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Phylogenetic positions of the rotifer *Brachionus plicatilis* species complex from Indonesian waters

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ABSTRACT

The *Brachionus plicatilis* species complex comprises a group of rotifers commonly found in marine and brackish water environments worldwide. This group has garnered substantial attention due to its ecological significance and its utility in aquaculture research. Notably, the *B. plicatilis* sp. complex is renowned for its remarkable genetic diversity, which has been extensively investigated through molecular and taxonomic studies. Various genetic markers, including ribosomal DNA and mitochondrial DNA, have been employed to identify several distinct genetic lineages within the *B. plicatilis* sp. complex. Understanding the taxonomy and phylogenetic relationships within the *B. plicatilis* sp. complex holds significant implications for its application in aquaculture and ecotoxicology research. In this study, we conducted an analysis of the mitochondrial DNA cytochrome c oxidase subunit I (COI) gene using a dataset of 305 sequences, including four Indonesian rotifers belonging to the *B. plicatilis* sp. complex. These rotifers were obtained from Sulawesi Island (MNBO and MNSL), Bali Island (AY785226), and Sumatra Island (LMPG). Our primary objective was to determine their phylogenetic positions relative to molecular data available in the GenBank database. We calculated genetic distances among these rotifers using the Tamura Nei model. The results of Maximum Likelihood tree analyses revealed distinct clustering patterns among the Indonesian rotifers. Those originating from Sulawesi Island (MNBO and MNSL) and Bali Island (AY785226) formed the SS1 group (*B. ratundiformis*), whereas those from Sumatra Island (LMPG) comprised the SM2 group (*B. koreanus*). This study underscores the importance of molecular phylogenetic analysis in advancing our comprehension of the diversity and evolutionary relationships within the *B. plicatilis* sp. Complex.

Introduction

The rotifers *Brachionus plicatilis* species complex plays a crucial role in aquaculture by serving as the primary live food source for various marine organisms, including larval fish and crustaceans (Hagiwara *et al*., 2017; Ogello *et al*., 2020; Hansen and Møller, 2021; Kagali *et al*., 2022). These rotifers offer several advantages over alternative live food sources, such us their small size facilitates easy consumption by larvae (Kotani., 2017; Snell *et al*., 2019), while their high nutritional value enhances the survival and growth rates of the larvae (Hemre, 2016; Eryalçın, 2019; Turgay *et al*., 2020). Additionally, the ease of cultivation ensures a consistent supply of live feed, thereby reducing overall aquaculture production costs (Hagiwara *et al*., 2017; Ogello *et al*., 2018). These *B. plicatilis* sp. complex also have remarkable ability to accumulate pollutants and contaminants from their environment, making them valuable tools for assessing the impact of environmental stressors on aquatic ecosystems (Piscia *et al*., 2016; Ma *et al*., 2022; Wang *et al*., 2023). Furthermore, they serve as model organisms for investigating various genetic processes, such as sex determination, meiosis, gene expression, and others (Hanson *et al*., 2013; Gribble and Welch 2017; Seudre *et al*., 2020; Han *et al*., 2023). Despite their numerous advantages, the *B. plicatilis* sp. complex presents certain challenges, including the potential for genetic variation within and between genetic lineages. These rotifers exhibit a high level of

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cryptic diversity, where morphological distinctions may be insufficient to differentiate among monogonont rotifer species (Papakostas *et al.*, 2016; Gabaldón *et al*, 2017).

Indonesia, with its extensive estuary regions, serves as a habitat for the rotifer *B. plicatilis* sp. complex. Hatcheries in Indonesia also employ the *B. plicatilis* sp. complex as a natural feed for rearing various marine fish larvae. Nevertheless, research regarding the biodiversity of rotifers and their phylogenetic positions, particularly members of the *B. plicatilis* sp. complex in Indonesia, remains significantly limited. In this study, phylogenetic data of the *B. plicatilis* sp. complex originating from Indonesia have been constructed through a molecular approach to determine their phylogenetic positions within the classification of this complex species.

Materials and Methods

Location and time of research

In this study, we selected a total of 305 COI gene sequences from members of the rotifer *B. plicatilis* sp. complex available in GenBank as of September 2023. These sequences include 3 COI gene sequences from the rotifer *B. plicatilis* sp. complex that is ecologically isolated in estuarine waters of Bali Island (AY785226), Sumatra Island (LMPG), and Sulawesi Island (MNBO) (Yoshinaga *et al.*, 2004). Additionally, this study utilizes the COI gene sequence of the rotifer *B. plicatilis* sp. complex isolated from the estuarine waters of South Minahasa, Sulawesi Island, Indonesia (MNSL) (Figure 1).

The rotifer *B. plicatilis* sp. complex (MNSL), isolated from the estuarine waters of South Minahasa, Sulawesi Island, Indonesia, has been monocultured for several generations to obtain a sufficient quantity of monospecies rotifer samples for DNA analysis, and subsequently sieved using a 40 µm mesh size plankton net. The Spin-Column Protocol was employed to extract genomic DNA, following the procedures outlined in the Qiagen DNeasy Blood & Tissue kit. The COI gene was amplified using a universal primer consisting of the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primer pairs. The amplified COI gene was subjected to sequencing services provided by FirstBase Co. in Selangor, Malaysia. Sequencing was carried out bidirectionally using the BigDye® Terminator v.3.1 Cycles Sequencing Kit (Applied Biosystems, United States). Subsequently, the generated sequences were read using an ABI PRISM® 377 automatic DNA sequencer. Following this initial analysis, the sequences underwent trimming, assembly, and manual editing using Geneious Prime version 2020 (http://www.geneious.com).

Data analysis

The MEGA X software (Kumar *et al.*, 2018) was utilized for performing phylogenetic analysis and the sequences were aligned using Clustal W. The most appropriate evolutionary model was determined and the best model was identified as K2+1. The Maximum Likelihood (ML) tree method was performed with MEGA X and was applied to construct the phylogenetic tree. The confidence and robustness of the nodes in the ML analysis were evaluated by conducting 100 bootstrap replications. Tamura-Nei model was used for substitutional model.

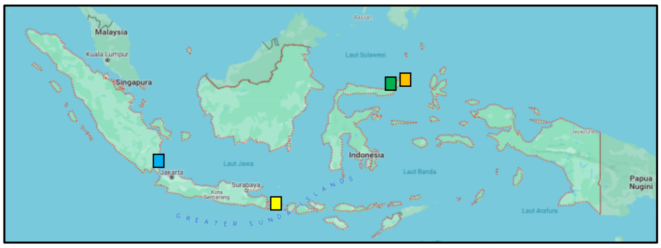

Figure 1. Map indicating the origin locations of rotifers *B. plicatilis* sp complex from Indonesia: Bali Island (yellow box), Sumatra Island-LMPG (blue box), Sulawesi Island MNBO (orange box), and MNSL (green box).

Results

In this analysis, a dataset comprising a total of 305 COI gene sequences from the *rotifer B. plicatilis* sp. complex was employed. This dataset included COI gene sequences from *B. plicatilis* sp. complex specimens originated from Bali Island (AY785226), Sumatera Island (LMPG), and Sulawesi Island (MNBO). Additionally, the COI gene sequences obtained from rotifers collected in South Minahasa of the Sulawesi Island (MNSL) during this study were integrated into the dataset. It is noteworthy to mention that this represents the sole available COI gene sequence data for the rotifer *B. plicatilis* sp. complex sourced from Indonesian waters. The alignment of these COI gene sequences resulted in the formation of a consensus dataset encompassing 661 nucleotide bases, featuring 337 conserved nucleotide sites. Notably, COI gene sequences of the *B. plicatilis* sp. complex type "L" were excluded from this analysis due to the absence of this particular rotifer type in Indonesian estuaries.

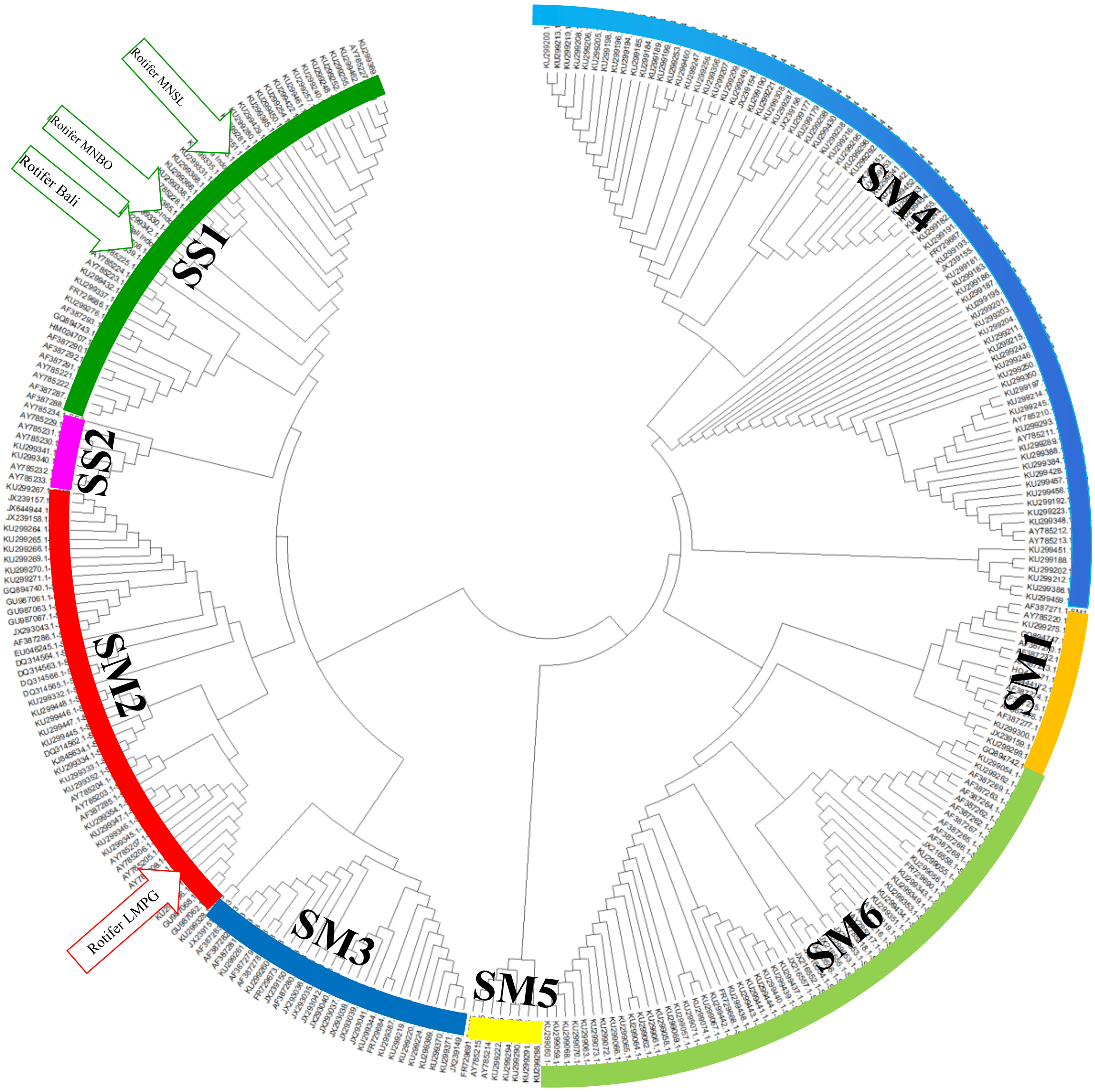

Figure 2. Molecular phylogenetic relationship of the rotifer *B. plicatilis* sp. complex from Indonesian waters based on the Cytochrome Oxidase Subunit 1 (COI) gene. The phylogenetic tree was constructed using the Maximum Likelihood (ML) tree method. The clades are represented by initials, i.e., SM1 - SM6, SS2, and clade SS1, while the rotifers *B. plicatilis* sp. complex from Indonesian waters were indicated by arrows pointing to the respective clades.

Sequences were aligned using Clustal W and the aligned dataset was trimmed at the ends for a total length of 661 nucleotide positions. The phylogenetic analysis outcomes demonstrate the existence of 8 separate clades within both types of the *B. plicatilis* sp. complex (Type SS and SM). Among these, the SS type encompasses 2 clades (SS1 and SS2), while the SM type encompasses 6 clades (Figure 2). The rotifer *B. plicatilis* sp. complex, originating from different regions of Indonesia, including Bali Island (AY785226), Sumatera Island (LMPG), and Sulawesi Island (MNBO and MNSL), exhibited distinct clustering patterns. Specifically, those from Bali Island (AY785226) and Sulawesi Island (MNBO and MNSL) were grouped within the SS1 clade (*B. rotundiformis*), whereas those originating from Sumatera Island were categorized into the SM2 clade (*B. koreanus*).

Discussion

The *B. plicatilis* sp. complex is one of the species within the phylum Rotifera that has garnered

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significant attention due to its various benefits and uniqueness (Hagiwara and Marcial, 2019; Walczyńska and Serra, 2022; Jezkova *et al.*, 2022; Walczyńska *et al.*, 2023). In aquaculture, this rotifer species is utilized as the primary natural feed for rearing various species of fish and marine invertebrate larvae (Imnetai *et al.*, 2019; Waqalevu *et al.*, 2019; Dhert *et al.*, 2020). The global hatchery industry still relies heavily on and continues to depend on the *B. pilicatilis* sp. complex as the primary nutritional source in their larvae production processes (Khoa *et al.*, 2021; Polumpung *et al.*, 2022). Advanced aquaculture technology has been applied to this type of rotifer to support the improvement of both quantity and quality in larval production (Waqalevu *et al.*, 2019; Dhert *et al.*, 2020). In this regard, a high-density rotifer cultivation technology has been developed utilizing various feed sources, enabling the rapid production of sufficient rotifer biomass in larval rearing (Polumpung *et al.*, 2022). Nutrient enrichment technology for rotifers has also become feasible, allowing for the specific transfer of nutrients to the target larvae in aquaculture (Dhert *et al.*, 2020; Khoa *et al.*, 2021). The hatchery industry in Indonesia is highly dependent on the availability of the *B. plicatilis* sp. complex rotifer supply. Interestingly, the available stock of *B. plicatilis* rotifers in Indonesian hatcheries generally originates from strains that are not native to Indonesian waters (Anita and Dewi., 2020; Dadiono *et al.*, 2021). However, Indonesia is a country with vast estuarine areas (Arifanti *et al.*, 2022), which serve as habitats for various species of zooplankton, including the *B. plicatilis* sp. complex. It is conceivable that within these estuarine regions, there may be a suitable complex of *B. plicatilis* species for aquaculture requirements, or even specimens with distinctive traits that could serve as valuable research model organisms. So far, there is limited information available regarding the biodiversity, especially from a molecular perspective, of the *B. plicatilis* sp. complex in Indonesia.

Initially, aquaculturists categorized the *B. plicatilis* sp. complex into two distinct rotifer types based on body size: a group with a large body size (L-type) and a group with a small body size (S-type). Subsequently, Serra and Fontaneto (2017) reported that there was a rotifer type with a medium body size, referred to as the SM-type (or *B. ibericus*). Further, Mills *et al* (2017) by using sequences of COI and ITS1 for 1273 isolates in the GenBank database, reported that there are at least 15 different species within this morphotype, with the L-type consisted of four different species, L1-L4 ("L1" (*B. plicatilis* s.s.), "L2" (*B. manjavacas*), "L3" (*B. asplanchnoidis*), and "L4" (*B. paranguensis*), the SM-type consisted of nine different species, SM1-SM9 (SM1 (*B. ibericus*), SM2 (*B. koreanus*), and SM3-9 (unnamed)), and the SS-type comprising two different species, SS1-SS2 ("SS1" (*B. rotundiformis*) and "SS2" (unnamed). The genetic perspective regarding the phylogenetic placement of the *B. plicatilis* sp. complex originated Indonesia remains uncertain, even though these rotifers are typically small in size. Nevertheless, there are limitations associated with exclusively depending on morphological characteristics for species differentiation. In this research, we conducted an examination of the phylogenetic placement of the *B. plicatilis* sp. complex from Indonesia by focusing on specific COI gene sequences from two rotifer types, namely SS and SM.

Table 1. Lokasi asal, type dan nama latin dari rotifer *B. plicatilis* sp. complex

Location	***B. plicatilis* sp. complex**	
	clade	**name**
Bali Island (AY785226)	SS1	*B. rotundiformis*
Sumatra Island (LMPG)	SM2	*B. koreanus*
Sulawesi Island (MNBO)	SS1	*B. rotundiformis*
Sulawesi Island (MNSL)	SS1	*B. rotundiformis*

Rotifer type SS is known to possess a smaller body size than type SM and is typically associated with tropical waters found in regions like Indonesia, Malaysia, Thailand, and other tropical areas. In our study, rotifers from the *B. plicatilis* sp. complex collected in Indonesian waters, specifically from Bali Island and North Sulawesi (MNBO and MNSL), were classified as rotifer type SS and formed a cluster within clade SS1. This cluster was previously recognized as the species *Brachionus rotundiformis*. Rotifer type SS, which falls into this category, is extensively utilized in the aquaculture of various fish larvae and marine invertebrates. Initially, *B. rotundiformis* was categorized into two distinct strains based on size, SS (super small) and S (small). However, research conducted by Mills *et al.*, (2017) reclassified *B. rotundiformis* as rotifer type SS within clade SS1. Meanwhile, rotifers from Lampung (LMPG) were classified as rotifer type SM and were placed in cluster SM2 under the name *Brachionus koreanus* (Mills *et al.*) (Table 1). In a previous study by Yoshinaga *et al.* (2004), this rotifer type had been grouped together with *Brachionus ibericus*, which later became known as cluster SM1 (Mills *et al.*, 2017)

Conclusion

The phylogenetic analysis, which utilized the COI gene of four members of the *B. plicatilis* sp. complex found in Indonesian waters, revealed that the *B. plicatilis* sp. complex from Bali Island (AY785226) and Sulawesi Island (MNBO and MNSL) were classified within the SS1 clade (*B. rotundiformis*), whereas the one from Sumatera Island (LMPG) waters was classified within the SM2 clade (*B. koreanus*).

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