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Molecular identification of *Oncomelania hupensis lindoensis*, snail intermediate hosts of *Schistosoma japonicum* from Central Sulawesi, Indonesia

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Abstract. *Sutrisnawati, Ramadhan A, Trianto M. 2022. Molecular identification of* Oncomelania hupensis lindoensis, *snail intermediate hosts of* Schistosoma japonicum *from Central Sulawesi, Indonesia. Biodiversitas 23: 5989-5994.* Schistosomiasis is a zoonotic parasitic disease with *Oncomelania hupensis lindoensis*, intermediate snail hosts of *Schistosoma japonicum*. The spread of *O. hupensis lindoensis* snail habitat was found in the three areas of the Napu, Bada, and Lindu Highlands with an infection rate above 1%. *Oncomelania hupensis lindoensis* is primarily cryptic species that are morphologically difficult to identify and distinguish from other species. Consequently, it can be confused with the naming of species. One of the molecular approaches that can be used to identify the *Oncomelania* spp. quickly and accurately is DNA barcoding using the COI mitochondrial gene. However, the research on identifying *Oncomelania* spp. in Indonesia is still very limited. Therefore, this study aimed to identify six *Oncomelania* spp. from Napu and Lindu, Central Sulawesi using COI mitochondrial gene as a molecular marker for DNA barcoding. The method used in this study was a Polymerase Chain Reaction (PCR) method with universal primers, LCO-F and HCO-R. The data obtained were then analyzed using GeneStudio, DNASTAR, BLAST, Identification Engine, Mesquite, MEGAX, and BEAST. The analysis was conducted to obtain similarity, genetic distance and reconstruct a phylogenetic tree. The result revealed that all six samples of *Oncomelania* spp. collected from Napu and Lindu were identified in one species, namely *Oncomelania hupensis lindoensis*. This research is very important to be carried out regularly periodically so that it can be used as a basis for *Schistosomiasis* control program data and related sectors to eradicate snails effectively, efficiently, and on target.

Keywords: Central Sulawesi, COI, DNA barcoding, *Oncomelania* spp.

INTRODUCTION

Schistosomiasis in Indonesia was first discovered by Muller and Tesch in 1937, it is caused by *Schistosoma japonicum* with an intermediate host of *Oncomelania*. The snail was first discovered in the rice field, lake Lindu in 1971 by Carney et al. and was identified as *Oncomelania hupensis lindoensis* (Gunawan et al. 2014). In Indonesia, schistosomiasis in humans is only found in Central Sulawesi, especially in Napu, Lindu, and Bada. Central Sulawesi is the only province in Indonesia that is endemic to schistosomiasis. This disease was found in 2 of 11 regencies (Sigi and Poso) in Central Sulawesi (Maksud et al. 2014). According to the data based on the measurements by the Kato-Katz method, there were 19 cases of schistosomiasis, 12 of them were current cases in 2015 and 7 others were new cases. This indicates that the medical treatments to patients were not going well. Praziquantel is an effective drug for the treatment of schistosomiasis. However, the treatment of schistosomiasis in this area was not done properly, especially in terms of taking medication. Microscopic measurements (Kato-Katz method) were done routinely by the local Public Health Officer every year to detect the presence of the eggs of Schistosoma, but the worm eggs were not detected under the microscope when they were in few numbers (Kosala et al. 2015).

Schistosomiasis is one of the most important human parasitic diseases caused by trematode worms from the genus *Schistosoma* and the phylum *Platyhelminthes* (Yang et al. 2013). More than 240 million people in 78 countries are infected and about 280,000 deaths are related to schistosomiasis per year (Qiu et al. 2014; Zheng et al. 2014). In addition to causing major intravascular infection, this disease also has a significant socio-economic impact (Wang et al. 2016; Shi et al. 2017). There are three main species that cause schistosomiasis in humans, namely *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. *Schistosoma japonicum* worms are distributed in Indonesia, the People's Republic of China and the Philippines. *Schistosoma mansoni* is distributed in Africa, Middle East, South America, and West Indies (Barakat 2013), while *S. haematobium* has a similar distribution to *S. mansoni* but is absent in South America and the West Indies (Kosala et al. 2015). In Indonesia, schistosomiasis cases are only found in the highlands of Lindu, Napu, and Bada, Central Sulawesi province (Ajakaye et al. 2016).

The intermediate host, which is the vector of transmission of schistosomiasis in Central Sulawesi, is the snail *Oncomelania hupensis lindoensis* (Araujo et al. 2018). The presence of the *O. h. lindoensis* snail and its habitat has an important role in schistosomiasis transmission. This is related to the stage of asexual

development of *S. japonicum* in *O. hupensis lindoensis* snails. Apart from being important for public health, the *O. hupensis lindoensis* snail is also important in the study of the history of biogeography and evolutionary radiation from Schistosoma found in Indonesia and Asia.

Based on tracing in GenBank, no sequencing data from *O. hupensis lindoensis* is found in the highlands of Lindu and Napu, Central Sulawesi, so it is not certain the position of the clade of this species among other *Oncomelania* clades found in Asia. As a comparison, in mainland China, based on the suitability of allozyme and mitochondrial Cytochrome Oxidase Subunit I (COI) gene sequences, there are three different clades of *Oncomelania hupensis* which are geographically separated, namely *O. hupensis robertsoni* (softshell, without varix), *O. hupensis tangi* (softshell, strong varix), and *O. hupensis hupensis* (smooth or striped shell, strong varix) (Angelo et al. 2018). In the Philippines, there is one clade, namely the snail *Oncomelania hupensis quadrasi* (Gastropoda: Caenogastropoda: Littorinimorpha: Pomatiopsidae), is a subspecies of *Oncomelania hupensis*, which also acts as an intermediate host for *Schistosoma japonicum* (Calata et al. 2019). Two subspecies are found in Taiwan and one subspecies is found in Japan (Chua et al. 2017). With the presence of molecular data, most subspecies designations based on geographic distribution data can be improved.

This study aims to study genomic differences based on the sequence of COI and the evolution of the subspecies of the *O. h. lindoensis* strain in two different locations, namely the Napu and Lindu regions of Central Sulawesi province. The use of the COI gene has been shown to provide a stable differentiation analysis (Inobaya et al. 2014; Panprommin et al. 2019). In addition, because the mitochondrial COI gene is inherited maternally without recombination, each distinct sequence is a haplotype. The phylogeny of the mtDNA gene tree reflects the past of a species and adds a historical perspective to the population structure. Therefore, this study aimed to identify *Oncomelania hupensis lindoensis* from Central Sulawesi, Indonesia, using the CO1 mitochondrial gene as a DNA barcoding marker.

MATERIALS AND METHODS

Sample collection

A total of six *Oncomelania hupensis lindoensis* (code OHL) were collected from Napu and Lindu in Central Sulawesi in August 2020. These stations were chosen by purposive sampling to represent the natural barriers. The sampling of *O. h. lindoensis* was performed using a hand and the sample was cleaned and documented. Each sample was then placed in a bottle, placed in a coolbox, and transported to the Laboratory of Biology, Brawijaya University. Sample to be preserved with 95% ethanol and stored at -20^{o}C until used in the following process.

DNA extraction, amplification, electrophoresis, and sequencing

The total genomic DNA was extracted from part of the tissue muscle from *O. h. lindoensis*. DNA isolation was carried out using the gSYNC^{TM} DNA Extraction Kit (Genaid GS300). The DNA Isolation steps follow its manufacturer's protocol for species barcoding. DNA Amplification was performed with PCR Thermocycler using two CO1 primers. We amplified and sequenced the mitochondrial DNA, specifically the CO1 gene using following forward primer OhqCOX1_22-41aF GCA TGT GAG CGG GGC TAG TA and reverse primer OhqCOX1_189-209aR AAG CGG AAC CAA TCA GTT GCC. PCR Master Mix, (2x) Dream Taq Green PCR Master Mix. The amplifications were carried out under the following condition: PCR conditions were the following: 95^{o}C for initial denaturation for 30s, 95^{o}C for denaturation for 5s, and 60^{o}C for annealing for 30s for 50 cycles. The electrophoresis of PCR products was run on a 1% agarose gel stained with Florosafe (Bioline) and buffered with Tris-acetate Ethylenediaminetetraacetic Acid (EDTA) (TAE) at 50 volts for 20 minutes. Visualization was conducted under UV light. All amplification samples were sent to First Base Sdn Bhd (Malaysia) by P.T. Genetika Science (Jakarta) for purification and sequencing in both forward and reversed directions using the Big Dye Terminator (Applied Biosystems) and the ABI 3730xl Genetic Analyzer (Applied Biosystems).

Data analysis

Information obtained from DNA sequencing results was altered in the GeneStudio program and approved with SeqMan and EditSeq on the DNASTAR program (DNASTAR Inc. Madison, USA). Sequencing responses were made on every individual utilizing both forward and switch groundworks. Chromatograms were assessed physically to actually look at vague bases and stop codons. The *O. hupensis lindoensis* CO1 arrangements were then adjusted utilizing Opal on Mesquite v.351 programs and ClustalW on the MEGAX program. The structure of the CO1 nucleotides was determined utilizing the MEGAX program. Genetic distance was dissected utilizing the MEGAX program with the Kimura-2 Parameter (K2P) model and summed up in a Neighbor-Joining (NJ) tree, which is the standard procedure utilized in barcoding studies with bootstrap 1000. The reproduction of the phylogeny tree was examined utilizing the Neighbor-Joining and Maximum Likelihood strategies with 1000 bootstraps utilizing the MEGAX program and Bayesian Inference utilizing the BEAST program (Suchard et al. 2018). The Bayesian Information Criterion (BIC) executed in jModelTest 2.1.10 (Darriba et al. 2012) was utilized to decide the best fit transformative model. This examination's most ideal grouping replacement model is HKY with invariant destinations (HKY+I) on the Bayesian Information Criterion (BIC). The Markov Chain Monte Carlo (MCMC) was run for 106 cycles ages to appraise the back probabilities conveyance with an inspecting recurrence set to each 1000. The agreement trees were envisioned in FigTree 1.4.4 (Rambaut 2019).

RESULTS AND DISCUSSION

Morphological characteristics of *Oncomelania hupensis lindoensis*

The type of snail that is the reservoir of schistosomiasis is *O. hupensis lindoensis* with the morphological shape shown in Figure 1. The results showed that the morphological shape of the snail *O. hupensis lindoensis* had a fairly small size with a length of 3-5 mm with a shell-shaped cone and slightly blackish brown, generally found on grassroots or attached to tree branches, is amphibious so it cannot live in areas waterlogged or in dry areas. The same thing was also reported by the snail *O. hupensis lindoensis* has a conical shell, smooth surface yellowish brown and slightly clear when cleaned with a circumference of 6.5-7.5 mm and a length 5.2*0.6 mm with an open mandible of, curved outer lip and protruding inner lip below the base of the shell, the operculum contains and slightly firm. Glands around the eyes called eyebrows are light yellow to bright yellow. When compared with other types of snails, such as *O. hupensis quadrasi* has a bright yellow color, and *O. hupensis chiui* is white. Snails are amphibious, meaning this snail can live in humid areas, not too much water and not too dry, if the snail habitat is dried or always inundated with water, the snail will die (Hadidjaja 1982).

In addition to human factors, animals, distribution, and infection rate of the *O. hupensis lindoensis* can also be affected by conditions natural such as floods, landslides or focus is buried by materials that cause the snail to die by drowning more than 30-50 cm from the ground. So that in controlling focus it is better implemented in an integrated manner by all relevant cross-sectors to reduce the number of foci and prevalence in humans and animals (Zheng et al. 2014). Early detection Schistosomiasis at a low prevalence rate using a method other than the survey stool will be more effective. Schistosomiasis control in an integrated manner by combining treatment and control of snail focus is the best strategy for eliminating schistosomiasis. *Oncomelania* snail habitat also influenced by temperature conditions, soil type, the type of vegetation, as well as the adequacy of water supports the development of snails and also cercariae movement. intermediate conch schistosomiasis found in focal areas schistosomiasis at normal pH (Zhang and Hanner 2012). Research in the schistosomiasis endemic area of Napu, indicates that snails are found in areas with a pH between 5.5-7.26 Research in endemic areas of Bada found that snails can live in an environment with a pH 6-8.15. Other results showed that schistosomiasis education among school children could improve knowledge, attitudes, and risk behavior on the control of schistosomiasis in among children in endemic areas can predict cases of schistosomiasis, effect and various future control measures required a mathematical model of transmission *Schistosoma.*

PCR amplification and sequence identification

The result showed that the enhancement of the CO1 mitochondrial quality of six *O. hupensis lindoensis* from Napu and Lindu created a piece length of around 565 bp (Figure 2). The agreement arrangement results from the chromatogram altering process were between 570-595 bp. As indicated by the outcomes, each of the six samples gathered from Napu and Lindu was distinguished in one sort, specifically *Oncomelania* and comprised of one animal type *O. hupensis lindoensis*. The similitude of the examples contrasted with the information in GenBank was 86.10-90.52% (Table 1).

Figure 1. Morphological characteristics of *Oncomelania hupensis lindoensis*

Table 1. Species identification based on GenBank database using BLAST and BOLD identification

Sample code	Identified species from GenBank / BOLD	Similarity (%)	Query cover (%)	Accession number	References
OHL-001	*O. hupensis hupensis*	86.10	86	KR002674.1	Direct submission
OHL-002	*O. hupensis hupensis*	86.21	87	KR002674.1	Direct submission
OHL-003	*O. hupensis hupensis*	86.15	86	KR002674.1	Direct submission
OHL-004	*O. hupensis hupensis*	90.36	90	KR002674.1	Direct submission
OHL-005	*O. hupensis hupensis*	90.30	90	KR002674.1	Direct submission
OHL-006	*O. hupensis hupensis*	90.52	91	KR002674.1	Direct submission

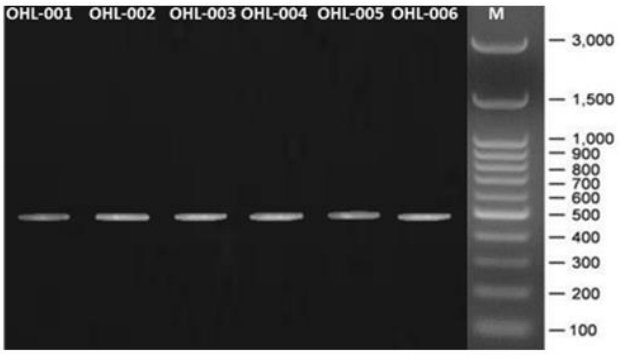

Figure 2. PCR amplification result of CO1 mitochondrial gene of *O. hupensis lindoensis* from Napu and Lindu migrated in 1% agarose electrophoresis. OHL-001 to OHL-006 005 is the sample code. M is marker visualized from DNA ladder 1 kb (GENEAID)

Sequence alignment

The alignment of six *Oncomelania* spp. arrangement tests from Napu and Lindu yielded a spotless grouping (a succession that came about after the arrangement and cutting course) of 565 bp. These clean CO1 quality successions of every species were then utilized for intraspecies investigation. The CO1 groupings arrangement of *Oncomelania* spp. from Napu, Lindu, and other Indonesian districts kept in GenBank brought about a section length of 525 bp. This outcome was then exposed to the intraspecies investigation (nucleotide creation and hereditary distance). For phylogenetic tree examination, the clean CO1 groupings (525 bp) of 16 examples addressed 1 genus and 4 species, in particular *O. minima, O. quadrasi, O. nosophora,* and *O. hupensis* from Napu, Lindu, and other Indonesian districts recorded on GenBank were utilized. Two CO1 arrangement of *Conus borgesi* (Accession number: KJ550572.1 and AY588160.1) was utilized as an outgroup (Table 2).

Phylogenetic analysis and genetic distance

The tree reproduction results yielded indistinguishable tree geographies (Figure 3). The consequences of the jModelTes2 examination uncovered that the ideal arrangement replacement model is HKY with invariant lo cales (HKY+I) on the Bayesian Information Criterion (BIC). The phylogenetic tree remaking of *Oncomelania* spp. from Napu, Lindu, and *Oncomelania* spp. from different areas in China and Japan recorded on GenBank framed three unique clades. The development of these 8 clades was upheld by a bootstrap worth of 87-100% in the Neighbor-Joining and Maximum Likelihood techniques. Also, the back likelihood esteem is 1.00 in Bayesian Inference. Bootstrap and back likelihood results showed that the development of these clades was strong and inflexible.

Our phylogenetic investigation in view of effectively enhanced CO1 qualities likewise showed *O. hupensis lindoensis* from Napu and Lindu, Central Sulawesi is settled inside a similar clade of another *O. hupensis hupensis* from China (Accession number: KR002674.1) (Figure 3) with an all-around upheld bootstrap and on the hubs. The bootstrap and back likelihood esteem demonstrate the consistency of information appearing in rates. Low qualities imply that the arrangement gives alternate tree geography on each test. The considered all-around upheld bootstrap incentive for the greatest probability examination is at >75%. In any case, in light of the perceptions of our arrangement, it was shown that there were varieties in a few locales, at this point, were deficient in separating the examples. As per Kress and Erickson (2012), the CO1 quality can recognize the taxa up to species level on account of the exceptionally rationed varieties of the locale. These varieties of nucleotides can be utilized by a person that recognizes the species. The high variety of nucleotides among a grouping of tests is a successful device for distinguishing *Oncomelania* spp. (Zhang and Hanner 2012).

In view of our genetic distance investigations of six examples (OHL-001 to OHL-006) of CO1 mtDNA qualities of *O. hupensis lindoensis* from Napu and Lindu, Central Sulawesi, and four examples *Oncomelania* spp. other China and Japan recorded on GenBank, it was shown that our examples have a high hereditary distance (at the worth of 4%) to the *O. hupensis hupensis* from China (Accession number: KR002674.1) (Table 3). It was viewed that there was hereditary disengagement happen, and hereditary design is all around kept up with to shape the single species (Braich and Akhter 2015). As per Zemlak et al. (2009), the limit for intraspecies hereditary distance in species is 3.5%. Assuming that it surpasses 3.5%, it is viewed as an alternate animal group. In view of the rule from Zemlak above, *O. hupensis lindoensis* from Napu, Lindu, and *O. hupensis hupensis* from China kept in GenBank were yet delegated no similar species.

Table 2. The CO1 sequences alignment of *Oncomelania* spp. from Napu and Lindu and other Indonesian regions recorded in GenBank

Samples	Locality	Accession number	References
O. hupensis lindoensis	Lindu, Central Sulawesi	-	This study
O. hupensis lindoensis	Lindu, Central Sulawesi	-	This study
O. hupensis lindoensis	Lindu, Central Sulawesi	-	This study
O. hupensis lindoensis	Napu, Central Sulawesi	-	This study
O. hupensis lindoensis	Napu, Central Sulawesi	-	This study
O. hupensis lindoensis	Napu, Central Sulawesi	-	This study
O. hupensis hupensis	China	KR002674.1	Direct submission
O. hupensis robertsoni	China	KR002675.1	Attwood et al. (2015)
O. hupensis robertsoni	China	AF531547.1	Attwood et al. (2003)
O. hupensis nosophira	Japan	KR002673.1	Attwood et al. (2015)
Conus borgesi	Cape Verde	KJ550572.1	Direct submission
Conus borgesi	Cape Verde	AY588160.1	Direct submission

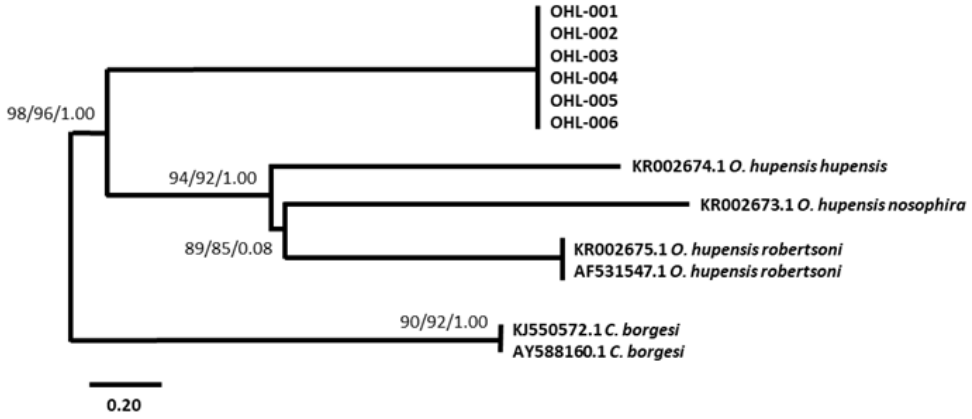

Figure 3. Phylogenetic tree reconstruction based on Neighbor-Joining (NJ), Maximum-Likelihood (ML), Bayesian Inference (BI) topology *Oncomelania* spp. and outgroup based on CO1 gene sequence (525 bp). The node represented the number bootstrap (NJ and ML) and Bayesian Posterior Probability (Bayesian Inference)

Table 3. Genetic distance of *Oncomelania hupensis lindoensis* from Napu and Lindu, Central Sulawesi and additional sample *Oncomelania* spp. From the GenBank

	OHL-001	OHL-002	OHL-003	OHL-004	OHL-005	OHL-006	KR002674.1	KR002673.1	KR002675.1	AF531547.1	KJ550572.1	AY588160.1
OHL-001	0.000											
OHL-002	0.000	0.000										
OHL-003	0.000	0.000	0.000									
OHL-004	0.000	0.000	0.000	0.000								
OHL-005	0.000	0.000	0.000	0.000	0.000							
OHL-006	0.000	0.000	0.000	0.000	0.000	0.000						
KR002674.1	0.040	0.040	0.040	0.040	0.040	0.040	0.000					
KR002673.1	0.047	0.046	0.045	0.042	0.047	0.045	0.062	0.000				
KR002675.1	0.054	0.054	0.053	0.055	0.054	0.054	0.077	0.065	0.000			
AF531547.1	0.062	0.064	0.153	0.154	0.152	0.154	0.169	0.159	0.129	0.000		
KJ550572.1	0.215	0.218	0.215	0.214	0.218	0.215	0.227	0.219	0.226	0.216	0.000	
AY588160.1	0.223	0.222	0.224	0.226	0.222	0.224	0.231	0.228	0.232	0.234	0.236	0.000

In conclusion, our molecular examination showed that the phylogenetic investigation additionally settled that the examples that were dissected are, without a doubt, types of *O. hupensis lindoensis* from Napu and Lindu, Central Sulawesi, Indonesia. At long last, we expect there are future investigations that focus on all-around settled phylogenies and, along these lines, eliminate extra clamor from the examination. The exploration information is supposed to add to the conservation and use of one of Indonesia's significant biodiversity assets.

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