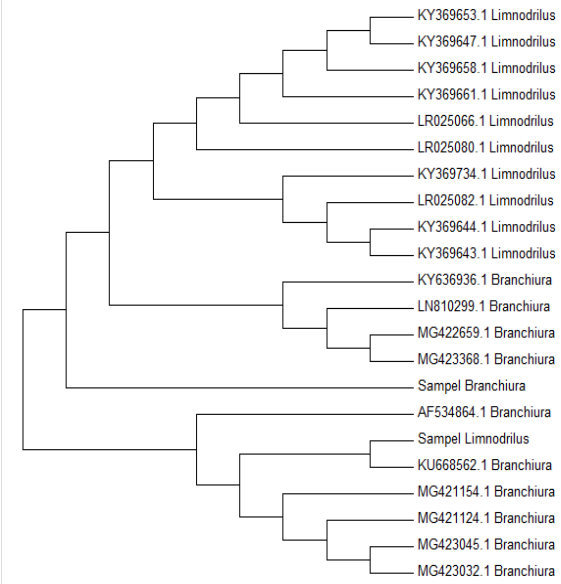
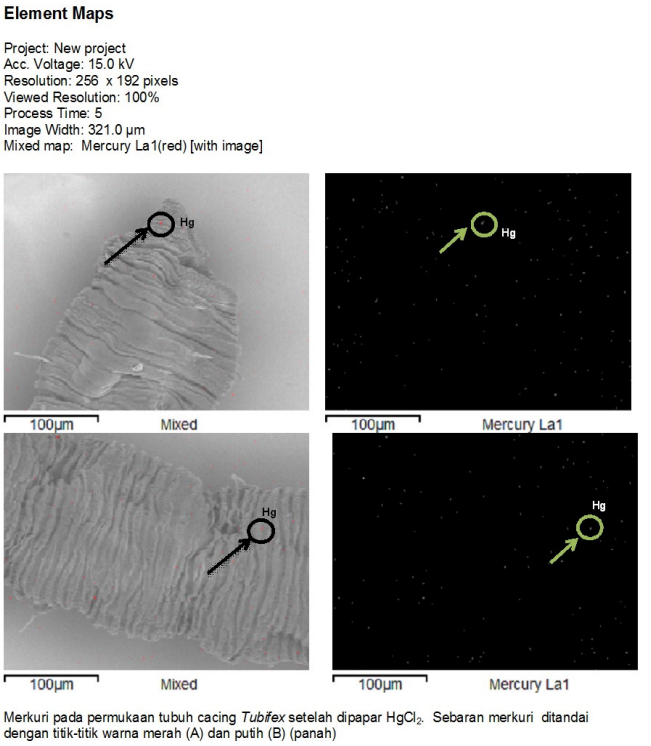


Figure 3. Reconstruction of the phylogenetic tree of Limnodrilus hoffmeisteri and Branchiura show erby worms based on COI.

Table 2. Results of morphological identification of worm specimens.

No. Specimen	Species	Family
1	*Limnodrilus hoffmeisteri*	Nadidae
2	*Branchiura sowerbyi*	Nadidae

The E-value of 0.0 indicates that the determination of its sequence was identical to the species level. Therefore, specimens of sample-1 and sample-2 were *Limnodrilus hoffmeisteris* and *Branchiura sowerbyi* because they have 99% and 86% similarities. Identification with a morphological approach for Oligochaete is difficult because there are still many aquatic species that are not clear and have not been detected morphologically (Anggraini *et al.*, 2022; Cheng *et al.*, 2019; Liu *et al.*, 2017). Several studies revealed that the mitochondrial COI gene is an effective barcode for the identification of aquatic and terrestrial species (Becker *et al.*, 2021; Folmer *et al.*, 1994; Geller *et al.*, 2013). COI barcodes have been assigned to several freshwater oligochaetes in China (Zhou *et al.*, 2021), Argentina (Erséus *et al.*, 2010), Korea (Kang *et al.*, 2017), America (Folmer *et al.*, 1994) and India (Naveed, 2012), but the taxonomic range and geographic origin of the specimens are relatively limited (Ali *et al.*, 2020; Ewald, 2006; Hikam *et al.*, 2021; Xu *et al.*, 2017) suggested a 10% COI divergence threshold for the separation between congeneric species in aquatic oligochaetes. Several studies also reported that the mitochondrial COI gene is effective in the identification of these organisms (Anggorowati *et al.*, 2019; Hebert *et al.*, 2003; Nneji *et al.*, 2020; Rodriguez and Reynoldson, 2011).



Merkuri pada permukaan tubuh cacing *Tubifex* setelah dipapar $HgCl_2$. Sebaran merkuri ditandai dengan titik-titik warna merah (A) dan putih (B) (panah)

Figure 4. Distribution of mercury in Oligochaeta after mercury exposure, the anterior and middle sections are marked with red and white dots (arrows). The image is the result of Mapping EDX.

They can also live in water conditions polluted by harmful organic materials and heavy metals (Mao *et al.*, 2016; Peng *et al.*, 2018), and are tolerant to hypoxic conditions (Bird and Ladle, 1981; Blakemore *et al.*, 2012; Christoffersen, 2012). *L. hoffmeisteri and B. sowerbyi* are found in Indonesian waters, including in Bedaai River, Deli Serdang Regency, North Sumatra. The results showed that *Limnodrillus* sp. is present in polluted waters (Klerks and Bartholomew, 1991), in the East Pontianak Canal (Setiawan *et al.*, 2015), and in the Musi River in Palembang (Zulkifli and Setiawan, 2012). The waters in Kejapanan, Pasuruan, East Java, contain household, industrial, and agricultural wastes, which indicates that they are contaminated with organic and inorganic materials including live heavy metals of several types of annelids, such as *L.*

hoffmeisteri. The organism is often used as a model in basic and applied sciences including phylogenetic studies due to its ability to resist limited conditions (Beauchamp *et al.,* 2001; Bird and Ladle, 1981; Blakemore *et al.,* 2012). It also mixes the material stored in sediments by digging, feeding, and breathing, hence, it is used as a biological indicator of organic pollution in environmental health (Rodriguez and Reynoldson, 2011; Zhang *et al.,* 2014). It is considered a single species dispersed in the sediments of various freshwater habitats (Liu *et al.,* 2017). Mercury released into the environment is often suspended or bound to organic compounds found in the soil or surrounding water (Lee *et al.,* 2018; Macirella and Brunelli, 2017; Shi *et al.,* 2010). Its organic form is obtained when it binds with carbon (Risjani *et al.,* 2022). All these forms occur naturally at low concentrations (ATSDR, 2022; Risjani *et al*., 2020).

Mercury accumulation in aquatic organisms is influenced by several factors including individual species, Hg source, physicochemical water quality, and pollutant levels (Abid Maktoof, 2020; Aly and Abouelfadl, 2020). Furthermore, the high dose used for exposure has a significant effect on its bioavailability and bioaccumulation in organisms, especially in freshwater (ATSDR, 2022; Kumar, 2012; Lee *et al.,* 2019; Shen *et al.,* 2001). The distribution of mercury in the worms exposed is presented in Figure 3. It spreads throughout the whole body of the worm, including the anterior, middle, and posterior parts. Mercury also spreads to the surface of the worm's body and is indicated by the presence of red dots. In Oligochaeta, it is absorbed through the layers of the skin during the process of gas exchange (respiration). The front part of the body is immersed in sediment, where the tail is left in the water body to increase the water

Table 3. Results of BLAST Specimen in GenBank NCBI (10 top).

Description	Max score	Total score	Query cover	E value	Identy	Accession
L. hoffmeistri isolate 1147_T17	1179	1179	92%	0.0	99%	LR025082.1
L. hoffmeistri isolate 1147_T17	1179	1179	92%	0.0	99%	LR025080.1
L. hoffmeistri isolate 1147_T17	1179	1179	92%	0.0	99%	LR025066.1
L. hoffmeistricomplex lineage VII isolate CNWQ42	1179	1179	92%	0.0	99%	KY369734.1
L. hoffmeistri complex lineage VII isolate CE22916	1179	1179	92%	0.0	99%	KY369661.1
L. hoffmeistri complex lineage VII isolate CE22911	1179	1179	92%	0.0	99%	KY369658.1
L. hoffmeistri complex lineage VII isolate CE22905	1179	1179	92%	0.0	99%	KY369653.1
L. hoffmeistri complex lineage VII isolate CE22898	1179	1179	92%	0.0	99%	KY369647.1
L. hoffmeistri complex lineage VII isolate 22894	1179	1179	92%	0.0	99%	KY369644.1
L. hoffmeistri complex lineage VII isolate 22893	1179	1179	92%	0.0	99%	KY369643.1
B. sowerbyi voucher S-4331	765	765	93%	0.0	87%	KU668562.1
B. sowerbyi voucher BI-OUG21000-H07	665	665	81%	0.0	86%	MG421154.1
B. sowerbyi voucher BI-OUG21865-F04	777	777	95%	0.0	86%	MG423368.1
B. sowerbyi voucher CE713	777	777	95%	0.0	86%	KY636936.1
B. sowerbyi isolate T5_163	777	777	95%	0.0	86%	LN810299.1
B. sowerbyi BRAsohr	776	776	92%	0.0	86%	AF534864.1
B. sowerbyi BIOUG21865-E01	774	774	95%	0.0	86%	MG422659.1
B. sowerbyi BIOUG21000-H05	680	680	84%	0.0	86%	MG422045.1
B. sowerbyi BIOUG21000-H04	680	680	84%	0.0	86%	MG423032.1
B. sowerbyi BIOUG21000-H03	680	673	84%	0.0	86%	MG421124.1

flow and accelerate gas exchange (Beauchamp *et al.,* 2001; Bird and Ladle, 1981; Giere, 1983; Peng *et al.,* 2018).

4. Conclusion

Based on the identification results, the worms found in the waters of Kejapanan Pasuruan, East Java, Indonesia include *Limnodrilus hoffmeisteri* (similarity indexes: 99%) and *Branchiura sowerbyi* (similarity indexes: 86%). No statement has been found that worms can be used as biomonitoring for mercury contamination. For this reason, the results of this study can provide a novelty that worms can be used as biomonitoring of water pollution using the barcode data.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author is as follow, IMW and MAA; collected the data, drafted the manuscript, and designed the figures. E; devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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