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Contributed Paper

Identification and Nutritional Value of Live Feeds for Ornamental Fish from Bangkok Metropolitan Markets in Thailand

Supenya Chittapun*[a], Darawan Ruamkuson [b] and Mariena Ketudat-Cairns*[b,c]

[a] Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Campus, Pathum Thani 12120, Thailand.

[b] School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand.

[c] Embryo Technology and Stem Cell Research Center, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand.

*Author for correspondence; e-mail: supenyac@tu.ac.th; ketudat@sut.ac.th

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ABSTRACT

The production of suitable and highly nutritional fish feed is important for the aquarium ornamental fish industry. In this study, the biochemical compositions of four common live feeds from ornamental fish markets in the Bangkok metropolitan area were examined. The highest energy and protein contents in feed were found in aquatic worms (57.42 Kcal×100 g^{-1} and 59.13%, respectively), followed by brine shrimp (25.56 Kcal×100 g^{-1} and 25.25%), blood worms (23.80 Kcal×100 g^{-1} and 26.06%) and freshwater fleas (21.52 Kcal×100 g^{-1} and 26.88%). The highest amount of astaxanthin per gram of sample was found in brine shrimp (170.22 ng×g^{-1}), followed by aquatic worms (10.90 ng×g^{-1}), freshwater fleas (8.34 ng×g^{-1}), and blood worms (5.11 ng×g^{-1}). The species present in these four live feeds were next identified both morphologically and molecularly. Partial sequences of the cytochrome c oxidase I (*COX*I) gene were used for the molecular identification of the four live feeds. The freshwater fleas, brine shrimp and blood worms were identified as *Moina macrocopa*, *Artemia franciscana* and *Chironomus circumdatus*, respectively, by both morphology and *COX*I gene sequence analysis. *COX*I sequence analysis identified the aquatic worm as *Branchiura sowerbyi*, while morphological analysis identified the same worm as *Limnodrilus hoffmeisteri*. To resolve this discrepancy, 18S rDNA was used, conclusively identifying the organism as *L. hoffmeisteri*, consistent with its morphology.

Keywords: live feed identification, nutritional composition, *COX*I gene, ornamental fish

1. INTRODUCTION

The aquarium ornamental fish export industry in Thailand is a well developed and growing industry, with the total export value for this industry expected to increase considerably [1]. The major factors currently affecting the expansion of the Thai fish export industry are the quality and quantity of ornamental fish obtained

from suppliers. Good quality and large quantities of fish depend mainly on the quality and availability of the feed. Suitable, high-quality feed significantly improves the growth and survival rate of ornamental fish. In addition, highly nutritional feed can improve the function of the immune systems of the fish and newly hatched fry [2], which are required to combat the stress endured during collection and transportation [3]. However, the lack of suitable feed for these fish during the production stage is one of the most significant problems in the ornamental fish development industry [4].

Live feeds have been shown to significantly improve the growth and survival of many ornamental fish compared to conventional feeds, particularly for starter feeds used for the onset of juvenile external feeding [4, 5]. Recently, rotifers, cladoceran, copepods, *Artemia*, *Chironomus* larvae and aquatic worms have been used as live feed for fish hatchery production worldwide [3-7]. Common live feeds in the Thai ornamental fish markets include freshwater fleas, brine shrimp, blood worms and aquatic worms. These live feed animals vary in size, providing a suitable food source for both fry and fish, and are an important component of the quality and quantity of Thai ornamental fish. Unfortunately, no studies on the scientific names, nutritional contents or morphologies of the most common live feeds in Thailand have been reported. Information on the size and nutritional quality of these feeds would be useful to Thai fish farmers for the selection of appropriate live feeds for each stage of the fish larvae. Moreover, the identification of these species will be useful as a reference for future studies.

In this work, mitochondrial cytochrome c oxidase subunit I (*COX*I) gene sequences were analyzed to identify the species of the four common live feeds. The *COX*I gene is a highly conserved protein-coding gene, favored in phylogenetic studies for its diversity [8, 9]. The identification of the four live feeds by microscopy and molecular biology, and the analysis of their nutritional contents, should be beneficial to the fish production industries for the selection of suitable live feeds for each stage of fish larva.

2. MATERIALS AND METHODS

2.1 Live Feed Sampling

Four common live feeds, including freshwater fleas, brine shrimp, blood worms and aquatic worms, were sampled 8 times from two main distributors of two different major ornamental Bangkok metropolitan fish markets, during April-August, 2009. Each sample was washed in distilled water and then divided into three fractions. One hundred and fifty g and 50 g of each sample were stored in plastic tubes at -20°C for biochemical and molecular analyses, respectively, and 20 g was preserved in 5% formaldehyde solution for 1-2 months prior to the morphological study.

2.2 Biochemical Composition Analysis

Nutritional analyses of the four samples were performed at the Food Quality Assurance Service Center, Kasetsart University, Thailand. The methods of each biochemical analysis are shown in Table 1.

2.3 Morphological Study

The four live feeds were mounted on slides, and the morphologies were examined and photographed using a ZEISS AXIO Imager according to Amat [16], Mura [17], Mura and Brecciaroli [18], Mura *et al.* [19], Paggi [20] and Epler [21]. In addition, some samples were prepared by stepwise

Table 1. Methods of each biochemical analysis.

Biochemical contents	Methodologies
Protein (%, factor 6.25)	TMC-03 Kjeldahl Method: Inhouse method based on AOAC [8]
Fat (%)	TMC-75 In house method based on AOAC [9]
Total Carbohydrate (%)	TMC-78 In house method based on AOAC [10] by Calculation
Ash (%)	TMC-01 In house method based on AOAC [11]
Total Calories (Kcal×100g^{-1})	TMC-78 In house method based on [9] by Calculation
Calories from fat (Kcal×100g^{-1})	TMC-78 In house method based on [9] by Calculation
Carotenoids (Astaxantin) (mg×100g^{-1})	TMC-57 In house method based on Meléndez-Martínez *et al*. [12]
Fatty acid compositions	TMC-05 In house method based on Compendium of Methods for Food Analysis, Thailand [13]

dehydration [22] in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90 %, 95% and absolute ethanol and photographs were subsequently taken using a JEOL 5800 LV Scanning Electron Microscope (SEM). The size of freshwater fleas was measured in mm and later converted to mm, while the other organisms were measured in μm. To calculate the average length and width of the organisms, 100 individuals from each sample were measured.

2.4 *COX*I Gene Analysis

2.4.1 DNA Extraction

Fifty mg of each sample were placed into 1.5 ml microcentrifuge tubes. Next, 380 μl of lysis buffer (2% CTAB, 0.35 M NaCl, 25 mM Tris-HCl pH 8.0 and 5 mM EDTA) and 200 μg Proteinase K were added. The solutions were mixed thoroughly and incubated at 56°C overnight. The tubes were allowed to cool to room temperature (RT), and 40 μg of RNAse were added and mixed thoroughly, followed by an incubation at 37°C for 2 hr. Next, 300 μl of 24:1 v/v chloroform/isoamyl alcohol was added, and the solution was mixed thoroughly and centrifuged at 16,000 × *g* for 10 min. Three hundred μl of the aqueous phase was transferred to new microcentrifuge tubes, and then 300 μl of isopropanol was added and mixed gently. The samples were then incubated at RT for 10 min and centrifuged at 16,000 × *g* for 10 min. The supernatants were discarded, and then the pellets were washed with 70% ethanol, gently mixed and centrifuged at 16,000 × *g* for 5 min. The supernatants were discarded, and the pellets were washed with 500 μl of absolute ethanol and mixed gently. The pellets were then collected by centrifugation at 16,000 × g for 5 min and air-dried at RT. Finally, the DNA was resuspended in 30 μl TE buffer and stored at -20°C until analysis.

2.4.2 *COX*I and 18S rRNA Gene Amplification and Identification

The *COX*I gene fragments from blood worms, aquatic worms, and freshwater fleas were amplified using primers LCO1490F 5'GGTCAACAAATCATAAAGATATTGG-3' and HCO2198R 5'-TAAACTTCAGGG

TGACCAAAAAATCA-3' [8, 23, 24, 25]. The *COX*I gene of brine shrimp was amplified using primers 1/2 Ar_COI_F 5'-ATTCTA CGAATCACAAGGAT ATTGG-3' and 1/2 Ar_COI_R 5' TACACTTCAGGATG GCCAAAAAATCA-3' [26]. The 18S rRNA gene fragment of the aquatic worms was amplified using NS0 5' TACCTGGTTGA TCCT GCC-3' and EF3 5'- TCCTCTAAA TGACCAAGTTTG-3' [27]. Each mixture (final volume 50 μl) contained approximately 4 ng template DNA, 0.4 μM primers, 0.2 mM dNTP, 2 mM $MgCl_2$, 1x $MgCl_2$-free buffer (Promega, USA) and *Taq* DNA polymerase (made in-house at SUT). PCR reactions were run according to the following protocols. Firstly, 1 cycle at 94°C for 5 min; then: 35 cycles at 94°C for 30 sec, 51.4°C for 30 sec followed by 72°C for 45 sec for the partial *COXI* gene amplification or firstly, 1 cycle at 94°C for 5 min; then: 35 cycles at 94°C for 30 sec, 53°C for 45 sec, and 72°C for 1 min and 30 sec; followed by a final stage of 72°C for 5 min for the partial 18SrRNA gene amplification.

2.5 Sequencing and Alignment

The purified partial *COX*I and 18S rRNA gene amplicons were ligated into the pGEM-T easy vector (Promega) and transformed into *E. coli* DH5α. Recombinant plasmid DNA samples were sent to Macrogen (Korea) for sequencing. The sequencing results were aligned by performing a BLAST [28] against the NCBI database.

3. RESULTS AND DISCUSSION

3.1 Nutritional Compositions of Live Feeds

Nutritional compositions of four common live feeds from ornamental fish markets around the Bangkok metropolitan area in Thailand were analyzed. These results indicated that of live feeds analyzed, aquatic worms have the highest energy content (57.42 Kcal×100 g^{-1}) followed by brine shrimp (25.56 Kcal×100 g^{-1}), blood worms (23.80 Kcal×100 g^{-1}) and freshwater fleas (21.52 Kcal×100 g^{-1}). The highest protein and carbohydrate contents were found in aquatic worms (59.13% and 11.24%, respectively), whereas the amounts found in blood worms (26.06% and 0.97%) and brine shrimp (25.25% and 1.36%) were lower and quite similar. The protein content in freshwater fleas was 26.88%, but the carbohydrate content in these organisms was undetectable. Suitable feeds with high nutritional values significantly improve the growth and survival rates of ornamental fish, while also promoting the immune systems of the fish [2, 7]. The majority of these feeds are suitable for adult fish, particularly aquatic worms, which have a high nutritional composition and high energy content. These four common live feeds are composed of both saturated and unsaturated fatty acids (Table 2); interestingly, essential fatty acids (EFA) such as linoleic acid and palmitoleic acid, which are essential for the normal growth and cellular structure and function of most fish, [29] were also found in these feeds. Aquatic worms showed the highest amount of EFA compared to the other feeds analyzed (Table 2).

Aside from nutritional values, the ability to promote pigmentation in fish is also an important consideration in the ornamental fish industry. The analysis of carotenoids indicated that brine shrimp have the highest amount of astaxanthin (170.22 ng×g^{-1}) compared to aquatic worms (10.90 ng×g^{-1}), freshwater fleas (8.34 ng×g^{-1}) and blood worms (5.11 ng×g^{-1}). Astaxanthin which is one of the biochemical intermediate in the carotenoid biosynthesis pathway should enhance pigment formation [30, 31, 32].

Table 2. Fatty acid composition ($g \times 100g^{-1}$) of four common live feeds in Thailand.

Fatty acid composition ($g \times 100g^{-1}$)	Freshwater fleas	Brine shrimp	Blood worm	Aquatic worm
Saturated fatty acid				
Lauric acid (C 12:0)	-	-	-	0.02
Myristic acid (C 14:0)	0.02		0.02	0.06
Pentadecanoic acid (C 15:0)	0.01	-	-	0.03
Palmitic acid (C 16:0)	0.10	0.10	0.08	0.14
Heptadecanoic acid (C 17:0)	-	-	-	0.04
Stearic acid (C 18:0)	0.02	0.04	0.04	0.07
Behenic acid (C 22:0)	0.02	-	-	0.03
Lignoceric acid (C 24:0)	-	-	-	0.04
Unsaturated fatty				
Palmitoleic acid (C 16:1)	0.12	0.09	0.06	0.08
Oleic acid (C 18:1, cis-9)	0.09	0.07	0.04	0.23
Linoleic acid (C 18:2n6)	0.05	0.07	0.07	0.11
Gamma Linoleic acid (C 18:3n6)	0.02	0.04	0.02	0.12
Cis-11, 14 Eicosadienoic acid (C 20:2)	-	-	-	0.03
Erucic acid (C 22:1)	-	-	-	0.07

Moreover, astaxanthin has been found to increase egg quality and fertilization ability in *Gadu morhua* [33].

3.2 Morphology and Species Identification

The four live feeds were identified using a combination of morphology and *COX*I gene sequencing. The size (Table 3) of the live feeds is the key factor in the selection and use of these feeds in the ornamental fish industry. Among the four common live feeds, freshwater fleas are the most appropriate feed for fry due to their small size (0.45-1.15 mm) and their slow movement, which facilitates their capture by the fry. The other live feeds analyzed in this study have also been used to feed fry, as well as young and adult fish. However, feeding fry, or even adult fish, with brine shrimp must be performed under strict measures because brine shrimp, being marine organisms, die quickly in fresh water. Additionally, brine shrimp pollute the water when they are used as feed. Feeding fry and fish with freshwater fleas and blood worms should also be of concern; the hygiene and water quality of fish farms should be monitored because the feeds are usually cultured in rich, organic manure water. Additionally, these four common live feeds sold in ornamental fish markets in Thailand have been reported to be contaminated with mycobacteria [34], which is the main cause of mycobacteriosis, a chronic progressive disease in aquarium fish.

The *COX*I gene were partially amplified from the four live feeds and sequenced. The *COX*I gene sequences were submitted to NCBI and aligned using both the BLASTn and BLASTx programs [28]. The freshwater flea was identified both by morphology (Figure 1) and *COX*I gene partial sequence (GenBank: HM236481) identity as *Moina macrocopa*. Although *M. macrocopa* was first discovered in Thailand in 1984 [35],

M. macrocopa can no longer be found in natural habitats in this country [36-38]. *M. macrocopa* was believed to be inoculated as live feed in intensive aquaculture systems. The presence of *M. macrocopa* in intensive aquaculture systems but not in natural habitats indicated that *M. macrocopa* is a sensitive species that cannot compete against other local species in the environment.

The brine shrimp, or *Artemia*, present in the Thai fishery markets is thought to be *A. salina*, which is endemic to the Mediterranean [34,39]. However, the morphological analysis (Figure 2) and the molecular analysis of the partial *COX*I gene sequence (GenBank: GU944723) of our sample identified this particular brine shrimp as *A. franciscana*. A natively American species, *A. franciscana* was

Table 3. Size variation in four live feed (n = 100).

Live feeds	Length (mm)			Width (mm)		
	Min-Max	Average	Mode	Min-Max	Average	Mode
Freshwater fleas	0.45-1.15	0.72	0.72	0.28-0.45	0.35	0.35
Brine shrimp	3-8	5	5	1-2.6	1.5	1
Blood worm	6-16	11	12	1-1.2	1.0	1
Aquatic worm	3-19	10	8	0.5-0.5	0.5	0.5

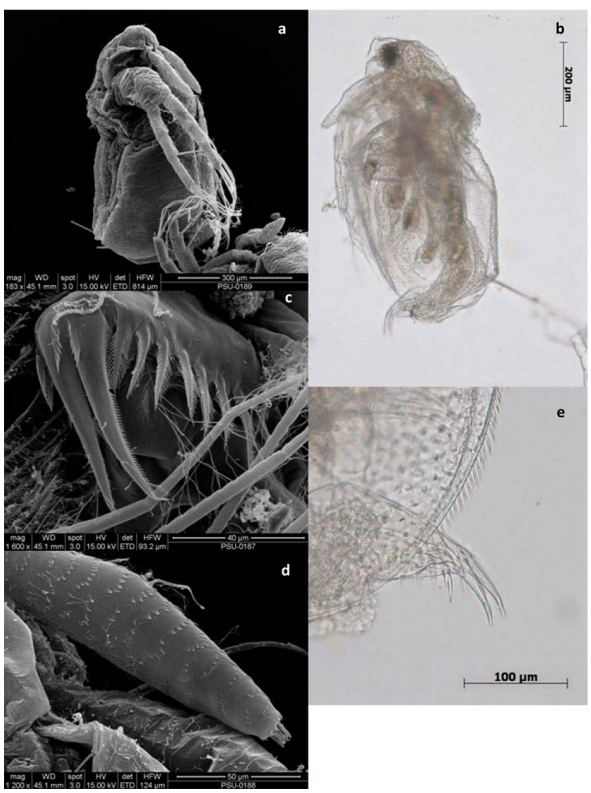

Figure 1. *M. macrocopa*: a,b: whole organism, c: post abdomen, d: antenna, e: carapace.

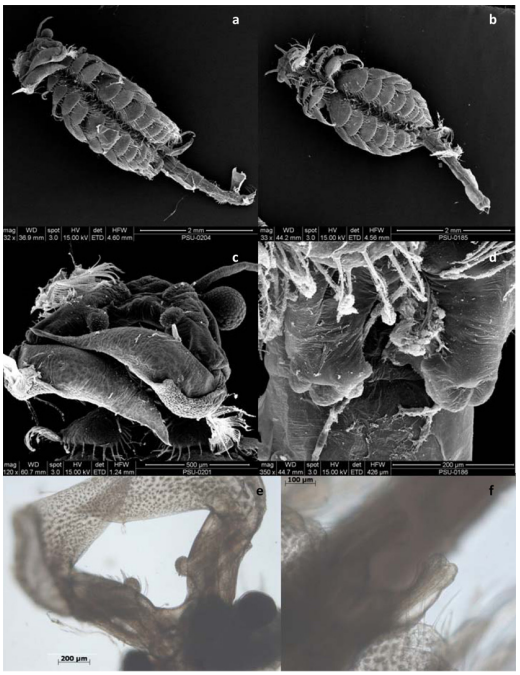

Figure 2. *A. franciscana*: a: male, b: female, c & e: male second antennae, d & f: male gonad.

believed to be introduced by aquarists into Thailand during the intensive aquaculture industry period [39].

The blood worms in this study were identified using both morphology (Figure 3) and the *COX*I gene partial sequence analysis (GenBank: GU944724) as *Chironomus circumdatus*. *C. circumdatus* has been found in many countries in Asia, including Korea, Japan, India and Micronesia [40,41]. In Thailand, *C. circumdatus* has been reported in several natural habitats of the Northeastern lakes [42].

Partial *COX*I gene sequence analysis of the aquatic worms (supplementary data Figure S1) demonstrated 99% similarity between *COX*I gene sequences of the worm and *Branchiura sowerbyi* (GenBank: AF534864). However, morphological analysis (Figure 4) based on chaetal structures and genital organs indicated that the worm belongs to the family Naididae (formerly Tubificidae [42]), subfamily Tubificinae, and species *Limnodrilus hoffmeisteri* Claparéde, 1862. *L. hoffemeisteri* is a common organism, often found in eutrophic or organically polluted waters. The organism is common to Southeast Asia, as well as most of the world [43]. Some studies [44, 45] have reported that *B. sowebyi* contains gill filaments on the posterior half of its body. According to the morphological analyses in this study, the aquatic worms were not *Branchiura* because the worms did not have long hair chaetae or gill filaments on their dorsal bundles. Even though, *B. sowebyi* is thought to be a native of tropic and subtropical regions of Asia. To date, sequence information on the *COX*I gene of *Branchiura* is limited. Therefore, partial sequences of 18S rDNA from the aquatic worms were amplified and sequenced. The sequence (GenBank: KC189002) and BLAST search results identified these aquatic worms as *L. hoffmeisteri*, consistent with the morphological analysis but inconsistent with the results from the *COX*I gene sequencing, which indicated that the sample was *B. sowebyi.* This finding demonstrates that molecular information

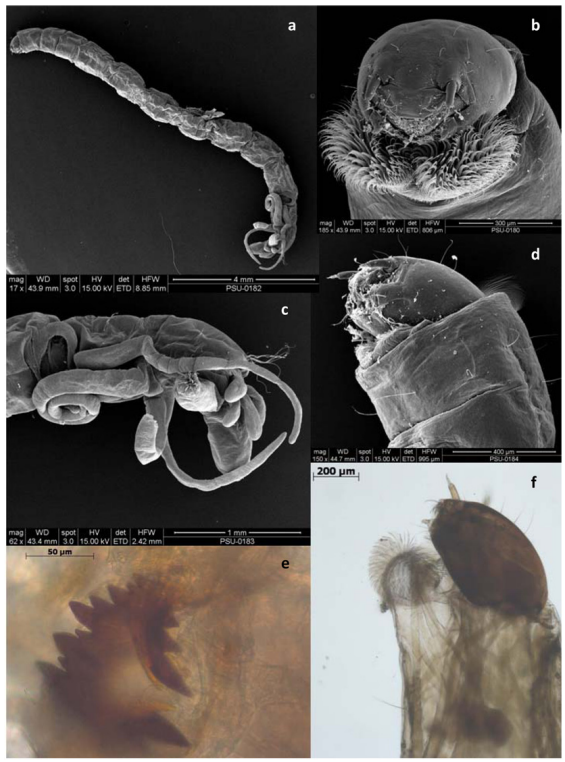

Figure 3. *C. circumdatus*: a: whole organism, b: ventral view of head part, c: posteria end, d & f: lateral view of head part, e: mantum.

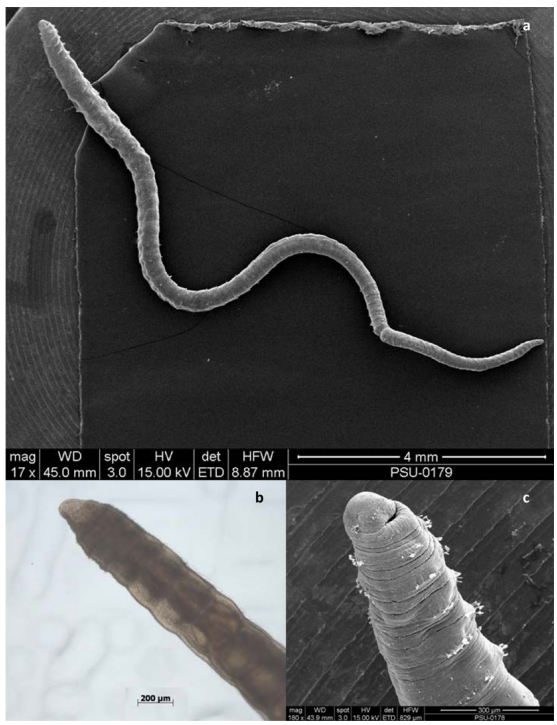

Figure 4. Aquatic worm: a: whole organism, b: anterior part, c: setae at anterior.

from the *COX*I gene alone should not be used to identify all organisms; rather, results from both molecular and morphological techniques must be analyzed collectively.

4. CONCLUSIONS

High nutritional values and energy stores are important for live feeds, which have been proven to significantly improve the growth and survival of many ornamental fish, compared to conventional feed such as yolk [4, 5]. In this work, four common live feeds were identified, and the nutritional values of these feeds were assessed. Among the four live feeds analyzed, freshwater fleas are recommended as a live feed for freshwater fish larvae, due to the small size of the fleas and their ease of capture by fry. However, the development of a freshwater flea culture system is needed to avoid contaminants, which will pollute the aquariums of ornamental fish.

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