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NEW RECORD OF PEANUT WORM *Sipunculus nudus* FROM THAILAND

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ABSTRACT: Modtanoy Beach (Trang, Thailand) is a commercial source of peanut worms, mainly collected for fishing bait, but species identifications have never been reported. Thus, peanut worm specimens from Modtanoy Beach were sampled and identified. They were all identified as *Sipunculus nudus* Linnaeus, 1776, based on morphology according to dichotomous keys of the Sipuncula. The specimens were highly similar in character to those described in the published records of *S. nudus*. However, morphological differences were found between the *S. nudus* from Thailand in this study and those reported from 11 other localities around the world. Comparative sequence analyses of 16S rRNA and cytochrome c oxidase subunit 1 (COI) gene fragments between our specimens from Thailand and those in GenBank revealed that our samples matched available sequences from *S. nudus*, while phylogenetic analyses, based upon maximum likelihood and Bayesian inference, using the concatenated 16S and COI gene fragment sequences, revealed that these Thai specimens fell within *S. nudus* but were separate from other geographic regions, including the Chinese and Vietnamese clades, supporting the morphological differences, yet limited by the absence of sequences for related species in the same genus, such as *S. robustus*, *S. thailandicus* and *S. gulfus*. Within the Thai samples collected, three and two haplotypes were observed from 11 and 12 individuals for 16S and COI gene fragments, respectively. Haplotype diversities for 16S rRNA and COI genes were 0.345 and 0.167, respectively. These results indicate a new record of *S. nudus* in Thailand.

Keywords: peanut worm, new record, *Sipunculus nudus*, Thailand

INTRODUCTION

Peanut worms are members of the Sipuncula, a distinct clade of exclusively marine invertebrate worms with a long cylindrical body plan without segmentation (Pan-Wen and Siang 2016). This Phylum consists of two classes, Phascolosomatidea and Sipunculidea, which differ in the arrangements of their tentacles. In Phascolosomatidea, the tentacles are arranged in an arc around the nuchal organ; in Sipunculidea, the tentacles are arranged around the mouth; in all sipunculans, tentacles are located on the end of a retractable introvert (Schulze *et al*. 2007).

Peanut worms have been utilized as human food and fishing bait worldwide, but their use as a research animal for monitoring marine environmental quality is potentially far more valuable. They live in tropical, temperate and polar regions, and usually burrow into sand, mud and rock crevices or in empty shells (Cutler 1994). Peanut worms are found in all the oceans from the intertidal zone to a depth of 900 m below sea level (Cutler 1994).

In Thailand, there are five families, ten genera, and twenty-four species of reported peanut worms (Stephen and Edmonds 1972; Frith *et al*. 1976; Cutler 1987, 1989; Hylleberg 1994a, 1994b, 2014). Studies on sipunculans date back to the early 20th century but are more recent in Thailand, where Frith *et al*. (1976) reported that *Phascolosoma arcuatum* was abundant in Thailand's mangroves. Stephen and Edmonds (1972) and Cutler (1987; 1989) then referred to eight valid species (six species from coral blocks and two species from

the seabed). More information was documented by Hylleberg (1994a, 1994b) who reported that eight species of sipunculans were encountered in heads of the live coral *Porites lutea*, and found nine new species in Thailand, including *Sipunculus gulfus* and *Sipunculus thailandicus* within the Genus Sipunculus, which along with *S. robustus* (Hylleberg, 2014) comprise the three known species from this genus in Thailand.

Sipunculus nudus is commonly found in the intertidal zone, especially in muddy sands and mud flats (Nguyen *et al*. 2007; Pan-Wen and Siang 2016) along the Atlantic coasts of Europe and Northern America, in the Mediterranean, and along the Pacific coasts of the USA, China, and Japan (Hylleberg 1994a, 1994b). However, no specimens of *S. nudus* had been recorded in Thailand.

Peanut worms have been exploited as an exotic cuisine and fishing bait in Asian countries including China and Vietnam. In Thailand, peanut worms are collected by local fishermen at Modtanoy Beach (Trang) on the Andaman Sea coast and sold as sport-fishing bait. In 2017, the marketable size was at least 10 g with a value of 0.30 USD. However, peanut worms can also be used as an ideal indicator species to monitor the quality of marine environments, and in particular, the bioaccumulation of heavy metals (Tan *et al*. 2013). For example, Yan and Wang (2002) reported the accumulation of three heavy metals (Cd, Cr and Zn) in the gut fluids extracted from *S. nudus* following sediment ingestion.

In total, 24 species of peanut worms have been reported in Thailand, but those at Modtanoy Beach have not been investigated. However, before they can be used as indicator species, the species identification of peanut worms must be completed, as well as the establishment of a laboratory-scale breeding system, since there are no commercial peanut worm farms in Thailand. The objective of this study was to identify the species of peanut worms at Modtanoy Beach, Thailand, using morphological taxonomy and molecular genetics.

MATERIALS AND METHODS

A total of 30 peanut worms were collected from the sandy substratum in the intertidal zone of Modtanoy Beach, Kantang District, Trang Province, Thailand (Lat. 7.307223′N, Long. 99.416662′E) at a depth of 40–50 cm. All specimens were preserved in 70% (v/v) ethyl alcohol and deposited in the Natural History Museum of Chulalongkorn University.

Morphological analysis

Morphological analysis was conducted by dorsal dissection and observation under stereoscopic light microscopy, scanning electron microscopy (SEM), and micro-computed tomography (Micro-CT). The following data were recorded: (1) number of longitudinal muscle bands (LMBs), counted at the base of the introvert retractors; (2) state of LMBs at the glans region (bifurcated versus non-bifurcated); (3) degree of nephridia attached to the body wall; (4) brain shape; and (5) shape of the digitate processes. These parameters were then compared to those in specimens of *S. nudus* from the other 11 localities reported around the world according to Kawauchi and Giribet (2013).

For the SEM analysis, the samples were preserved in 2.5% glutaraldehyde in 0.1 M phosphate buffer. Then, the specimens were post-fixed in 2% osmium tetroxide and were subsequently dehydrated by ethanol and isoamyl acetate, respectively. Then, the specimens were mounted on aluminum stubs and were coated with gold. After that, all specimens were visualized under a scanning electron microscope (JSM 5410LV; JEOL Ltd., Tokyo, Japan).

For Micro-CT analysis, the peanut worms were dehydrated in 100% ethanol and kept at a temperature of 20°C. The peanut worm sample was then sent for analysis and visualization by Bruker Biospin AG. These techniques allow detailed virtual re-constructions of the morphology and anatomy of specimens to support research in systematics and taxonomy.

Molecular analysis

Total genomic DNA was extracted from 12 randomly selected museum specimens. The tissues were incubated for 1–2 hours in lysis buffer with proteinase K at 65°C before DNA extraction using phenol-chloroform was performed in accordance with Wang *et al*. (2006). The mitochondrial 16S rRNA and cytochrome c oxidase subunit 1 (COI) gene fragments were PCR amplified in a total volume of 50 µL comprising 1 µL DNA extract and using the 16SAR (5’-CGCCTGTTTATCAAAAA-CAT-3’) and 16SBR (5’-CGGTTTGAACTCA-GATCATG-3’) primers for 16S (Xiadong *et al*. 2008), and the LCO1490 (5’-GGTCAACAAAT-

CATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers for COI (Folmer et al. 1994) amplification. The obtained PCR products were purified using the Montage PCR Cleanup Kit (Millipore) and then sequenced using an ABI 3730 automated DNA sequencer.

Multiple sequence alignments of the 16S rRNA and COI gene fragments were performed using ClustalW as implemented in the MEGA version 6.0 software (Tamura *et al*. 2013), while haplotype diversity was analyzed using the DnaSP version 4.50.3 software (Rozas *et al*. 2003). Phylogenies based on maximum likelihood (ML) and Bayesian inference (BI) analyses were performed as reported (Kawauchi and Giribet 2013) using the 103 published sequences of *Sipunculus* spp. (51 sequences for 16S rRNA and 52 sequences for COI) obtained from NCBI's GenBank (www.ncbi.nlm.nih.gov/nucleotide/) along with the sequences from the samples of this study. In addition, the sequences from a member of each of three families of Sipunculans (*Themiste pyroides*, *Golfingia elongata* and *Phascolopsis gouldii*) were used as outgroup taxa (Table 1).

The phylogenetic analyses were first performed on the 16S and COI datasets separately, and then on the combined 16S plus COI datasets. The ML analysis used the General Time Reversible (GTR) with rates among sites being Gamma distributed to Invariant sites (G+I), as determined by finding the best DNA/Protein model, while bootstrap analysis was based on 1,000 iterations and implemented in the MEGA 6.0 software. The BI analysis was executed in MRBAYES v.3.1.2 (Ronquist and Huelsenbeck 2003) after using the jModelTest version 0.1.1 software (Darriba et al. 2012) to estimate the best model for this dataset (GTR + Γ + I). The BI posterior probabilities (PP) were approximated by sampling trees using a variant of the Markov Chain Monte Carlo (MCMC) method and using 1 million generations and sampling every 100 generations. The BI analysis reached stationarity and so 1,000,000 generations (25%) were discarded as burn-in. The BI analyses additionally used the following parameters: nst = 6, rates = invgamma, and trees were summarized using the parameters sump burnin = 250 and sumt burnin = 250. Phylogenetic trees were visualized using the program Treeview (Page 1996).

Table 1. *Sipunculus nudus* and outgroup 16S and COI gene fragment sequences used in the phylogenetic analysis and their GenBank accession numbers.

Sample no.	Species	COI	16S
DNA 100234	*Sipunculus nudus* (Puerto Rico)	DQ300160	JN864959
DNA 100468	*Sipunculus nudus* (Bermuda)	DQ300162	JN864962
DNA 100993	*Sipunculus nudus* (Belize)	DQ300164	JN864964
DNA 101882	*Sipunculus nudus* (Brazil)	JN865108	JN865000
DNA 102316	*Sipunculus nudus* (Brazil)	JN865107	JN865004
DNA 103527-1	*Sipunculus nudus* (USA, Florida)	KF042451	KF042397
DNA 103527-2	*Sipunculus nudus* (USA, Florida)	KF042452	KF042398
DNA 103527-3	*Sipunculus nudus* (USA, Florida)	KF042453	KF042399
DNA 103527-4	*Sipunculus nudus* (USA, Florida)	KF042454	KF042300
DNA 103527-5	*Sipunculus nudus* (USA, Florida)	KF042455	KF042301
DNA 103527-6	*Sipunculus nudus* (USA, Florida)	KF042456	KF042302

Sample no.	Species	COI	16S
DNA 100629-1	*Sipunculus nudus* (Panama)	DQ300163	JN864963
DNA 100629-2	*Sipunculus nudus* (Panama)	KF042448	KF042394
DNA 103549-1	*Sipunculus nudus* (Solomon Islands)	KF042457	KF042303
DNA 103549-2	*Sipunculus nudus* (Solomon Islands)	KF042458	KF042304
DNA 103549-3	*Sipunculus nudus* (Solomon Islands)	KF042459	KF042305
DNA 103549-4	*Sipunculus nudus* (Solomon Islands)	KF042460	KF042306
DNA 103549-6	*Sipunculus nudus* (Solomon Islands)	KF042461	KF042307
DNA 103549-7	*Sipunculus nudus* (Solomon Islands)	KF042462	KF042308
DNA 103549-8	*Sipunculus nudus* (Solomon Islands)	KF042463	KF042309
DNA 103550-1	*Sipunculus nudus* (Solomon Islands)	KF042464	KF042310
DNA 103550-5	*Sipunculus nudus* (Solomon Islands)	KF042465	KF042311
DNA 103550-7	*Sipunculus nudus* (Solomon Islands)	KF042466	KF042312
DNA 100246-1	*Sipunculus nudus* (Vietnam)	DQ300161	JN864961
DNA 106941	*Sipunculus nudus* (South Africa)	KF042445	–
DNA 100245-1	*Sipunculus nudus* (France)	JN865105	JN864960
DNA 100245-2	*Sipunculus nudus* (France)	KF042446	KF42392
DNA 100245-3	*Sipunculus nudus* (France)	KF042447	KF42393
DNA 101884-1	*Sipunculus nudus* (France)	KF042449	KF042395
DNA 101884-2	*Sipunculus nudus* (France)	KF042450	KF042396
DNA 103730-1	*Sipunculus nudus* (Spain)	KF042467	KF042313
DNA 103730-2	*Sipunculus nudus* (Spain)	KF042468	KF042314
DNA 103730-3	*Sipunculus nudus* (Spain)	KF042469	KF042315
Beihai-2	*Sipunculus nudus* (China)	FJ788908 1	EU260101 1
Beihai-3	*Sipunculus nudus* (China)	FJ788909 1	EU260102 1
Beihai-4	*Sipunculus nudus* (China)	FJ788910 1	EU260103 1
Beihai-5	*Sipunculus nudus* (China)	FJ788911 1	EU260104 1
Beihai-6	*Sipunculus nudus* (China)	FJ788913 1	EU260108 1
Sanya-1	*Sipunculus nudus* (China)	FJ788914 1	EU260109 1
Sanya-2	*Sipunculus nudus* (China)	FJ788915 1	EU260110 1

Sample no.	Species	COI	16S
Sanya-3	*Sipunculus nudus* (China)	FJ788916 1	EU260111 1
Sanya-4	*Sipunculus nudus* (China)	FJ788917 1	EU260112 1
Sanya-5	*Sipunculus nudus* (China)	FJ788918 1	EU260113 1
Xiamen-1	*Sipunculus nudus* (China)	FJ788919 1	EU260114 1
Xiamen-2	*Sipunculus nudus* (China)	FJ788920 1	EU260115 1
Xiamen-3	*Sipunculus nudus* (China)	FJ788921 1	EU260116 1
Xiamen-4	*Sipunculus nudus* (China)	FJ788922 1	EU260117 1
Xiamen-5	*Sipunculus nudus* (China)	FJ788923 1	EU260108 1
DNA 101003	*Golfingia elongata*	DQ300123-1	JN864985-1
DNA 100199	*Phascolopsis gouldii*	DQ300134-2	JN864952-1
DNA 101084	*Themiste pyroides*	DQ300171-1	JN864995-1

RESULTS

TAXONOMY

Class Sipunculidea E. Cutler and Gibbs, 1985
Order Sipunculiformes E. Cutler and Gibbs, 1985
Family Sipunculidae E. Cutler, 1994
Sipunculus nudus Linnaeus, 1766

Sampling location. The sand flats at the intertidal zone of Modtanoy Beach, Kantang District, Trang Province, Thailand (Lat. 7.307223′N, Long. 99.416662′E) at a depth of 40–50 cm.

Diagnosis. Tentacles were arranged around the mouth, on a short introvert without hooks but with large papillae. The trunk body was smooth and cylindrical in shape. (Fig. 1A, B) There were 25–27 LMBs. Two nephridiopores were located anteriorly to the anus, partially attached to the body wall. The spindle muscle originated on the body wall anterior to the anus. There were four introvert retractor muscles. The longitudinal muscles formed distinct, individual bands along the trunk.

External character. The trunk was 140–170 mm in length, 5–12 mm in width while the colour was red-brown. The mouth was located at the anterior end of the introvert and the tentacles were arranged around the nuchal organ (Figs. 1A, B). The body wall was smooth, without epidermal structures, and was formed from layers of circular and longitudinal muscles (Figs. 2A–C). For the skin bodies without epidermal organs, the surface was divided into rectangular areas. Introvert hooks were absent and triangular papillae were present on the distal part of the introvert (Figs. 3A, B). The anus was 37 mm from the anterior end of the trunk. The nephridiopore was anterior to the anus.

Internal character. The body wall musculature was composed of 26–27 LMBs and about 100-110 circular muscle bands. Two pairs of retractor muscles were attached to the body wall and connected to the apical portion of the introvert. The brain was slightly bi-lobed and the digitate process was a sponge-like tuft. The body cavity formed a large undivided coelom. The intestine was U-shaped and helical (Fig. 4), which is characteristic of most sipunculans. The spindle muscle was attached anteriorly to the body wall, anterior to the anus, supporting the intestine. Two nephridia were attached to the anterior lateral body wall, yellow in color, 35-40 mm long, hanging freely in the coelom, partially attached to the body wall and open to the exterior via the nephridiopores. The

caecum was present. Two contractile vessels were attached to the surface of the esophagus in the introvert. The post-esophageal loop was loose before entering the coil. This general pattern was seen only in *Sipunculus*.

Habitat. Their burrows are star-like traces in the intertidal zone to 30–60 cm depth. Modtanoy Beach is a sandy beach of fine grade sand where the surface sediment is mainly composed of 95–97% sand by weight. The sediment grain size is about 0.2 mm, salinity is 30–35 psu and average organic matter content is 0.53%. Modtanoy Beach is surrounded by mangroves and sea grass beds.

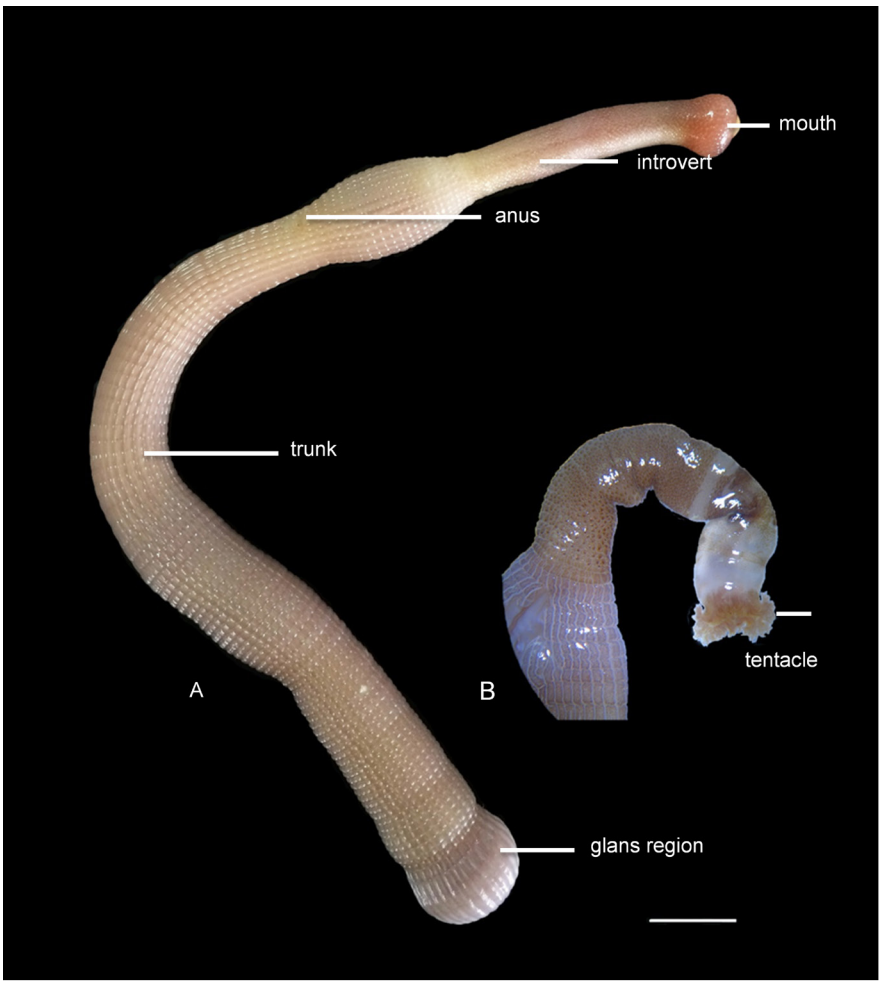

Figure 1. Photograph showing the morphology of *Sipunculus nudus* from Modtanoy Beach: A. lateral view, introvert everted, note that tentacles at the tip of the introvert cannot be seen if the introvert is even slightly retracted; B. head with tentacular apparatus (scale bar = 1 cm).

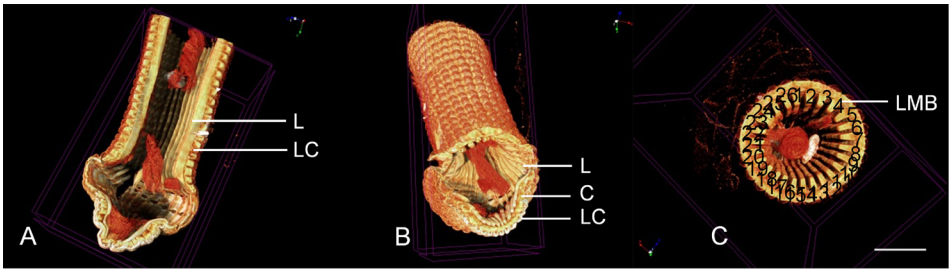

Figure 2. Micro-CT scan of *Sipunculus nudus*: A. Longitudinal section view. The inside body wall shows the longitudinal muscles (L) and longitudinal canals (LC); B. Part of the glans region showing the circular muscle (C); C. Cross-section of the trunk. There are 26 LMBs. (scale bar = 1 cm).

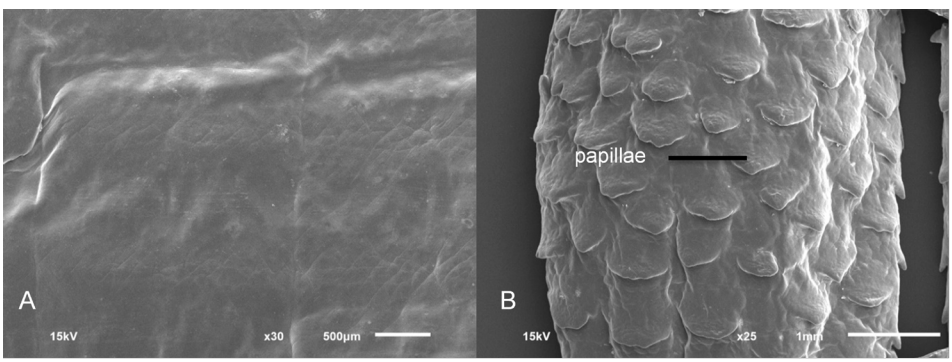

Figure 3. Representative SEM images of *Sipunculus nudus*: A. External appearance of the trunk wall (30x magnification); B. Papillae of the introvert (25x magnification).

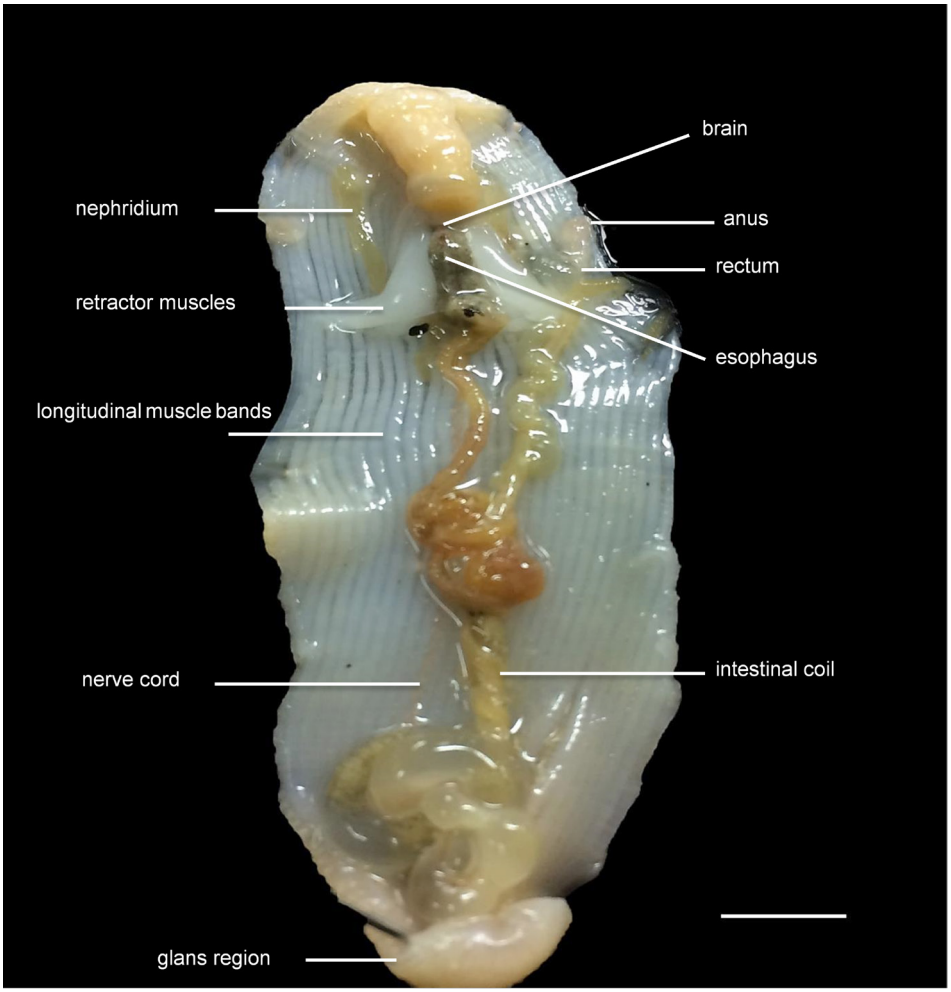

Figure 4. Dissection photograph of the internal organs of *Sipunculus nudus* (scale bar = 1 cm).

Mitochondrial DNA polymorphism

For the 16S rRNA and COI gene sequences, three haplotypes (462 bp) of the 16S rRNA gene with only two transition point mutations were observed from 11 individuals (PCR failure was found in one sample), while two haplotypes (651 bp) of the COI gene with only one transition point mutation were observed from 12 individuals (Table 2). The haplotype diversities for the 16S rRNA and COI genes were 0.345 and 0.167, respectively.

Table 2. DNA haplotypes in *Sipunculus nudus* at Modtanoy Beach (Thailand) in the 16S (11 samples) and COI (12 samples) gene fragments and the respective GenBank accession codes for these sequences

Haplotype	Number of samples/ total (frequency)	DNA sequence change[a]	GenBank accession codes
16S-1	1/11 (9.1%)	A300G	MF373109.1
16S-2	1/11 (9.1%)	C225T	MF373113.1
16S-3	9/11 (81.8%)	–	MF373110.1, MF373111.1, MF373112.1, MF373114.1, MF373115.1, MF373116.1, MF373117.1, MF373118.1, MF373119.1
COI-1	1/12 (8.3%)	C272T	MF382105.1
COI-2	11/12 (91.7%)	–	MF382106.1, MF382107.1, MF382108.1, MF382109.1, MF382110.1, MF382111.1, MF382112.1, MF382113.1, MF382114.1, MF382115.1, MF382116.1

[a]Shown as A300G for A at position 300 changing to G

Comparison of these 16S and COI gene fragment sequences with those in the GenBank database (Table 1) revealed low divergence (1.1–3.1%) at the 16S gene but high divergence (18.8–25.0%) at the COI gene. The percent divergences at the 16S and COI genes within these samples were 0.0–0.2% and 0.0–0.3%, respectively.

Molecular phylogenetic analyses of 16S and COI gene fragments with ML and BI gave trees with similar topologies (not shown) and so the combined dataset (concatenated 16S and COI) was analyzed. The ML and BI trees showed the same topology and so for simplicity the ML tree is shown here (Fig. 5), but with the BI PP shown below each node and ML bootstrap % above the node. All the Thai samples formed a single group (BS = 100%, PP = 1.00) within the *S. nudus* species complex. The Thai specimens were separated from the southern Chinese and Vietnamese sister clades (Fig. 5).

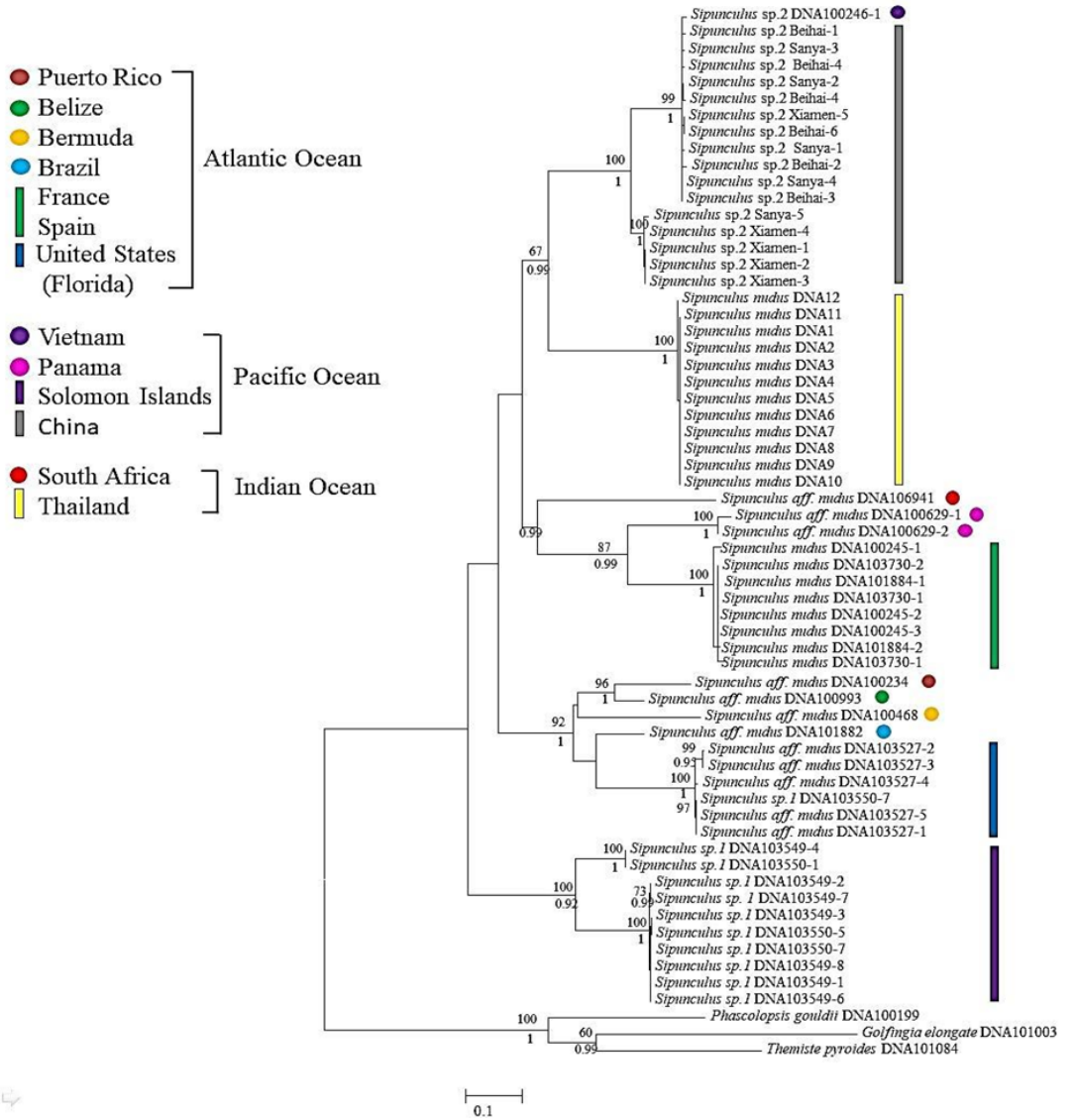

Figure 5. ML phylogenetic tree for the combined (concatenated) 16S and COI gene fragment (1113 bp) dataset. Numbers above and below nodes are the ML bootstrap (%) and BI PP (posterior probability) support values, respectively, where bootstrap values below 50% are not shown.

Molecular data for other *Sipunculus* species, including those previously reported in Thailand, are not available, which limits the ability of our phylogenetic analyses to ascribe species designations and thus help to ascertain if *S. nudus* is a single geo-polymorphic species or a cryptic species complex, and the correct standing of these Thai specimens. The morphological comparison between the three *Sipunculus* species previously reported in Thailand (*Sipunculus gulfus*, *S. robustus* and *S. thailandicus*) and the newly collected samples from Modtanoy Beach in this study, nominally designated as *S. nudus*, is summarized in Table 3. The number of LMBs, ventral, and dorsal retractors of the peanut worm samples in this study (nominal *S. nudus*) was distinct from *S. gulfus* and *S. thailandicus*. Although *S. robustus* is reported to have the same number of LMBs (25–27) as the *S. nudus* samples in this study, they differed in the other morphological characteristics, such as the digitate processes of the

brain (*S. nudus* has anterior sponge-like tuft while *S. robustus* has lateral string-like processes (Ditadi 1982a).) and that the nephridia in *S. robustus* are not attached to the body beyond the nephridiopore (Cutler and Cutler 1985). Thus, the morphological analysis does not support the samples from Modtanoy Beach as belonging to any of the three previously recorded species (*S. gulfus*, *S. robustus* or *S. thailandicus*).

Table 3. Comparison of the morphological characteristics of *Sipunculus* spp. in Thailand.

Species	VR	DR	LMB	Habitat
S. gulfus	2–4 (1–5)	8–9	24	Sea depth ~ 50 m
S. robustus	3–4 (2–6)	8–12	26	Fine sand at intertidal flat zone.
S. thailandicus	1–6	10–14	34–39	Intertidal coral sand with small rocks, boulders, gravel.
***S. nudus* (This study)**	1–6 (1–7)	8–12 (7–12)	25–27	Fine sand at intertidal flat zone.

Note. LMBs = longitudinal muscle bands, VR = ventral retractor (The ventral retractors begin close to the ventral nerve cord), DR = dorsal retractor. For measurement, see Fig. 6.

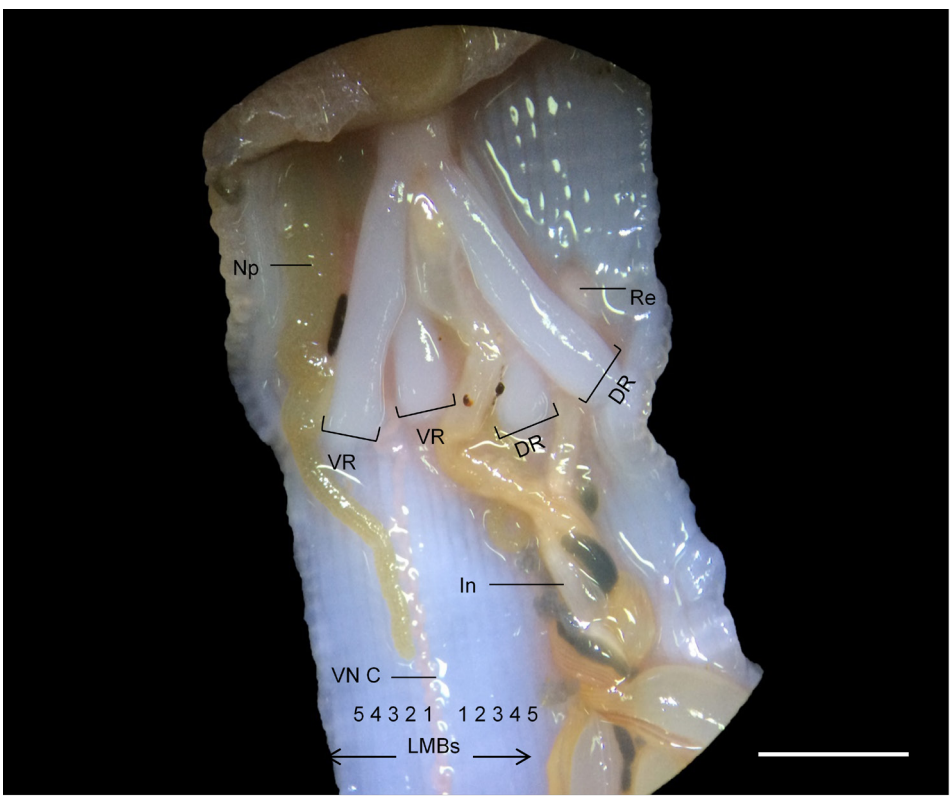

Figure 6. Dissection photograph showing the internal organs of *Sipunculus nudus* and the method for measurement of the LMBs, ventral retractor (VR), dorsal retractor (DR), intestine (In), rectum (Re), nephridium (Np) and ventral nerve cord (VNC) (scale bar = 1 cm).

Morphological comparison between these Thai and other *S. nudus* specimens

The morphological examination of the peanut worm samples collected from Modtanoy Beach (Trang, Thailand) in this study revealed that they have similar characteristics to those of *S. nudus*, particularly the nephridia, which were partially attached to the body wall, the brain, which is bi-lobed, and the brain processes, which are dorsal and digitate or a short, sponge-like mass (Cutler 1994). The Micro-CT scan showed that the digestive system is basically a recurved gut twisted into a double helix and the number of LMBs matched that of *S. nudus*. However, phylogenetic analyses revealed different clades, suggesting either variation within *S. nudus* or different species that may be closely related. Thus, comparison of the morphological characteristics among the Thai nominal *S. nudus* in this study with *S. nudus* from 11 other localities (Kwauchi and Giribet 2013) was performed, and the results are summarized in Table 4.

Table 4. Comparison of the morphological characteristics of *Sipunculus nudus* (Thailand) in this study with those reported from other localities.

IZ Accession Number	Locality	LMBs			Retractor muscle		Brain	
		n	State at glans region	Nephridia attachment	VL/VR	DL/DR	Shape	Digitate process
130419	Puerto Rico	28	All split	*	*	*	*	*
130420	France	31	*	10%	*/1–8	*/8–16	Bilobed	Fringed
		31	Not all split	20%	2–6/*	8–14/*	Bilobed	Fringed
		31	Not all split	17%	*/1–6	*/9–16	Bilobed	Fringed
130435	Spain	31	Not all split	18%	1–7/1–6	*/8–14	*	*
		33	Not all split	23%	1–7/1–6	*/9–14	Bilobed	Fringed
130422	Bermuda	23	Not observed	*	*	*	*	*
130423	Panama	32	All split	Free	*	*	*	*
		32	All split	Free	*	*/8–10	*	*
130424	Belize	28	All split	*	Membrane	Membrane	Bilobed	Solid tuft
130426	Brazil	29	*	41%	*	*	*	*
130430	USA, Florida	30	All split	25%	Membrane	Membrane	Bilobed	Solid tuft
		30	All split	39%	Membrane	Membrane	Bilobed	Solid tuft
		28	All split	15%	Membrane	Membrane	Bilobed	Solid tuft
		30	All split	37%	Membrane	Membrane	Bilobed	Solid tuft
		30	All split	43%	Membrane	Membrane	Bilobed	Solid tuft
130432	Solomon Islands	26	All split	13%	2–5/1–5	*/8–11	Bilobed	Short

IZ Accession Number	Locality	LMBs			Retractor muscle		Brain	
		n	State at glans region	Nephridia attachment	VL/VR	DL/DR	Shape	Digitate process
		26	All split	Free	3–4/3–4	7–9/7–9	Bilobed	Absent
		25	All split	Free	3–4/3–4	*/9–10	Bilobed	Absent
		28	All split	Free	3–4/3–4	8–10/9–11	Bilobed	Absent
		21	All split	14%	1–5/1–5	7–11/7–11	Bilobed	Absent
130433	Solomon Islands	25	All split	Free	1–4/2–6	*/7–10	Bilobed	Absent
		25	All split	Free	3–4/3–4	9–10/*	Bilobed	*
		26	All split	Free	1–4/1–4	6–10/7–10	Bilobed	Short
130421	Vietnam	30	Do not split	54%	1–7/1–7	*/7–14	Bilobed	Solid tuft
130440	South Africa	28	All split	Free	Membrane	Membrane	Bilobed	Solid tuft
CUMZ(H)2017.1-1	Thailand	26	All split	58.82	1–6/1–6	9–13/8–13	Bilobed	Solid tuft
CUMZ(H)2017.1-2		26	All split	71.43	1–5/1–5	6–11/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-3		26	All split	52.17	1–5/1–5	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-4		26	All split	66.67	1–6/1–6	7–11/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-5		26	All split	41.67	1–6/1–6	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-6		27	All split	46.67	1–6/1–6	7–9/7–11	Bilobed	Solid tuft
CUMZ(H)2017.1-7		26	All split	31.43	1–6/1–5	8–13/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-8		26	All split	40.00	1–7/1–6	7–13/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-9		26	All split	50.00	1–6/1–6	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-10		26	All split	37.50	1–5/1–5	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-11		26	All split	44.74	1–6/1–6	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-12		27	All split	26.67	1–6/1–6	7–12/7–11	Bilobed	Solid tuft
CUMZ(H)2017.1-13		26	All split	40.48	1–5/1–6	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-14		26	All split	37.21	1–7/1–6	9–13/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-15		26	All split	37.50	1–6/1–6	8–13/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-16		26	All split	53.57	1–6/1–6	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-17		26	All split	39.58	1–6/1–6	7–12/8–12	Bilobed	Solid tuft

IZ Accession Number	Locality	LMBs			Retractor muscle		Brain	
		n	State at glans region	Nephridia attachment	VL/VR	DL/DR	Shape	Digitate process
CUMZ(H)2017.1-18		26	All split	45.00	1–6/1–6	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-19		26	All split	40.00	1–6/1–6	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-20		26	All split	37.50	1–6/1–6	7–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-21		26	All split	34.29	1–6/1–6	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-22		26	All split	30.00	1–6/1–6	7–12/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-23		26	All split	40.00	1–6/1–6	7–12/7–11	Bilobed	Solid tuft
CUMZ(H)2017.1-24		26	All split	38.24	1–5/1–6	8–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-25		27	All split	32.00	1–6/1–6	7–12/8–12	Bilobed	Solid tuft
CUMZ(H)2017.1-26		27	All split	43.33	1–7/1–6	7–11/7–12	Bilobed	Solid tuft
CUMZ(H)2017.1-27		26	All split	48.57	1–5/1–6	7–12/7–12	Bilobed	Solid tuft

Note. Characteristics not observed are marked with an asterisk. LMBs = longitudinal muscle bands; VL/VR = ventral left/ventral right; DL/DR = dorsal left/dorsal right.

DISCUSSION

Morphological and molecular data support the placement of peanut worms examined from Modtanoy Beach in southern Thailand within the *S. nudus* complex, and therefore establish a new record of *S. nudus* for Thailand. Cutler's (1994) taxonomic key, using the number of LMBs, origin of the retractor muscles, shape of the digitate processes of the brain, nephridia attachment and state of LMBs at the glans region, is useful for identification of the *Sipunculus* species.

In this study, we draw comparisons with the work of Kawauchi and Giribet (2013) which separates the three best-represented clade morphologies. The *Sipunculus nudus* from Trang province, Thailand, when compared with the specimens from France, Spain, the Solomon Islands, and Florida (USA), have morphological characteristics that differ in the origin of the retractor muscles, the brain process, and the splitting of the LMBs at the glans region. This was supported by our phylogenetic tree (Fig. 5), which showed that the Thai specimens were distinct from the other clades (ML bootstrap value of 100% and BI PP of 1).

From the morphology comparison of the *Sipunculus nudus* samples from Thailand and Vietnam, it was found that they had similar morphologies. However, the differences were in the number of LMBs (25–27 LMBs in the samples from Thailand compared with 28–32 LMBs in those from Vietnam), and in the nephridia of *S. nudus* from Vietnam, which had about half of the nephridial length attached to the body wall and the LMBs undivided at the glans region, while the specimens from Thailand had a wider range of nephridial attachment (25–70%) and all of LMBs were divided at the glans region (Adrianov and Maiorova 2012). This was consistent with our data from the phylogenetic tree showing that *S. nudus* from Thailand and *S. nudus* from Vietnam were classified into different groups, and with each of those groups showing closer affinities to *S. nudus* from other countries (Fig. 5).

Gene flow is the exchange of genetic material between populations caused by the movement of

adult individuals or their larval stages (Klinbunga *et al*. 1998). The estimated planktonic larval stage of *S. nudus* is 7.5–27 days at 21.5–34.0°C (Lan *et al*. 2007). The reason why *S. nudus* larvae have not dispersed from Vietnam and Southern China may be because their planktonic larvae only spend a short period of time in sea water (~30 days), notable differences between stream flows (Thai specimens of this study were collected from the Andaman Sea of the Indian Ocean, while those from China and Vietnam were collected from the South China Sea of the Pacific Ocean), and the existence of some natural barriers between their populations.

Although our samples were collected from the Indian Ocean, as was the case for South Africa, the distance between the two locations may account for the difference in their morphologies at the two localities; South Africa had the nephridia free from the body wall and the base of the retractor muscles was not subdivided into fascicles connected to separate LMBs (Kawauchi and Giribet 2013). Therefore, phylogenetic analyses further illustrated that these two clades were separated, and clearly distinct from one another.

CONCLUSION

Peanut worms collected from Modtanoy Beach (Trang province, Thailand) were nominally identified as *S. nudus* based on their internal and external morphological characteristics, which was supported by molecular phylogenetic analyses. The body and tissue characteristics of specimens in this study were highly similar to those characteristics described in the published records of *S. nudus*. Comparative sequence analysis of the DNA between our specimens from Thailand and sequence data in GenBank also revealed that the Thai samples of this study matched the confirmed sequences of *S. nudus*. The peanut worm presented in this study has not been recorded previously in Thailand, and thus represents a new record of *Sipunculus nudus*. However, it remains plausible that *S. nudus* is in fact a species complex, and so the exact taxonomic status of this Thai population and its relationship to other nominal "*S. nudus* species" remains unclear.

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