A SUPPLEMENT TO MOLECULAR DATA FOR FIVE FREE-LIVING MARINE NEMATODE SPECIES OF THE FAMILY COMESOMATIDAE FILIPJEV, 1918 (NEMATODA: CHROMADORIDA) FROM NORTH VIETNAM

Nguyen Dinh Tu[1]*, Nguyen Thanh Hien[1], Nguyen Vu Thanh[1], Phan Ke Long[2], A. V. Tchesunov Alexei[3]

[1]Institute of Ecology and Biological Resources, VAST, *ngdtu@yahoo.com
[2]Vietnam National Museum of Natural, VAST
[3]Moscow Lomonosov State University, Russia

ABSTRACT: Five species of Comesomatidae: *Dorylaimopsis halongensis*, *Hopperia dolichurus*, *Paracomesoma lissum*, *Sabatieria doancanhi* and *Sabatieria praedatrix* were identified based on morphological characters of males. To expose the systematic position of these comesomatids, we obtained nucleotide sequences of nuclear ribosomal DNA (D2/D3 and ITS region). The results showed the clear molecular differences between species in the Comesomatidae that proved to the morphology data.

Keywords: Comesomatidae, D2D3, ITS, marine nematodes, Ba Lạt.

INTRODUCTION

The systematics of Comesomatidae was reviewed by several authors as de Coninck (1965) [1]; Vitiello (1969) [19]; Jensen (1979) [11]; Platt (1985) [16]; Lorenzen (1994) [14]; Smolyanko & Belogurov (1991) [17] and Hope & Zhang (1995) [10]. According to Jensen (1979) [11] the Comesomatidae included three subfamilies, such as Sabatierinae Filipjev, 1934; Dorylaimopsinae de Coninck, 1965 and Comesomatinae Filipjev, 1918. In a molecular comparison of the D3 expansion segment (26/28S ribosomal RNA gene), Litvaitis et al. (2000) [12] concluded that the Comesomatidae comprised a sister group to the Monhysterida, yet they placed them in the Chromadorida because they considered their molecular trees to be equivocal. In a recent review of nematode systematic position conducted by De Ley and Blaxter (2004) [2] and based on new results on combining morphological and molecular characteristics and phylogeny evaluation of the Comesomatids these authors assigned Comesomatidae to the order Areolaimida. In this paper, first results combining morphological traits and molecular characteristics of four marine species of the family Comesomatidae family recently described in Vietnam, *Dorylaimopsis halongensis* Nguyen Dinh Tu et al., 2008, *Hopperia dolichurus* Gagarin & Nguyen Vu Thanh, 2006, *Paracomesoma lissum* Gagarin & Nguyen Vu Thanh, 2009, *Sabatieria doancanhi* Nguyen Dinh Tu et al., 2008 and one *Sabatieria praedatrix* de Man, 1907 are presented.

MATERIALS AND METHODS

Sampling: Sediment samples from intertidal area in the Xuan Thuy national park areas in 2011 and 2012 were taken by PONNAR grab (20 cm × 20 cm surface). Sediment from each site was taken with a depth of 10 cm with Perspex core (3.5 cm in diameter and 40 cm in length) and immediately fixed in DESS solution (dimethylsulfoxide (20%) diluted in distilled water, with EDTA salt 0.25 m, NaOH and saturated with NaCl).

Sample processing: Sediment was sieved through 1 mm mesh size (to separate the coarse shells and plant remains from the sediment). The samples then were rinsed with tap water in a 5 liter beaker. After settlement (10 seconds) the supernatant was poured through a 63 µm. The rinsing and decantation were repeated 3 times until the water became clear.

After decantation, the sample consisting of a small amount of material was carefully washed bringing the extracted portion of the sediment to one side of the sieve. Then it was washed into a large beaker using LUDOX TM50 specific gravity of 1.18 g/ml. At least 3 times the sample

volume of Ludox solution was added, and stirred. Then it was left to settle for at least 40 minutes. Finally, the supernatant was carefully poured through a 40 µm sieve. This process was repeated 3 times. The extracted nematodes was washed thoroughly with tap water and then preserved with DESS solution in a suitable container.

Nematode isolation and vouchering: Identification of species and genera was done by an expert nematode taxonomist using an Axioscope Plus II research microscope. Digital photographic vouchers representing head, body surface and tail regions of each specimen were taken at small, intermediate and immersion oil magnification. Immediately after the vouchering procedure, nematodes were collected from the temporary slide, put in lysis buffer and stored at -20^{o}C until further processing.

Molecular analyses of captured specimens: *DNA extraction*: Immediately after vouchering, DNA was extracted by cutting each nematode into several pieces in 20 µl of Worm Lysis Buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl2, 0.45% NP, 0.45% Tween 20), transferring them to one or two sterile 0.5-mL centrifuge tubes and digesting them for 1 h at 65^{o}C and for 10 min at 95^{o}C with 2 µl of Proteinase K (10 mg⁄ml). Tubes were centrifuged at maximal speed (20817 g) for 1 min and stored at 80^{o}C.

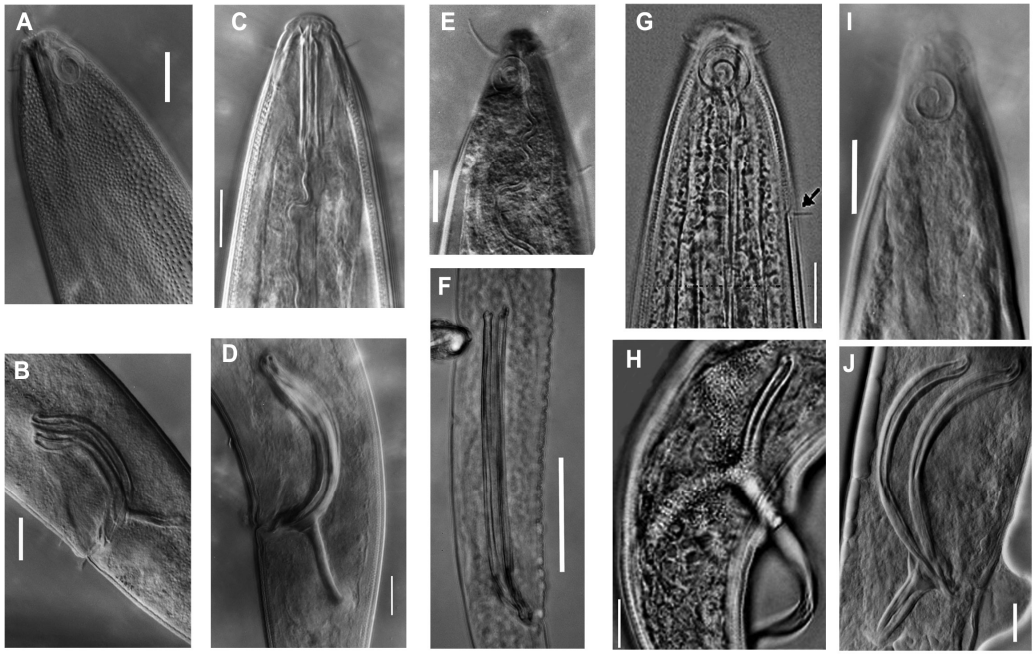

Figure 1. Head and spicule region of a male of *Dorylaimopsis halongensis* Nguyen Dinh Tu et al., 2008 (A, B), *Hopperia dolichurus* Gagarin & Nguyen Vu Thanh, 2006 (C, D), *Paracomesoma lissum* Gagarin & Nguyen Vu Thanh, 2009 (E, F), *Sabatieria doancanhi* Nguyen Dinh Tu et al., 2008 (G, H) and *Sabatieria praedatrix* de Man, 1907 (I, J).
Scale bars: A- E, G - J = 10 µm; F = 50 µm.

PCR for phylogenetic analyses: The D2D3 region of the 28S ribosomal subunit was amplified with primers D2A (5'- ACA AGT ACC GTG AGG GAA AGT TG) and D3B (3' - TCC TCG GAA GGA ACC AGC TAC TA) as in Derycke et al. (2008) [2]. The Toptaq PCR mix was used, and thermocycling conditions were: 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 s, 56^{o}C for 30 s and 72^{o}C for 2 min; and 72^{o}C for 10 min. A fragment of the ITS region of the 28S ribosomal subunit was amplified with primers Vrain 2F (5' - CTT TGT ACA CAC CGC CCG

TCG CT) and Vrain 2R (3'- TTT CAC TCG CCG TTA CTA AGG GAA TC) as in Derycke et al. (2008) [2]. The Toptaq PCR mix was used, and thermocycling conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s; and 72°C for 10 min.

Data analysis: Sequences of comesomatid species from Vietnam were aligned using Clustal X 1.64. Equally weighted maximum parsimony (MP) analysis was performed using PAUP* (4.0 beta version). A heuristic search procedure was used with the following settings: ten replicates of random taxon addition, tree-bisection reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap analysis was carried out with 100 replicates.

RESULTS AND DISCUSSION

Morphological data and DNA sequence data were obtained for five comesomatid species, *Dorylaimopsis halongensis* Nguyen Dinh Tu et al., 2008; *Hopperia dolichurus* Gagarin & Nguyen Vu Thanh, 2006; *Paracomesoma lissum* Gagarin & Nguyen Vu Thanh, 2009; *Sabatieria doancanhi* Nguyen Dinh Tu et al., 2008 and *Sabatieria praedatrix* de Man, 1907.

*Table 1.*Morphometric data and accessions number on GenBank of the five species of the family Comesomatidae from Vietnam (all measurements in µm except ratios)

Accessions number on GenBank	Species				
	Dorylaimopsis halongensis	*Hopperia dolichurus*	*Paracomesoma lissum*	*Sabatieria doancanhi*	*Sabatieria praedatrix*
	JX040634	JX512280	JX512278	JX512281	JX512279
Species measurement (Max - Min; n = 3)					
Total body length	2057-2098	2150-2301	1468-1504	2135-2276	2786-2957
a	57.5-58.8	48.0-51.0	32.4-33.6	46.6-47.7	57.5-61.6
b	6.6-9.9	11.4-12.0	9.0-9.2	11.0-12.0	9.2-9.6
c	5.5-5.7	9.5-10.6	10.0-10.1	14.3-16.0	11.1-12.6
c'	13.7-14.7	6.2-6.7	4.6-4.9	3.2-3.6	4.8-5.5
Head diameter	10-11	10.2-12	8.2-9	12.5-14.5	12.6-14.1
Cephalic setae	4.8-5	4.2-4.5	6.8-7.5	5-5.3	4.8-5.6
Amphid width	8.5-10.2	10.8-11.3	10.3-11	8.6-9.8	7.8-8.4
Pharynx length	211.5-312	189-196	159-167	184.5-199.2	289-318
Maximal body diameter	35-36.5	44.8-45.2	44.8-45.3	45.8-47.7	47.9-49.8
Spicule length	45.3-46.8	56.2-57.1	95.6-98.3	89.9-92.4	64-69
Gubernaculum length	14.5-16.1	21.1-22.5	23.5-26.1	41.2-43.3	32.4-35.2
Tail length	368.5-375.6	215-226	146-150	142.4-149.8	235-256
Anal diameter	25.6-27	33.6-34.8	30.6-32.8	41.6-45.2	46.5-49.3

Sequence analyses of D2/D3 region

The D2D3 region of species in the family Comesomatidae ranged from 721bp (*P. lissum*) to 738bp (*S. doancanhi*) in which species in the *Sabatieria* genus ranged from 735bp (*S. praedatrix*) to 738bp (*S. doancanhi*) (table 2). The D2D3 region exhibited the base composition as follow: A - 24 (21-27), C - 24 (21-28), G - 32 (28-35), T - 20 (16-24).

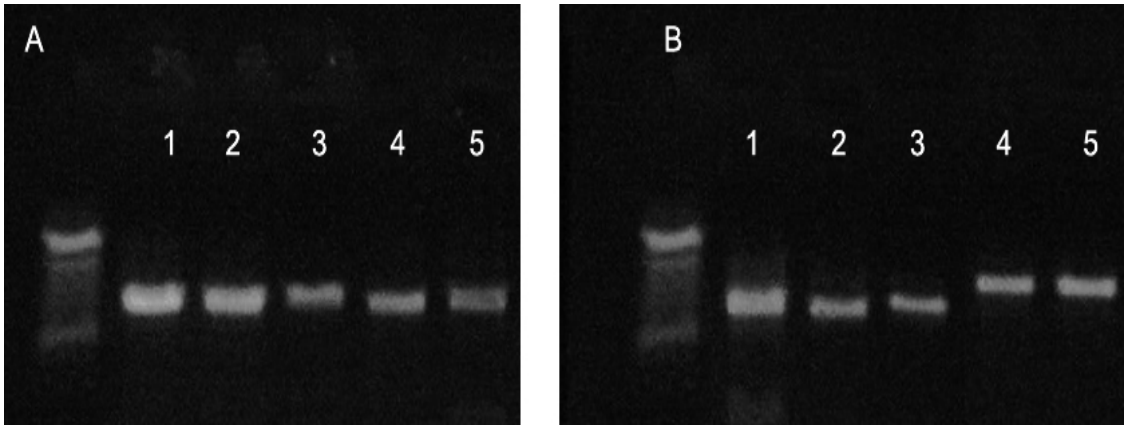

Figure 2. PCR product of amplified D2D3 (A) and ITS (B) region of *Dorylaimopsis halongensis* (lane 1), *Hopperia dolichurus* (lane 2), *Paracomesoma lissum* (lane 3), *Sabatieria doancanhi* (lane 4) *and Sabatieria praedatrix* (lane 5).

Table 2. The base composition and the length of the D2D3 region of the species in the family Comesomatidae in Vietnam

Species	Base composition (%)				D2D3 length (bp)
	A	C	G	T	
D. halongensis	23	23	32	22	731
S. praedatrix	21	28	35	16	735
S. doancanhi	23	26	35	17	738
P. lissum	27	21	28	24	721
H. dolichurus	26	23	30	22	730
Average	24	24	32	20	731

Table 3. Pairwise distance between species in the Comesomatidae family in Vietnam based on D2D3 sequences (below diagonal: total character differences, above diagonal: mean character differences adjusted for missing data)

No	Species	1	2	3	4	5
1	*H. dolichurus*	-	21.0	30.9	28.3	16.7
2	*S. praedatrix*	153	-	28.5	13.0	19.3
3	*P. lissum*	221	204	-	32.9	31.3
4	*S. doancanhi*	205	95	235	-	26.3
5	*D. halongensis*	122	141	224	191	-

S. doancanhi differed from *S. praedatrix* by 95 nucleotides. *P. lissum* differed from *S. doancanhi* by 235 nucleotides. The divergence between taxa ranged from 13-32.9% (table 3).

The MP analysis of D2D3 region indicated that among 750 characters, 127 were parsimony informative and obtained a single tree (tree length = 750) (fig 3). *S. praedatrix* clustered with *S. doancanhi* with high bootstrap support (100%) and had sister relationship with *P. lissum* (bootstrap 98%).

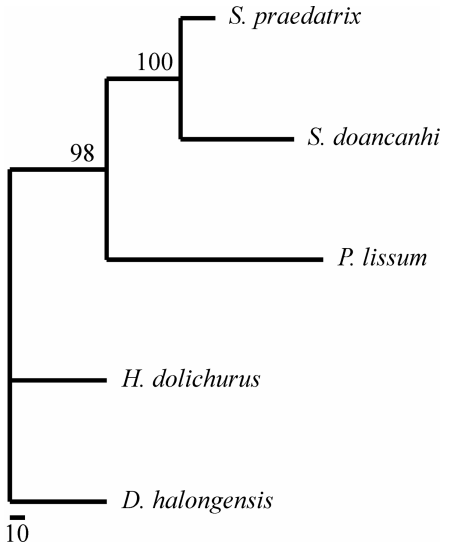

Figure 3. The phylogenetic relationship of species in the Comesomatidae family in Vietnam based on D2D3 sequences. The single MP tree (tree length = 750)

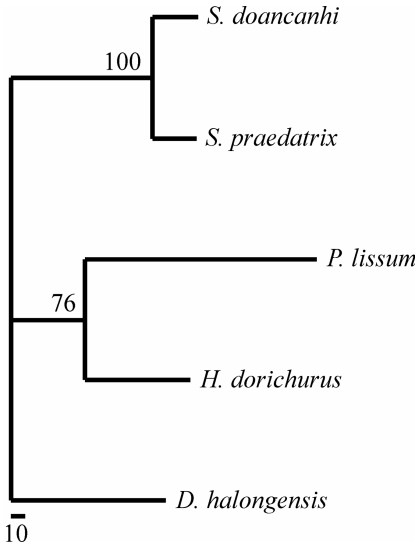

Figure 4. The phylogenetic relationship of species in the Comesomatidae in Vietnam based on ITS sequences. The single unrooted tree (tree length = 874)

Sequence analyses of ITS region

The length of partial 18S, ITS1, 5.8S, ITS2 and partial 28S of species in the Comesomatidae family ranged from 837bp (*P. lissum*) to 861bp (*S. praedatrix*); the species in the genus *Sabatieria* were 856bp (*S. doancanhi*) and 861bp (*S. praedatrix*) (table 4).

The Adenine composition in the ITS region of species in the Comesomatidae family ranged from 22% (*S. doancanhi*) to 28% (*P. lissum*); Cytosine composition was lowest in *P. lissum* (22%), highest in *S. doancanhi* and *S. praedatrix* (28%); Guanine composition was lowest in *P. lissum* (24%), highest in *S. praedatrix* (31%); Thymine composition was lowest in *S. praedatrix* (19%), highest in *P. lissum* (26%) (table 4).

Table 4. The base composition and the length of partial 18S-ITS1-5.8S-ITS2-28S partial of the species in the Comesomatidae family in Vietnam

Species	Base composition (%)				ITS length (bp)
	A	C	G	T	
D. halongensis	24	27	27	23	858
S. doancanhi	23	28	30	20	856
S. praedatrix	22	28	31	19	861
P. lissum	28	22	24	26	837
H. dorichurus	26	25	25	24	841
Average	24	26	28	23	850.6

Two species in the *Sabatieria* genus (*S. doancanhi* và *S. praedatrix*) had lowest divergence (7.1%) that equivalent to 61 nucleotides. The highest divergence was 30.8% between *P. lissum* and *D. halongiensis* (255 nucleotides). *H. dolichurus* had lowest divergence (20.8%) compare with *D. halongiensis* (173 nucleotides) and highest divergence (28.1%) compare with *P. lissum* (233 nucleotides).

Table 5. Pairwise distance between species in the Comesomatidae family in Vietnam based on ITS sequences (below diagonal: total character differences, above diagonal: mean character differences adjusted for missing data)

No	Species	1	2	3	4	5
1	*D. halongensis*	-	23.9	23.4	30.8	20.8
2	*S. doancanhi*	202	-	7.1	29.7	23.8
3	*S. praedatrix*	199	61	-	29.5	23.7
4	*P. lissum*	255	246	246	-	28.1
5	*H. dorichurus*	173	198	199	233	-

Maximum Parsimony (MP) analysis of ITS of species in the Comesomatidae family in Vietnam indicated that among 874 characters, 141 characters were pasimony informative. In the single MP unrooted tree (fig. 4), *S. praedatrix* clustered with *S. doancanhi* (bootstrap 100%) and *H. dolichurus* clustered with *P. lissum* (bootstrap 76%) and *D. halongiensis* located in the base of the tree.

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ĐẶC ĐIỂM PHÂN TỬ CỦA NĂM LOÀI TUYẾN TRÙNG BIỂN SỐNG TỰ DO THUỘC HỌ COMESOMATIDAE FILIPJEV, 1918 (NEMATODA: CHROMADORIDA) Ở MIỀN BẮC VIỆT NAM

Nguyễn Đình Tứ[1], Nguyễn Thanh Hiền[1], Phan Kế Long[2], A. V. Tchesunov Alexei[3], Nguyễn Vũ Thanh[1]

[1]Viện Sinh thái và Tài nguyên Sinh vật, Viện Hàn lâm KH & CN Việt Nam
[2]Bảo Tàng thiên nhiên Việt Nam, Viện Hàn lâm KH & CN Việt Nam
[3]Đại học tổng hợp quốc gia mang tên Lômônôxop (MGU), Matxcova, CHLB Nga

TÓM TẮT

Năm loài tuyến trùng biển sống tự do ở vùng nước ven bờ ở các tỉnh phía Bắc Việt Nam là những phát hiện mới cho khu hệ Việt Nam và đã được mô tả gần đây là các loài: *Dorylaimopsis halongensis* Nguyen Dinh Tu et al., 2008; *Hopperia dolichurus* Gagarin & Nguyen Vu Thanh, 2006; *Paracomesoma lissum* Gagarin & Nguyen Vu Thanh, 2009; *Sabatieria doancanhi* Nguyen Dinh Tu et al., 2008 và *Sabatieria praedatrix* de Man, 1907 dựa trên các đặc trưng cơ bản về các sai khác rất lớn trong hình thái học của chúng với các loài đã biết. Nhằm xác định chính xác vị trí phân loại của các loài tuyến trùng nói trên trong phả hệ của nhóm tuyến trùng Comesomatids, chúng tôi đã tiến hành các nghiên cứu chuỗi đặc trưng phân tử nhóm nucleotides ribosome DNA (D2/D3 28S và ITS). Kết quả nghiên cứu về sinh học phân tử một lần nữa khẳng định các loài bắt gặp ở Việt Nam hoàn toàn là ghi nhận mới và khác biệt so với các loài đã biết trong họ Comesomatidae

Từ khóa: Comesomatidae, D2D3, ITS, tuyến trùng biển, Ba Lạt.

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