

**MOLECULAR IDENTIFICATION OF FREE-LIVING MARINE NEMATODE SPECIES OF THE FAMILIES DESMODORIDAE AND MICROLAIMIDAE (NEMATODA: DESMODORIDA) FROM THE TIEN YEN MANGROVE, QUANG NINH PROVINCE**

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Morphological identification of free-living nematodes is fragrantly difficult (Derycke *et al.*, 2010) because of the morphological variation, the paucity of easily scorable diagnostic morphological characteristics or a complex evolution origin of these organisms (De Ley *et al.*, 2005; Derycke *et al.*, 2010). As an alternative to the morphological determination, molecular information is expected to provide better resolutions and typification of marine nematodes, especially molecular barcoding, a method using one or more parts of the genome as an identifying tag (Hebert *et al.*, 2003). Molecular barcodes can be preferable to morphological identification because they allow the analysis of all stages of all species, leading to unequivocal “molecular species” assignation and identification of novel taxa (Jörger & Schrödl, 2013). The data can be used for downstream analyses of phylogeny, taxonomy, abundance and diversity estimation, and biomonitoring.

Although the important roles of molecular information of free-living marine nematodes are undeniable, their molecular data are still limited. In Vietnam, there was only a molecular study of the family Comesomatidae was published (Nguyen *et al.*, 2013). In order to contribute to the molecular data of free-living marine nematodes communities in the mangroves of Vietnam, this study was implemented at the Tien Yen district mangrove which is under the administration of the Quang Ninh Province, Northeastern Vietnam. In this study, DNA barcoding approach using 18S rDNA and the mitochondrial cytochrome oxidase c subunit 1 was developed to assess the morphological based identification success of free-living marine nematode species of the families Desmodoridae and Microlaimidae from this mangrove.

**I. METHODS**

**1. Sampling**

Sediment of the samples from intertidal area in the Tien Yen mangrove was randomly taken using Perspex hand cores in April, 2015. After collecting, all samples were decanted and immediately fixed in the field with DESS solution (DMSO 20%, 0.25 M disodium EDTA, and saturated with NaCl, pH 8.0 (Yoder *et al.*, 2006).

**2. Ludox Extraction and digital photographic vouchers**

In the laboratory, the samples were extracted by floatation and centrifugation adding decantation with Ludox – TM50 (Heip *et al.*, 1985). The extracted nematodes were washed carefully with tap water and fixed again with DESS solution.

In each sample, nematodes were picked out randomly and rinsed (3x) with sterile water to remove traces of DESS before subsequent identification and measurement.

After vouchering, each nematode was collected from the temporary slide, then transferred to a sterile micro-centrifuge tube containing 20 µl of Worm Lysis Buffer (WLB) (50 mM KCL, 10mM Tris pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.45% NP 40 (Tergitol Sigma), and 0.45% Tween 20) and stored at -20°C for further processing.

### 3. DNA extraction, amplification, and sequencing

Proteinase K (10mg/ml) was used to extract DNA. The I3-M11 partition of the mitochondrial Cytochrome Oxidase c subunit 1 was amplified with primer set of forward primer JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (Bowles *et al.*, 1992) and reverse primer JB5 (5'-AGCACCTAAACTTAAACATAATGAAAATG-3') (Derycke *et al.*, 2005) as they showed high amplification success (Derycke *et al.*, 2012).

The F04 partition ca. 900 bp of the 18S rDNA (small subunit or SSU) was amplified using the primer set of G18S4 forward (5'-GCTTGTCTCAAAGATTAAGCC-3') and 4R reverse (5'-GTATCTGATCGCKTCGAWC-3') (Creer *et al.*, 2010).

Each 25 µl PCR reaction mixture for each primer set consisted of 1 µl template DNA and 24 µl of mixture containing 2.5 µl of 10x PCR buffer (15 mM MgCl<sub>2</sub> (Qiagen), 2.5 µl loading dye (Qiagen), 2 µl MgCl (25 mM), 0.5 µl dNTP (10 mM), 0.125 µl of each primer (25 nM), 0.125 µl TopTaq DNA polymerase (Qiagen), 16.125 µl PCR grade water. PCR mixtures were mixed, centrifuged and put in thermo-cycler.

PCR products were loaded on agarose gels to test amplification success. After verification in agarose gel, PCR products were sent to MACROGEN Inc for bidirectional sequencing using the same primers as for the PCR.

### 4. Sequence quality control

Sequences were checked for ambiguities and errors using DNASTAR Lasergene SeqMan Pro v7.1.0. The sequences were subsequently aligned in MEGA v6.0. In case of COI sequences, translating sequences into amino acids provide a quality control of the obtained sequences. Translated sequences were checked for the presence of frame shift mutations or stop codons. In case of 18S rDNA sequences, Gblocks program was employed to eliminate poorly aligned position and divergent regions of an alignment of DNA (gaps). Only positions where 50% or more of the sequences have a gap were treated as a gap position and deleted. Thus, positions with a gap in less than 50% of the sequences can be selected in the final alignment if they were within an appropriate block.

In order to validate the sequences, the neighbour-joining (NJ) trees were constructed in MEGA v6.0 using the pairwise distance method. The NJ trees were validated with bootstrap analysis of 1000 replicates.

### 5. Intra- and interspecific genetic distances

Intra - and interspecific genetic distances were estimated using pairwise distance implemented in Species Identifier of TaxonDNA v1.6.3.

## II. RESULTS

### 1. Amplification and sequencing success

Based on morphological identification, 24 specimens of 7 putative species of 4 genera belonging to two families Desmodoridae and Microlaimidae were screened and tested for amplification and sequencing success (Table 1).

The primer set G18S4-4R showed a relatively high success rate of 67% in terms of amplification. Of the 24 specimens, 8 specimens did not produce any product while others showed consistent amplification with strong bands, for instance *Metachromadora* sp., *Calomicrolaimus* sp.. Sequencing success of 18S rDNA was 93.8% (15 of 16 specimens).

The amplification success with the primer set JB3-JB5 was low with 29.1% (7 of 24 sequences). No amplification was observed in *Desmodora* spp. The I3-M11 partition was sequenced in both directions with a success rate of 85.7%.

Table 1

**List of species including specimen's number and their amplification success (x)**

Family	Species name	18S	COI	DNA code	Species name	18S	COI	DNA code
Desmodoridae	<i>Desmodora</i> sp.1	x		4TY	<i>Desmodora</i> sp.2			7TY
	<i>Desmodora</i> sp.2	x		9TY	<i>Desmodora</i> sp.2	x		16TY
	<i>Desmodora</i> sp.2	x		18TY	<i>Desmodora</i> sp.2	x		19TY
	<i>Desmodora</i> sp.2			20TY	<i>Desmodora</i> sp.2	x		21TY
	<i>Desmodora</i> sp.2			23TY	<i>Desmodora</i> sp.2			24TY
	<i>Metachromadora</i> sp.1			5TY	<i>Metachromadora</i> sp.1	x	x	6TY
	<i>Metachromadora</i> sp.1	x	x	8TY	<i>Metachromadora</i> sp.1	x	x	10TY
	<i>Metachromadora</i> sp.1	x	x	11TY	<i>Metachromadora</i> sp.1	x	x	12TY
	<i>Metachromadora</i> sp.2			13TY	<i>Metachromadora</i> sp.2			14TY
	<i>Metachromadora</i> sp.2			15TY	<i>Metachromadora</i> sp.3	x		17TY
	<i>Metachromadora</i> sp.3	x		22TY				
Microlaimidae	<i>Calomicrolaimus</i> sp.	x	x	2TY	<i>Calomicrolaimus</i> sp.	x	x	3TY
	<i>Microlaimus</i> sp.	x		1TY				

## 2. Phylogenetic relationships

The neighbor-joining (NJ) tree of 18S rDNA (Figure 1) showed a topology congruent with that known for families Desmodoridae and Microlaimidae. Two genera *Metachromadora* and *Desmodora* of the family Desmodoridae were clustered, and two genera *Microlaimus* and *Calomicrolaimus* were found in a clade of Microlaimidae.

The NJ tree of COI (Figure 2) was recovered (more than 90%) almost all morphological based species. However, four specimens 6TY, 8TY, 11TY and 12TY which were identified as *Metachromadora* sp1. were split into two clades.

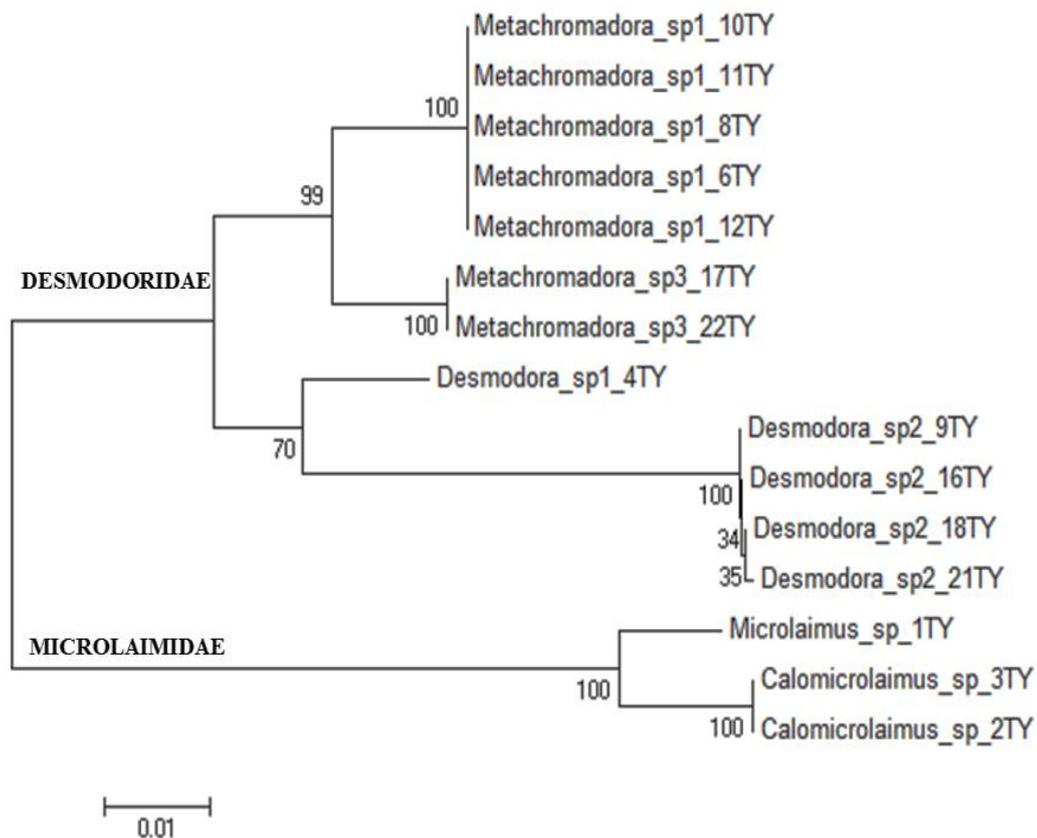
## 3. Genetic distances and variability of the 18S rDNA and COI partition

Variability of 18S was calculated using 15 sequences with the length of 685 nucleotides. The average proportions of nucleotides were 26.7% T, 18.6% C, 27.5% A, and 27.1% G. of 685 nucleotides, 105 were variable, accounting for 15.3%.

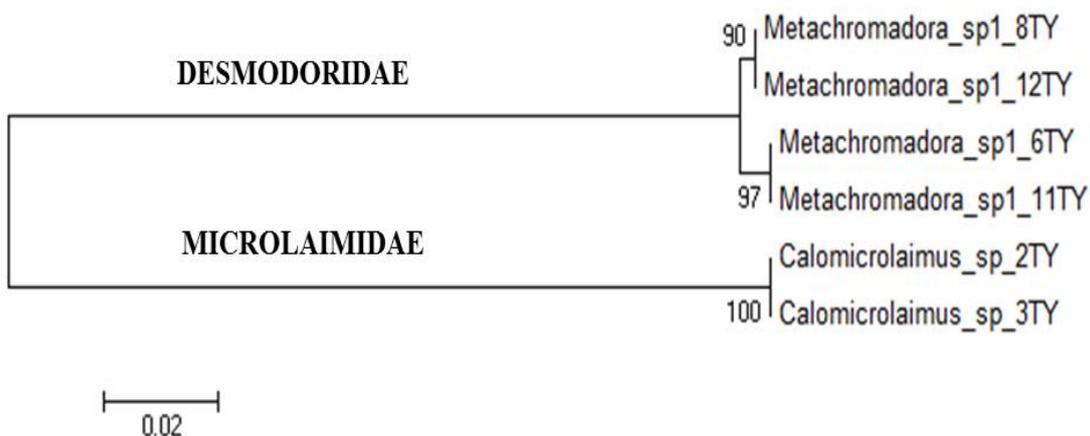
All maximum pairwise distances within species (intraspecific distance) of 18S were 0.0%. Minimum pairwise distances between species (interspecific) of 18S ranged from 2.0% to 5.5%. Therefore, 100% of all interspecific comparisons were higher than 1.0%.

Variability of COI was observed at the nucleotide level with 104 out of 405 nucleotides (25.7%) being variable. I3-M11 partition was highly AT-rich (A: 27.0%, T: 41.1%, G: 20.4% and C: 11.5%).

Maximum pairwise distances within species (intraspecific distance) varied between 0.0%-0.49%. *Calomicrolaimus* sp. showed the lowest pairwise distance of 0.0%. *Metachromadora* sp.1 showed an intraspecific distances of 0.49%.



**Figure 1: Bootstrap neighbor-joining tree of 18S sequences based on pairwise distance. Sequences are named by combining the name of species and DNA code**



**Figure 2: Bootstrap neighbor-joining tree of COI sequences based on pairwise distance. Sequences are named by combining the name of species and DNA code**

### III. DISCUSSION

#### 1. Effectiveness of using G18S4-4R and JB3-JB5 primer set on the families Desmodoridae and Microlaimidae

The results of our study on two families Desmodoridae and Microlaimidae showed that the amplification success of 18S was relatively high but lower than that of Bhadury *et al.* (2008) which covered the wider range of marine nematode species. The amplification of COI using JB3-JB5 primer set showed a much lower success rate (29.1%) in comparison with that of 87.8% in the study of Derycke *et al.* (2010). Particularly, no amplification was observed in *Desmodora* spp. and *Microlaimus* sp. although we have several specimens of those genera with good amplification success for 18S. It indicates that the primer set JB3-JB5 may not be suitable for these genera and capturing different nematodes taxa require different primer sets. Notably, no amplification of either 18S or COI was observed in *Metachromadora* sp.2 pointed out that their cuticle might inhibit the effect of proteinase K. Both 18S rDNA gene and the I3-M11 partition in our study showed equally high sequencing success rate. Therefore, in terms of amplification and sequencing success, G18S4-4R primer set is better to cover a wider range of species since COI showed significantly low amplification success. It was also confirmed in the studies of Bhadury *et al.* (2006), Creer *et al.* (2010) and Porazinska *et al.* (2010).

#### 2. Species identification of the families Desmodoridae and Microlaimidae using 18S rDNA and I3-M11 partition of COI

The majority of apparent nematode specimens identified using diagnostic morphological characters were relatively consistent at both 18S rDNA and COI level. The NJ tree based on both 18S and COI clearly recovered two distinct lineages corresponding to two families Desmodoridae and Microlaimidae in our data set. Both genera of those families were recovered as monophyletic groups. At species level, 18S did not fully support morphological based identification in case of *Desmodora* sp.2. Notably, 18S recognised all specimens of *Metachromadora* sp.1. as one species but COI recognised them as two different species. Therefore, based on morphological identification and 18S, four specimens 6TY, 8TY, 11TY and 12TY were recognized as *Metachromadora* sp.1. whereas based on COI, they might belong to two different populations. This showed that sequence variation in a partial segment of the mitochondrial COI gene was more effective for identifying species within *Metachromadora* than that of 18S.

The applicability of COI to recognize and identify closely related marine nematode was studied by Derycke *et al.* (2010). In our study, 100% of all intraspecific comparisons showed genetic distances <0.05. This result was similar to that of Derycke *et al.* (2010). However, we need more specimens of nematode species to calculate interspecific distances to have more adequate results.

Barcoding marine nematodes would clearly benefit from a multilocus approach where the large database of 18S gene would provide a solid taxonomic framework and where I3-M11 partition would allow identification to species level (Derycke *et al.*, 2010). Depending on the purposes of the study which considers species or higher level, 18S or COI can be used.

### IV. CONCLUSION

When considering amplification success, sequencing success, and phylogenetic relationship in our study, 18S is still a reliable marker for phylogenetic resolution at the genus and family level while I3-M11 partition of COI efficiently distinguished nematode species.

Our results asserted that both 18S and COI supported effectively the morphological identification, and the data generated here form an important contribution to a reliable reference database that will advance the identification of marine nematodes through DNA barcoding for future analysis of biodiversity.

**Acknowledgements:** *We would like to express our great gratitude to the Vietnam Academy of Science and Technology (VATS) and the Institute of Ecology and Biological resources (IEBR) under grant number IEBR.CBT.ThS07/2015 and Vietnam National Foundation for Science and Technology Development (Nafosted) under grand number 106.12-2012.31.*

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**ĐỊNH DANH BẰNG PHƯƠNG PHÁP PHÂN TỬ MỘT SỐ LOÀI  
TUYẾN TRÙNG BIỂN THUỘC HỌ DESMODORIDAE VÀ MICROLAIMIDAE  
(NEMATODA: DESMODORIDA) TẠI  
RỪNG NGẬP MẶN TIÊN YÊN, TỈNH QUẢNG NINH**

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**TÓM TẮT**

Phương pháp mã vạch phân tử sử dụng 18S ADN<sub>r</sub> và vùng I3-M11 của gen COI được áp dụng trong việc nghiên cứu tuyến trùng biểnn thuộc hai họ Desmodoridae và Microlaimidae tại rừng ngập mặn Tiên Yên. Mẫu được thu vào tháng 4/2015 tại vùng triều của rừng ngập mặn, lọc và cố định trong dung dịch DESS. Sau khi được định danh bằng phương pháp hình thái học, các mẫu được tách chiết ADN, khuếch đại và giải trình tự. Kết quả PCR cho thấy, các loài thuộc họ Desmodoridae và Microlaimidae có tỉ lệ thành công của 18S cao hơn COI nhưng đều thấp hơn các kết quả nghiên cứu trước đó về tuyến trùng biểnn. Tỉ lệ thành công của việc giải trình tự đều rất cao ở cả 18S và COI. Dựa trên việc phân tích cây phả hệ, việc giải trình tự 18S cho thấy độ tin cậy trong việc xem xét phát sinh chủng loài ở mức độ họ và giống trong khi COI lại cho thấy hiệu quả trong việc phân loại đến cấp độ loài. Nhìn chung 18S và COI đều khẳng định kết quả phân loại dựa trên hình thái trừ trường hợp của *Metachromadora* sp.1.