The Molecular Identification of *Nephtys* species (Polychaeta: Phyllodocida) from Songkhla Lake, Southern Thailand

Manasawan Saengsakda Pattaratumrong[1,2]* and Koraon Wongkhamhaeng[3]

[1] Marine and Coastal Resources Institute, Prince of Songkla University, Hat-Yai, Songkhla, 90112, Thailand

[2] Coastal Oceanography and Climate Change Research Center, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand

[3]Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand

* Corresponding author. E-mail address: manasawan.s@psu.ac.th

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Abstract

Polychaetes are macrobenthic fauna which play an important role in ecosystems by changing the physical nature of the seabed and nutrient cycling. They are the most dominant group in the benthic community, an important component in the food chain and bioindicators of environmental pollution. Songkhla lake has over fifty species of polychaetes and the most abundance family is Nephtyidae. Morphological identification of individual species is difficult and time-consuming, requiring expertise. This paper examined the phylogenetic relationships of the common nephtyids present in the outer sector of Songkhla Lake, Southern Thailand, *Nephtys polybranchia*, Nephtys oligobranchia and *Nephtys* sp., were studied using partial nuclear ribosomal first internal transcribed spacer (ITS1) nucleotide sequences. The results of the molecular phylogenetic analysis showed that the Nephtys can be grouped into two clades: (1) an unresolved clade including Nephtys polybranchia and Nephtys sp. as sister taxa and (2) Nephtys oligobranchia. The results indicated that ITS1 sequencing should be a useful taxonomic marker for Nephtys species in the ecological survey and routine identification.

Keywords: Nephtyidae, polychaete, molecular marker, Songkhla lake, ITS1

Introduction

Polychaetes are segmented invertebrates in the phylum Annelida, class Polychaeta. They are common benthic fauna and the dominant component of macrobenthos in tropical intertidal habitats such as the Andaman Sea, Coast of Thailand (Frojan et al., 2006) and Songkhla lake (Angsupanish and Kuwabara, 2006). Benthic fauna is an important component of the ecosystem and play a key role in the environment as food sources for bottom feeding fin and shellfish, bait organisms in the fishing industry, environmental indicators (Frojan et al., 2006; Pagliosa & Barbosa, 2006), primary energy sources during environmental stress (Arndt & Schiedek, 1997) and recycling nutrients with their erratic movements (Piot, Rochon, Stora, & Desrosiers, 2008). They make up the most frequent prey of catfishes *Osteogeneiosus militaris* and *Arius maculatus* (Angsupanich, Somsak, & Phrommoon, 2005).

The Nephtys are free-living polychaetes in phylum Annelida, class Polychaeta, order Phyllodocida, family Nephtyidae which contains five genera, *Aglaophamus*, *Destinephtys*, *Inermonephtys*, *Micronephtys* and *Nephtys* (Rizzo & Amaral, 2007). They occur in most marine environments, shallow water and intertidal zones. A previous report from Songkhla Lake noted the occurrences of over 50 species of polychaetes. Most species were in the Nereididae family and the most abundant appearing at almost all sampling stations was *Nephtys*. The Nephtyidae in Songkhla Lake consists of two genera, *Aglaophamus* and *Nephtys* (Angsupanich Kuwabara, 2006). The most abundant species is *N. polybranchia* Southern. The other common species are *N.*

paradoxa Malmgren, *N. oligobranchia* and *Nephtys* sp. (Angsupanich & Kuwabara, 1999; Angsupanich Siripech, & Charoenpornthip, 2005).

The distinguishing characters of Nephtyidae are the clear presence of interramal branchiae on the ventral notopodial edge called "recurved cirrus" or "interramal cirrus", the flattened prostomium and small antennae (Fauchald, 1977). However, traditional morphological identifications of polychaetes are often confused with other benthic animals such as Nemertea, Echiura, Priapulida, Phoronida and Sipuncula. The peristomial segments are often difficult to identify. The nephtyids may superficially be confused with the sigalionids (family Sigalionidae) as both groups have long, straight-sided bodies abruptly tapering anteriorly, and rather more gently posteriorly (Fauchald, 1977). The consideration of 145 polychaetes species originally described from New Zealand up to and including 1950 shows the particular taxonomic revisions or misidentifications (Glasby & Read, 2010). Some species of *Nephtys* had intraspecific variation in some characters which resulted in the redescription of closely related species. For example, the redescription of *N. Assimilis* and *N. kersivalensis* (Rainer 1989), and redescription of *N. hystricis* McIntosh and *N. incisa* Malmgren whose diagnostic features were confused since the original description (Rainer, 1990).

Recently, molecular techniques have been successfully applied in species identification studies. Various molecular markers including mitochondrial DNA or ribosomal DNA are now used to differentiate organisms. One commonly used ribosomal DNA marker appropriate for distinguishing populations within a species is the first internal transcribed spacer (ITS1). Previous research has demonstrated the usefulness of the ITS1 in taxonomic studies of parasitic nematodes (Trichostrongylus) (Hoste, Chilton, Beveridge, & Gasser, 1998) and marine animals (Cnidaria, Ctenophora, Mollusca, Annelida and Crustacea) (Chow, Ueno, Toyokawa, Oohara, & Takeyama, 2008). In addition, the phylogenetic investigation has resulted in the description of new species, new genera, combination of genera and also redescription and taxonomic revisions (Boggemann, Bienhold, & Gaudron, 2012; Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994; McHugh, 2005).

This study aimed to improve upon the limitations of morphological identification by determining the suitability of the ITS1 to differentiate *Nephtys* species. Three common and abundant species of *Nephtys*: *N. oligobranchia, N. polybranchia* and *Nephtys* sp. were collected from the outer sector of Songkhla Lake. The ITS1 region was amplified, sequenced and analysed using the neighbour-joining method with bootstrap analysis.

Methods and Materials

2.1 Study Area and Sampling Stations

Songkhla Lake is located between latitudes 7° 08′ and 7° 50′N and longitudes 100° 07′ and 100° 37′E (Sompongchaiyakul and Sirinawin, 2006) and covers three provinces in Southern Thailand: Songkhla, Phatthalung and Nakhon Si Thammarat. Fifteen sampling stations were located in the outer part of Songkhla Lake connected to the Gulf of Thailand (Figure 1). Samples were collected between February and December 2012 using an Ekman grab sampler. The sediment samples were sieved in the field on a 0.5 mm mesh. Collected organisms were washed thoroughly with water, then preserved in 70% ethanol and transferred to the laboratory. The polychaetes were sorted under microscope, enumerated and identified following the keys of Fauchald (1977).

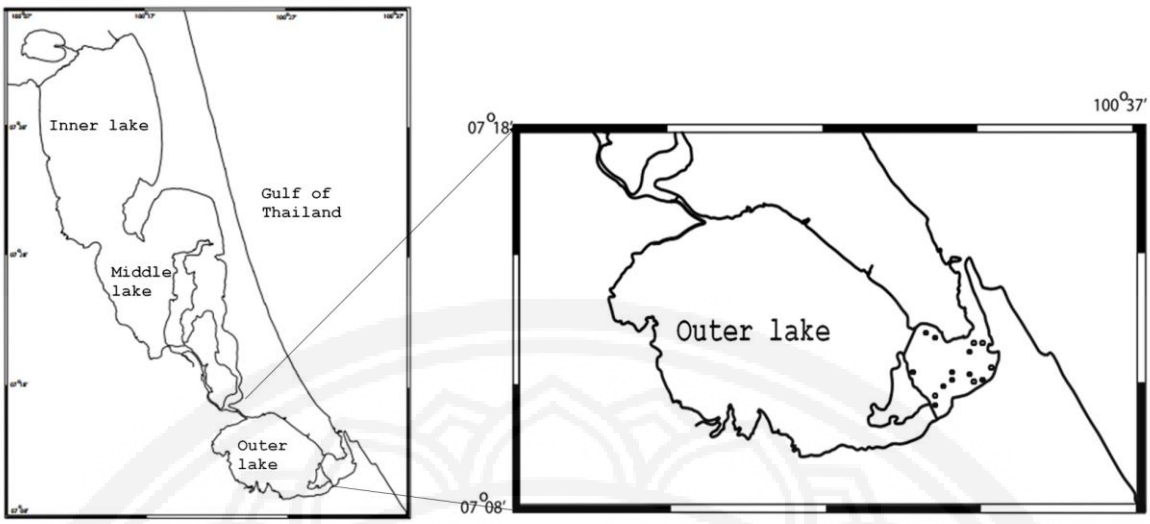

Figure 1 Location of sampling stations in outer Songkhla lake. Black circle indicate sampling sites.

2.2 DNA Extraction and Sequencing

The genomic DNA of 15 samples of *N. oligobranchia*, *N. polybranchia* and *Nephtys* sp. were extracted using a DNA extraction kit (Stratagene, USA). Each whole body was ground individually in lysis buffer to prevent the mixing of distinct genotypes. The samples were treated with pronase (100 μg/ml) and RNase (20 μg/ml) followed by ethanol precipitation (Green & Sambrook, 2012).

The partial region of the ITS1 gene was amplified using the primers ITS1f (5′-TCCGTAGGTGAA CCTGCGG-3′) and ITS1r (5′-CGCTGCGTTCTTCATCG-3′) (Chow et al., 2008). The PCR reaction mixtures were carried out in a volume of 50 μl with 500 ng genomic DNA, 1x PCR buffer minus Mg (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.2 mM dNTPs, 1.5 mM MgCl, 1.0 U of Platinum Taq DNA Polymerase (Invitrogen, Brazil), and 0.2 μM of each primer. The PCR conditions were as follow: 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were purified using gel extraction kits (QIAGEN, Germany) and sequenced using the BigDyeTM terminator cycle sequencing kit (PE Applied Biosystems, USA).

2.3 Multiple Alignment and Phylogenetic Analysis of ITS1

Nucleotide sequences were aligned using BLAST programs. A phylogenetic tree was generated from the alignment using the neighbour-joining method and bootstrap analysis was sampled 1,000 times.

Results and Discussion

3.1 Distribution and Morphological Analyses

The salinity of Songkhla Lake is influenced by seawater from the Gulf of Thailand and freshwater influx from many rivers and streams. The salinity varies depending on the season with seawater conditions during the dry season (February to May), brackish water during the light rains (May to October) and fresh water during the rainy season (October to January). During the study period (February to December), salinity in the outer sector of the lake ranged from 0 to 32 psu. During the freshwater period, no nephtyids were found. In the brackish and seawater periods, *N. polybranchia* was the dominant species and widely distributed, while *N. oligobranchia* and *Nephtys* sp. were less evident. Although previous studies indicated that

N. polybranchia was distributed throughout the year (Angsapanich et al., 2005), it has recently been suggested that the distribution depending on salinity.

Habitat preferences varied among the species, *N. polybranchia* and *Nephtys* sp. were common in muddy habitats, whereas *N. oligobranchia* was more abundant in sandy habitats. This habitat selection agreed with the abundant information concerning *Nephtys* species in the German Bight (North Sea) (Meibner, Darr, & Rachor, 2008) and on the South Indian coast (Musale & DeSai, 2011). Sediment characteristics, grain size, interstitial space and organic matter are important parameters for habitat selection and affect the structure of the benthic community. The distribution of macrobenthic fauna is related to the type of sediment which is linked to food, detritus food chain and hydrodynamics (Rodil, Lastra, & Lopez, 2009). Lake bottom sediment characteristics in this study were mainly clayey-sand, sandy-clay and sand-silt-clay (Pradit, Wattayakorn, Angsupanich, Baeyens, & Leermakers, 2010). The clayey-sand sediment was found in the north-western part of the study area. The sandy-clay sediment was found near river mount of Songkhla Lake. Additionally, the northern part of the study area was sand-silt-clay sediment (Pradit et al., 2010).

The samples were morphologically identified as *Nephtys* sp. using the following characteristics: (1) one pair of antenna, (2) one pair of very short palps, (3) one pair of lateral jaws, (4) biramous parapodia with well-developed rami, and (5) recurved interramal cirri. The main diagnostic characteristics of *Nephtys* sp. are the recurved interramal branchiae with outward coils (also called recurved cirrus or interramal cirrus) (Fauchald, 1977). The difference within *Nephtys* species is determined by the shape and morphology of the branchia on the parapodium. The parapodium of *N. oligobranchia* consists of well-developed branchia on chaetigerous segments 6-26, while *N. polybranchia* and *Nephtys* sp. have cerriform branchia (Table 1)(Figure 2).

Figure 2 The Nephtyidae found in the outer part of Songkhla Lake.

Table 1 Morphological comparisons within the *Nephtys* group: *Nephtys oligobranchia*, *Nephtys polybranchia* and *Nephtys* sp.

Morphology	*Nephtys oligobranchia*	*Nephtys polybranchia*	*Nephtys* sp.
Head	-Paired antenna -Paired pulp -18 bifid papillae -1 middorsal papilla	-Paired antenna -Paired pulp -18 bifid papillae -1 middorsal papilla	-Paired antenna -Paired pulp -18 bifid papillae -1 middorsal papilla
Parapodia	-chaetiger 2-5 w. small branchiae -chaetiger 6-26 w. well developed branchiae -chaetiger 27- w/o. branchiae	-chaetiger 2-6 w. small branchiae -chaetiger 7-30 w. cerriform branchiae -chaetiger 30- w. foliaceous branchiae	-chaetiger 2-5 w. small branchiae -chaetiger 6-30 w. cerriform branchiae -chaetiger 30- w. foliaceous branchiae

3.2 DNA Sequences and Phylogenetic Trees

Fifteen individual samples from 3 *Nephtys* species were subjected to PCR amplification of ITS1. The ITS1 sequences of *Nephtys* sp. from Songkhla Lake ranged in size between 240-273 bp. The length of the ITS1 sequence was 240 bp for *N. oligobranchia*, 267 bp for *Nephtys* sp., and 273 bp for *N. polybranchia*. The length of ITS1 for the three species of *Nephtys* (240-273 bp) was shorter than reported for other polychaetes in the order Phyllodocida, with families Syllidae (415-516 bp), Nereididae (351-562 bp) and Phyllodocidae (609-635 bp). This ITS1 length variation has also been observed in a wide variety of marine animal species (108-1,118 bp) (Chow et al., 2008), Biomphalaria Mollusks (549-778 bp) (DeJong et al., 2001) and ivory shells (515-520 bp) (Hualkasin, Tongchuai, Chotigeat, & Phongdara, 2008). The length variation may be correlated with genome size and the effect of the repetitive elements in the ITS region (Chow et al., 2008; Schulenberg et al., 2001). The sequences of three species were compared over an alignment. The greatest sequence differences were found between *N. oligobranchia* and the two other species. Sequence differences between *Nephtys* sp. and *N. polybranchia* occured at 7 positions, comprising transversions (substitution between purine and pyrimidine) at positions 4-6, 9, 16-17 and 216.

Phylogenetic trees produced from the neighbour-joining method analyses of ITS1 alignment are shown in Figure 3. The tree was outgroup rooted with polychaete *Pettibonella multiuncinata*, in the order Scolecida. In addition, representatives of three other Phyllodicid families (Phyllodocidae, Syllidae and Nereididae) (Table 2) were included in this analysis. The results indicated that the three populations comprised two clades; (1) an unresolved clade including *N. polybranchia* and *Nephtys* sp. as sister taxa and (2) *N. oligobranchia*. A sister relationship between *Nephtys* sp. and *N. polybranchia* was strongly supported (BP=100) which agreed with morphological studies indicating that these two species had similarly shaped branchia on the parapodium. The distance between clades (1) and (2) showed a divergence of about 2.095%. The distance between *Nephtys* sp. and *N. polybranchia* was 0.014%. Thus, unidentified *Nephtys* sp. may be closely related to *N. polybrabchia*. *Nephtys* sp. may have been formed from morphological abnormalities, adaptation due to the environment or sympatric speciation as mentioned in benthic Foraminifers (Ballent & Carignano, 2008).

The nuclear ribosomal ITS1 is accepted as a powerful marker, with sufficient variation for inferring phylogenetic relationships among major clades and good potential for inferring phylogenetic relationships below the genus level among the most closely related species (Chu, Li, & Ho, 2001). However, due to the limited sequence data available for the ITS1 of the Nephtyidae family compared with our results, further study is required for the development of taxonomic markers.

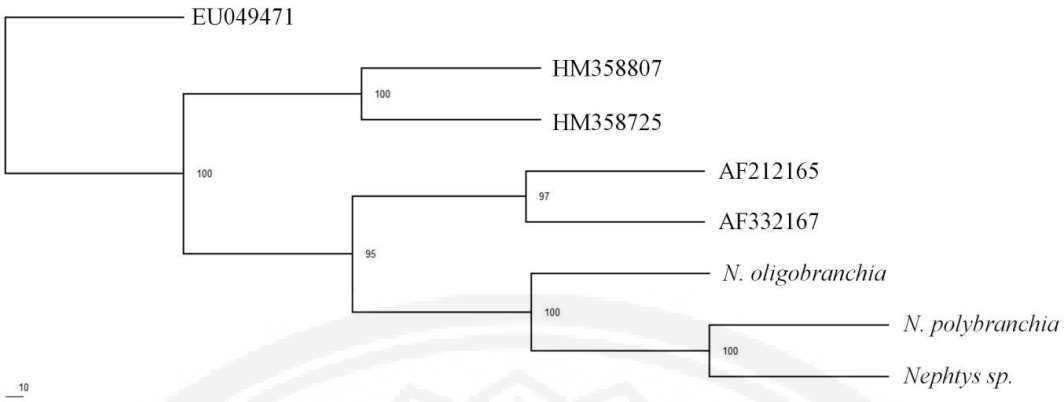

Figure 3 Phylogenetic analysis of sequences of ITS1 fragments. Reference sequences are shown with their Genbank accession numbers. The tree was constructed by bootstrap 1,000 using neighbor-joining method. The bootstrap values were shown to the right of the node.

Table 2 Accession numbers for polychaete included in the ITS1 sequence analysis

Class	Order	Family	Scientific name	Accession numbers
Polychaeta	**Scolecida**	Orbiniidae	*Pettibonella multiuncinata*	EU049471
	Phyllodocida	Phyllodocidae	*Eumida merope*	HM358807
			Sige fusigera	HM358725
		Syllidae	*Proceraea cortuna*	AF212165
		Nereididae	*Perinereis sp.* Chuwei	AF332167

Conclusion and Suggestion

The sequences of ITS1 of the Nephtyidae from Songkhla Lake revealed that *N.oligobranchia* and *N. polybranchia* were distinct and *N. polybranchia* and *Nephtys* sp. were sister taxa. However, this study was only a preliminary analysis of nuclear markers. Further research is necessary to determine whether molecular classification requires more markers using techniques such as well-conserved mitochondrial gene markers, SSRs (Microsatellite or simple sequence repeats) which have high polymorphic information content, or the single-copy sequence characterised amplified regions (SCAR). Finally, we hope that the information presented here will be useful as a molecular marker for *Nephtys* sp.

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