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Barcoding of ornamental freshwater shrimp, *Neocaridina denticulata* (De Haan, 1844) from the aquatic ornamental market in Purbalingga, Central Java, Indonesia

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Abstract. *Kusmintarsih ES, Mahmoud HHA, Sastranegara MH, Syakuri H, Nuryanto A, Ambarningrum TB. 2023. Barcoding of ornamental freshwater shrimp,* Neocaridina denticulata *(De Haan, 1844) from the aquatic ornamental market in Purbalingga, Central Java, Indonesia. Biodiversitas 24: 3766-3773.* Indonesia is one of the world's leading aquatic ornamental shrimps producers and exports. One of the aquatic ornament shrimp species is a *Neocaridina denticulate* (De Haan, 1844). There is a wide range of colors including red, bright red, yellow, orange, green, blue, violet, black, and color combinations. It is a landlocked Atydiae family species comprising 31 species and subspecies. Due to taxonomic difficulties, this species is continually under revision, and the validation of a few species is currently questionable and uncertain for the relationships among the various colors of *N. denticulata*. The Cytochrome Oxidase gene I (CO1) gene sequences of mitochondrial DNA were used for species DNA barcoding studies. This study aimed to validate the taxonomic status of different colors of *N. denticulata* species using the mitochondrial DNA or mtDNA Cytochrome oxidase (CO1) gene sequences. Neighbour Joining trees were constructed based on 545 bp of CO1 gene from white, black, red, yellow, blue colors, and their combination. Several sequences were derived from GenBank for relevant species from different countries and the outgroup. The result showed that all samples are homolog, even to *N. denticulata* from China NC_023823.1.

Keywords: Barcoding, CO1 gene, Indonesia, ornamental shrimp, sequences

INTRODUCTION

The shrimps of *Neocaridina* species (De Haan, 1844) discovered by Kubo, in 1938, are a genus of landlocked species of the Atydiae family that comprises 31 species and subspecies (De Grave and Fransen 2011; Shih et al. 2017). Genus *Neocaridina* originated from East and South Asia (Yixiong 1996; Hanan et al. 2020) and due to taxonomic difficulties, this genus is continually under revision, and the validation of a few species is currently questionable (De Grave and Fransen 2011; Klotz et al. 2013). Kemp 1918 reported *Neocaridina denticulata* is one of the species that inhabit the freshwater of different Southeast Asian countries, Hawaii and Japan (Klotz et al. 2013). These species are exported to Europe and Singapore through international trade (Nur and Christianus 2013; Pantaleão et al. 2015; Patoka et al. 2016; Jabłońska et al. 2018). *N. denticulata* shrimp can be differentiated based on colors, such as bright red, yellow, blue, and color combinations ex. dark red, bright red, black blue etc. (Figures 1).

Besides consisting of different colors species of *Neocaridina* also include several species such as *N. denticulata, N. dividi, N. palmata, N. saccam, N. ketagalan, N. ikiensis, N. spinosa, N. aff. koreana,* and *N. aff. fukiensis. N. hetropoda,* and *N. zeylanica,* etc (Klotz et al. 2013). Most of the relationships between color and species are not highly biologically supported, but *N. dividi* and *N. denticulata* are sister species with small interspecific distances that form a clade, with *N. koreana* and *N. palmata.*

Although *N. ikiensis* is sister to *N. aff. fukiensis* (Shih and Cai 2017) the taxonomy of this species remains partly contradictory and unclear. In the case of taxonomic similarities between species, correct species identification is very critical. Furthermore, conventional taxonomy does not differentiate against certain species due to many factors. The study of *N. denticulata* is important because it plays a role in the economy and trade between countries. However, a recent study has not even presented the identification of *N. denticulata* growing in aquariums (Mitsugi and Suzuki 2018; Barmats et al. 2019).

In Purbalingga, one of the traditional fish markets, by the domestic farmer sells aquarium freshwater ornamental shrimps, including the genus *Neocaridina*. In this market, there are various *Neocaridina* species raised in different aquariums and ornamental fish are kept in both aquariums and cement tanks. The species of *Neocaridina* cultivated in this market include *N. denticulata*, which is found in various colors, including red, blue, yellow, and transparent.

Figure 1. A. *Neocaridina denticula* yellow color (blue arrow), red-white color (black arrow). B. Blue color (yellow arrow). C. White color (red arrow)

There is little detailed information available concerning the taxonomy, morphology, and molecular data of the *Neocaridina* species, considering its significant economic and trade value as an ornamental shrimp (Tropea et al. 2015). Morphological identification of species only is difficult because *Neocaridina* species sometimes cannot be identified as the right species. Moreover, morphological identification becomes more complicated when the species are damaged due to rough handling. These identifying problems can be overcome by using molecular sequences, checking their similarity with those already deposited in the NCBI (Tahri et al. 2016), and analyzing for taxonomy by DNA Barcoding.

Cytochrome Oxidase Subunit 1 gene sequences of mitochondrial DNA were used for species identification and studies of genetic taxonomy of the species conducted by using CO1 sequence from unidentified DNA barcode in comparison to identified taxonomic species from the global reference sequence (Persis 2009; Kenny et al. 2014). Implementation of this approach provides for the use of complete information for species identification to complement taxonomic data and global validation of systemic location and evolution (Imtiaz et al. 2017).

DNA sequences and molecular systematics are used for this differentiation/comparison. Variation of nucleotides has been considered to be the best way to explore inheritable differences. Sequence analysis reveals the encoding potential of species complexes, phytogeography, population genetics, and DNA Barcoding studies (Hebert et al. 2003; Kekkonen and Hebert. 2014; Gamit et al. 2018). Phylogenetic trees are branching diagrams illustrating the evolutionary relationships among species, and the phylogeny is based on similarities and differences between the genetic characteristics of the species. Moreover, the phylogenetic trees provide an efficient structure of organized knowledge of biodiversity and allow the development of an accurate diagnosis overcoming the deficiencies of microscopic examination. Phylogeny aims to assemble a tree structure that represents a hypothesis on the evaluative source of a sequence of genes, species, and other phyla as well as the relations that several types of organisms share between specific genes

MATERIALS AND METHODS

Sample collection site

Samples of *N. denticulata* species were collected from the Purbalingga ornamental fish market, which is located in Kembaran Kulon Village, Purbalingga, Central Java, Indonesia. The collection of samples was conducted in August 2019 from the Purbalingga fish market and transported in a small glass aquarium filled with water to the laboratory. Purbalingga fish market is one of the main suppliers of ornamental shrimp in Central Java.

Genomic DNA extraction DNA mini kit (Geneaid)

DNA extraction from abdominal tissue of *N. denticulata* species was conducted by using the DNA Mini kit (Geneaid). According to the manufacturer's specifications, approximately 30 mg of the tissue of *N. denticulata* was placed into a 1.5 mL microcentrifuge tube, added 200 µL of GT buffer, was ground and mixed using micropestle, and then added 20 µL of proteinase mixture. After that, it was shaken vigorously by hand and incubated for at least 30 minutes at 60°C. A 200 µL of GBT buffer was applied to the mixture, shaken for 5 seconds by vortex, and incubated for at least 20 minutes at 60°C to make sure the lysate became clear. Subsequently, the elution buffer was preheated (100 µL per sample) to 60°C. Absolute ethanol (200 µL per sample) was applied to the lysate and shaken for 10 seconds in a vortex at low speed. The lysate was then moved and placed inside a collection tube of 1.5 mL, and centrifuged for 2 minutes at 14,000 rpm for 2 minutes. The collection tube of 1.5 mL was discarded, and then a GS column was moved to a new collection tube of 1.5 mL. In the GS column, 400 µL of W1 buffer was applied and then centrifuged for 1 minute at 14,000 rpm. The flow-through was discarded and a GS column was inserted back into the 1.5 mL collection tube; then 600 µL of washing buffer was applied to the GS column and centrifuged at 14,000 rpm for 3 minutes to dry the column matrix. The dried GS column was then moved to a clean 1.5 mL tube and 100 µL of preheated elution buffer was added and allowed to stand for 5 minutes, 14,000 rpm centrifuged for 1 minute and DNA was put at -20°C.

PCR conditions

A 50 ng of genomic DNA, 25 2x reaction mix of each primer, and 2.0 µL DNA polymerase were included in each 50 µL PCR reaction.

PCR amplification (My Taq Red Mix)

Primer was designed from the Primer3Plus design program using the mitochondrial DNA gene of *N. denticulata* uploaded in the GenBank. A specific primer was designed. To amplify a fragment of CO1 gene of the *N. denticulata*. Ten samples of ornamental shrimp of different colors were extracted and then sequenced. The primer designed was 5'-GTAGGAGAGGAGTCGGCAAC -3' (F) as a forward primer and 5'- TCTTAGTATCCCCGT CGTGC-3' (R) as a reverse primer generating around 545bp fragment. PCR was performed by initial denaturation at 95°C for 2 min, followed by 35 cycles each at 95°C for 30-sec denaturation, 50°C for 30 sec as annealing, 72°C for 2 min as an extension, and final extension at 72°C for 7 min.

Electrophoresis and sequencing

The amplified PCR products were separated by electrophoresis in 1% (w/v) agarose gel and visualized using Ethidium bromide. Only 5 µL of PCR product was loaded in the gel after properly mixed with 3 µL loading dye (DM2100), run at 70 Voltage for an hour, and then visualized under ultraviolet light. DNA sequencing was conducted for a positive band. Sequencing**,** Clear and strong amplicons were shipped to 1st BASE (www.base.asia.com) for sequencing. PCR products were sequenced in both forward and reverse directions using PCR primers on an automated sequencer by 1st base (www.base-asia.com) DNA sequencing services.

Molecular analysis

Sequence alignment

All sample sequences were assembled and merged by Contig using BioEdit (Hall, 1999), and multiple sequence alignment was carried out by MSA Viewer, MAFFT software program (Yachdav et al. 2016). The similarity of the sequences generated in this study was compared to other published sequences in the performed GenBank database (BLAST; https: //blast.ncbi.nlm.nih.gov/Blast).

DNA barcoding

The aligned DNA sequences were compared and checked and BLASTed on the NCBI platform for the species confirmation and gathering of essential sequences for the phylogenetic analysis. Samples were estimated by the Neighbor-Joining (NJ) Method, Jukes-Cantor Substitution Model (Yachdav et al. 2016) and branch support was measured by bootstrap values with 100 replicates. Published sequences from GenBank used in phylogenetics are shown in Table 1.

RESULTS AND DISCUSSION

PCR conditions

Molecular analysis of the Cytochrome oxidase 1 (CO1) gene from a total of 10 samples of different colors in Purbalingga aquarium shrimps was studied to identify those species. The species identification was based on sequence analysis. The PCR amplification results showed a clear amplicon of approximately 545 bp in all DNA samples of *N. denticulata* as shown in Figure 2. The results of PCR amplification confirm the presence of *N. denticulata* in the Purbalingga fish market aquarium.

Sequence results

Raw sample sequences of freshwater ornamental shrimp of different colors, *N. denticulata* forward and reverse were checked by eye in Bio Edit (Hall, 1999). The sequence length of the forward was found between 508 and 512 bp, while the length of the reverse was found between 506 and 511 bp. Forward and reverse Contig were assembled using Bio Edit (Hall, 1999), and the mean length of the Contig sequence was found between 541 and 545 bp.

Blast results

While performing Blast, the sequence of all sampled species showed almost the same level of similarity with existing data in the NCBI database. Samples number 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 showed 98-99% similarity with species of accession number NC_023823.1 uploaded in the GenBank. BLAST results showed the maximum homology (99.29%) of the *N. denticulata* and the minimum homology (98.24%) was in comparison with the species with accession number NC_023823.1 from China (Yu et al. 2014) as shown in Table 2.

Table 1. Published sequences from NCBI used for phylogenetic tree reconstruction

Species	Country	Accession number	Authors
Neocaridina denticulata	China	NC_023823.1	Yu et al. (2014)
N. denticulata	Israel	MN336483.1	Levitt et al. (2019)
N. denticulata	Hungary	MH780821.1	Weiperth et al. (2019)
N. palmata	Japan	LC324769.1	Shih et al. (2017)
N ketagalan	Japan	AB300182.1	Shih et al. (2017)
N. ikiensis	Japan	LC324775.1	Shih et al. (2017)
Eudistoma vaannamei	Brazil	MG515488.1	Paiva et al. (2017) (Outgroup)
N. saccam	Taiwan	AB300177.1	Shih and Cai (2007)
N. palmata	China	MN701612.1	Chen et al. (2019)
N. davidi	Poland	MG816776.1	Shih et al. (2017)
N. sinensis	Taiwan	AB300185.1	Shih and Cai (2007)

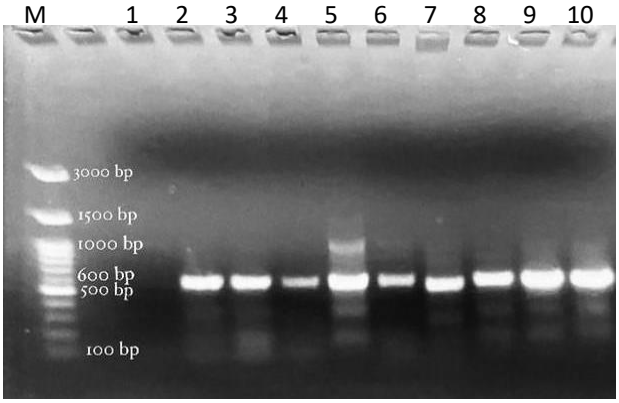

Figure 2. Gel electrophoresis of different color sample PCR amplification results of the CO1 gene segment showed a clear amplicon of approx. 545 bp in almost all DNA samples. Lane M: DNA Marker (DM2100 Excel Band 100 bp DNA ladder, range between 100-1.500 kb). Lane 1. Blue color (no band), lane 2. Dark-Red color, lane 3. Dark-Red color contained yellow eggs, lane 4. Bright-Red color, lane 5, Bright-Red color contains yellow eggs. Lane 6. Black color, lane 7. White color, lane 8. Black-blue color, lane 9. Blue-black color, lane 10. The Blue-black color contains yellow eggs

DNA barcoding results

The construction of phylogenetic tree was carried out using the NJ Method, Jukes-Cantor Substitution Model. Alignment of Contig sample sequences was performed by MSA Viewer, MAFFT software program (Yachdav et al. 2016), with one of the sequences of outgroup species and relevant species from different countries uploaded in the GenBank added to the alignment compared to the sequence samples in the software MSA Viewer, MAFFT. The alignment results analyzed using the Neighbor-joining method, Jukes-Cantor Substitution Model are shown in Figure 3.

Discussion

After conducting a DNA barcoding analysis of *N. denticulata* which has different molecular colors, it is known that all samples belong to *N. denticulata* with a monophyletic group, namely a group of organisms in the same taxon and originating from the same ancestor. Based on the results of the phylogenetic tree (study samples from 2 to 11) they are homologs, even to *N. denticulata* from China NC_023823.1. found in the GenBank uploaded by (Yu et al. 2014). Meanwhile, the *Neocaridina* sequence derived from GenBank formed another clade.

The color difference in the *N. denticulata* samples was due to the presence of chromatophores (Flores and Chien 2011; Lu et al. 2022; Lin et al. 2022). Chromatophores are pigment cells in animals that are responsible for color changes in the skin. The erythrophores pigments give a red color, the yellow color is due to the xanthophores pigments, the blue color is due to the iridophore pigments. The yellow color is the xanthophores pigment. The black color is the presence of melanophores pigment, the white color is due to the presence of leucophores pigment, and the color is transparent or often called a ghost because it has no pigment (Weber et al. 2016). This condition is called polymorphism, which is when two or several different phenotypes are present in the population of a species and are in the same habitat. This is in accordance with the samples used in the study, taken from the same location, namely the aquarium at the ornamental fish seller. The general function of polymorphism is to maintain color and/or shape variations in populations that are in a variety of environments.

Polymorphism in *Neocaridina* is also found in other species, for example, the species *Neocaridina davidi* (Weber et al. 2016) which has various colors. More than one color in one individual is caused by a mixture of chromatophores, namely xanthophores (yellow/orange), erythrophores (red/orange), and melanophores (black/ brown), the samples used for research also have mixed colors, namely blue black and black blue. The CO1 sequence of the *Eudistoma vannamei* which carried accession number MG515488.1 uploaded by (Paiva and Rocha 2017) was chosen as an outgroup.

Table 2. The results of CO1 gene sequence similarity of *Neocaridina denticulata* obtained using the BLAST program from the GenBank

Color	Description	bp	Query	Max score	Total score	Identity	Accession
Dark-red	*N. denticulata* mitochondrion, complete genome	15561	100%	996	996	98.41%	NC_023823.1
Dark-red contain yellow eggs	*N. denticulata* mitochondrion, complete genome.	15561	100%	992	992	98.24%	NC_023823.1
Bright-red	*N. denticulata* mitochondrion, complete genome.	15561	100%	1000	1000	98.59%	NC_023823.1
Bright-red contain yellow eggs	*N. denticulata* mitochondrion, complete genome.	15561	100%	1000	1000	98.41%/	NC_023823.1
Black	*N. denticulata* mitochondrion, complete genome	15561	100%	1022	1022	99.29%	NC_023823.1
White	*N. denticulata* mitochondrion, complete genome.	15561	100%	996	996	98.41%	NC_023823.1
Black-blue	*N. denticulate* mitochondrion, complete genome	15561	100%	1022	1022	99.29%	NC_023823.1
Blue-black	*N. denticulate* mitochondrion, complete genome	15561	100%	1014	1014	98.94%	NC_023823.1
Blue-black contain yellow eggs	*N. denticulate* mitochondrion, complete genome.	15561	100%	1018	1018	99.12%	NC_023823.1
Yellow	*N. denticulata* mitochondrion, complete genome	15561	100%	961	961	98,71%	NC_023823.1

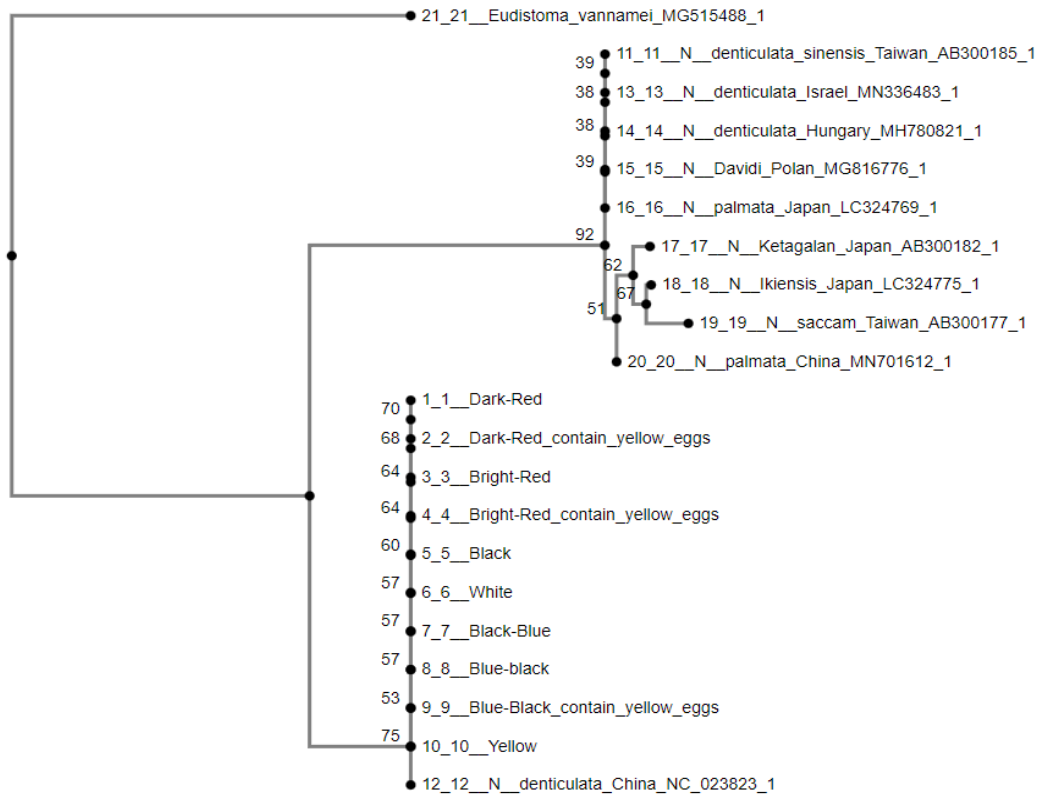

Figure 3. Phylogenetic tree construction by MAFFT version software using the Neighbor-joining method (CO1 gene that was sequenced in the present study is numbered from *N. denticulate.* The study samples of *N. denticulata* of different colors 10 study samples and the relevant *Neocaridina* species from different countries with a different accession number that is used. MG515488.1 *Eudistoma vannamei was* used as an outgroup to allow comparison with them in a group and for the phylogeny to be rooted

An outgroup is a reference group or taxon outside the group of interest and further related to the ingroup. The outgroup is used with the aim of knowing a primitive character/trait that has long been formed and spread across all members of the main branch (plesiomorph) and derived characters (an apomorphy)/ characteristics that are derived from and differ from the ancestral state of the ingroup and to determine points the beginning of the formation of a phylogeny tree. Moderate groups of closely related taxa are investigated for evolutionary relationships. Meanwhile, *Neocaridina* sequence derived from GenBank formed another clade.

The genetic distance value within *N. denticulata* study samples and relevant species from different countries was performed by pairwise distances using Mega software version X. The results show that the distance from 10 different color samples is zero. This indicates that all samples are species of *N. denticulat*a, although they are in different colors they are homologous. Research on the phylogeny and distance analysis of *N. denticulata* originating from aquariums traded from the Purbalingga area was the first to be carried out. The result shows that even though they are different colors, they are in the same species. Genetic distance is the distance that separates two genes in the same chromosome. Genetic distance is measured by various parameters. A small genetic distance indicates a close genetic relationship and conversely, a large genetic distance indicates a distant genetic relationship. Genetic distance can be used to compare genetic similarities between different species.

After comparing it to other relevant species, it shows that in all samples and *N. denticulata* from China, the distance is zero. Meanwhile, the comparison between samples to *N. denticulata* from Taiwan (AB300185.1) is 1.990. For the comparison to *N. denticulata* from Israel (MN336483.1), the distance is 2.080; for the comparison to *N. denticulata* from Hungary (MN780821.1), the distance is 2.080; for the comparison to *N. davidi* from Poland (MG816776.1), the distance is 1.990; For the comparison to *N. palmata* from Japan (LC324769.1), the distance is 1.990. For the comparison to *N. ketagalan* from Japan (AB300182.1), the distance is 2.226; for the comparison to *N. ikiensis* from Japan (LC324775.1), the distance is 2.415; for the comparison to *N. saccam* from Taiwan (AB3000177.1), the distance is 2.598. For the comparison to *N. palmata* from China (MN701612.1), the distance is 1.990. For the comparison to out group of *Eudistoma vannamei* (MG5115488.1), the distance is 3.103. In general, a distance greater than 2% indicates a very high probability that sequences belong to different species. For *N. ketagalan* from Japan (AB300182.1), with the distance is 2.226; *N. ikiensis* from Japan (LC324775.1), the distance is 2.415; *N. saccam* from Taiwan (AB3000177.1), the distance is 2.598. they are from different species, including out group of *Eudistoma vannamei* (MG5115488.1), with the distance is 3.103 (Table 3).

Table 3. Genetic distances among *Neocaridina* based on Kimura 2 parameter model

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1. Dark-red																				
2. Dark red contain yellow eggs	0.00																			
3. Bright red	0.00	0.00																		
4. Bright red contain yellow eggs	0.00	0.00	0.00																	
5. Black	0.00	0.00	0.00	0.00																
6. White	0.00	0.00	0.00	0.00	0.00															
7. Black-blue	0.00	0.00	0.00	0.00	0.00	0.00														
8. Blue black	0.00	0.00	0.00	0.00	0.00	0.00	0.00													
9. Blue-black contain yellow eggs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00												
10. Yellow	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00											
11. *N. denticulata sinensis* Taiwan AB300185.1	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99										
12. *N. denticulata* China KP168816.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
13. *N. denticulata* Israel MN336483.1	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08								
14. *N. denticulata* Hungary MH780821.1	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08	2.08							
15. *N. davidi* Polan MG816776.1	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99						
16. *N. palmata* Japan LC324769.1	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99					
17. *N. ketagalan* Japan AB300182.1	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22				
18. *N. ikiensis* Japan LC324775.1	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41			
19. *N. saccam* Taiwan AB300177.1	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59		
20. *N. palmata* China MN7016121.1	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	1.99	
21. *Eudistoma vannamei* MG 5154488.1	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10

In conclusion, samples were taken from the aquarium at the Purbalingga fish market with various colors belonging to one species, namely *Neocaridina denticulata*. The difference in colors is due to the chromatophores. The red color is caused by the presence of erythrophores. The yellow color is caused by the presence of xanthophores. Blue is caused by the presence of iridophores. Black by melanophores. White is caused by leucophores, and the color is transparent or often called a ghost because it has no pigment. Some colors are a mixture of chromatophores. The genetic distance between each sample and *N. denticulata* from China is 0, This indicates that all samples and *N. denticulata* from China are included in one species of *N. denticulata*. All samples showed that they are homolog, even to the species *N. denticulata* from China NC_023823.1

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