

SPECIES STATUS OF RAGWORM POPULATIONS
(NEREIDIDAE: *TYLORRHYNCHUS* SP.) EXPLOITED
AS A HUMAN SPECIALTY BASED ON THEIR
MOLECULAR ANALYSES IN THE NORTHERN
COAST OF VIETNAM

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Abstract

In the traditional culinary culture of Vietnam, the ragworms (Nereididae) are an ancient and well-known delicacy (*Chả rươi in Vietnamese*) of great nutritional and economic value. In order to determine their species status, distribution, and sustainable control, a comparative molecular genetic analysis was carried out in 2018–2019. It comprised of ragworm populations along the northern coast including Quang Ninh, Hai Phong, Hai Duong, Thai Binh and Nam Dinh, 20.0, 14.5, 34.0, 26.0 and 17.0 km from the sea, respectively. We found that all of them belong to one and the same species named *Tylorrhynchus heterochaetus* (Quatrefages, 1866). The ragworm in the Tu Ky area of Hai Duong province has the farthest distribution in the mainland, and was among the ragworm groups with the largest morphological measurements. Salinity and inland environmental conditions are likely factors influencing the morphological development of ragworm populations in this area. This may be the reason Tu Ky ragworms have been labelled a specialty dish in Vietnam dating back to ancient history.

Key words: ragworm *Tylorrhynchus heterochaetus*, molecular analysis, species status, distribution, Vietnam north coast

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Introduction. Ragworms or clam worms of the family Nereididae, belong to the polychaete group and are the most studied in the coastal and marine area around the world [1-5]. Their reproductive behaviour as well as role in the food chain of coastal-marine ecosystems has been studied [6,7]. There have been several studies about their role in the food chain of estuarine ecosystems, and especially as a nutritious food and traditional medicine for humans [8-10]. Ragworms play an important role in assessing water environment quality. They have also been investigated as a bioindicator for climate change, sea level rise and environmental water quality [11,12].

Vietnam has a coastal boundary of around 3260 km, featuring a dense estuarine system and a tropical monsoon climate. This area of the country is also highly affected by sea level rise and environmental salinity [13]. Ragworm populations have been studied widely for their taxonomical biodiversity, reproductive behaviour, and as an important component of the food chain of the benthic marine, estuarine and soil ecosystems. Of the hundreds of ragworm species (Polychaeta) identified in the coastal area of Vietnam, several have been recorded to migrate along the river and into the mainland. Among them, the ragworm *T. heterochaetus* is one of the first species studied [14-17]. This ragworm species is a well-known, traditional delicacy with great nutritional and economic value. It is widely exploited in the northern coastal area of Vietnam. Since ancient times, it has always been considered a delicious and nutritious specialty dish (*Chả rươi in Vietnamese*) in Tu Ky district of Hai Duong province (“*Classified discourse from the library*” *Ancient Encyclopedia by Le Quy Don 1773*) [8]. Most of the studies on ragworms used as a traditional specialty are implicitly assumed to belong to the species *T. heterochaetus*. Our morphological analysis of ragworms (*Tylorrhynchus* sp.) along the northern coast of Vietnam found that they belong to the same species. However, the study also revealed some morphological differences between them [15].

This study focuses on ragworm populations that are being overexploited as a specialty dish in Vietnam, in order to gain its sustainable control. The aims of the study are (1) to perform molecular genetic analysis to determine the species status of the ragworm populations, (2) to identify whether these ragworm populations belong to one and the same species or not, and (3) to survey their distribution.

Materials and methods. Study sites and DNA extraction. The ragworms (*T. heterochaetus*?) were collected from five provinces and cities along the northern coastal area of Vietnam: 1) District Tien Yen of Quang Ninh province, coordinates 21°27'N, 107°24'E, Tien Yen river, the distance to the sea is 20 km, 2) District Kien Thuy of Hai Phong city, 20°49'N, 106°41'E, Lach Tray river, 14.5 km, 3) District Tu Ky of Hai Duong province, 20°44'N, 106°24'E, Thai Binh river, 34 km, 4) District Thai Thuy of Thai Binh province, 20°29'N, 106°28'E, Tra Ly river, 26 km, and 5) District Nghia Hung of Nam Dinh province, 20°12'N, 106°11'E, Ninh Co river, 17 km (Fig. 1).

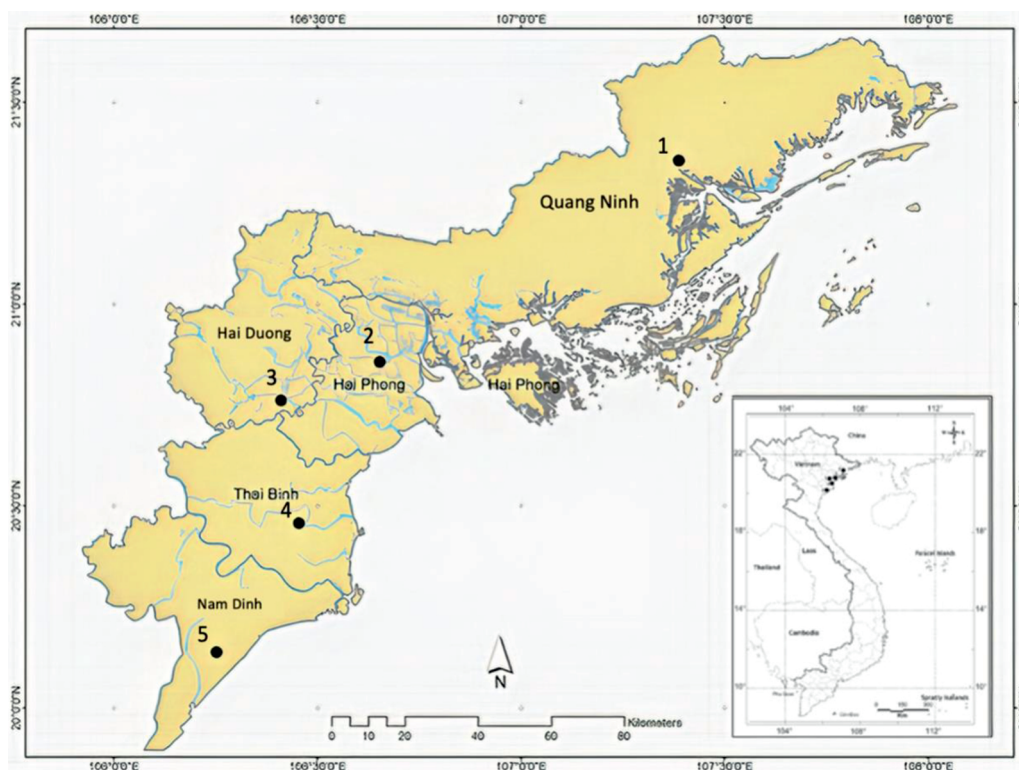


Fig. 1. Map showing the study sites: 1. Quang Ninh; 2. Hai Phong; 3. Hai Duong; 4. Thái Bình; 5. Nam Dinh

Ragworms were collected in 2018–2019 by using collection nets placed horizontally in their reproductive migration path. Live ragworm samples were initially kept in clean water. Then, they were processed in alcohol solutions with increasing concentrations. Finally, they were kept in 90° alcohol. Ragworms were stored in cold EtOH 90% for DNA isolation. Then, total DNA of the samples was extracted using the DNeasy Blood & Tissue Kits (Qiagen, Germany) according to the manufacturer's instructions.

Amplification and sequence of COI gene. In this study, the gene (around 70 bp) encoding for the cytochrome oxidizes subunit I (COI) and was used as a marker to distinguish closely related species. To amplify the COI gene from the extracted DNA, the primer was designed by using the Primer 3 application (<http://bioinfo.ut.ee/primer3-0.4.0/>). The sequence of the primer was presented as 5'- TCA ACA AAY CAT AAR GAT ATY GGA A- 3', reverse: 5'- GAAGAAGGCAGTATTTAGGTTTCG - 3'.

The PCR reaction was prepared in a 20 µL volume containing 10 µL of 2X Taq PCR Master mix, 0.5 µL of 5 mM each primer, 1 µL of 10 ng/µL template DNA, and 8 µL of Nuclease-Free Water. The PCR amplification was performed with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C

for 25 s, 56 °C for 25 s, and 72 °C for 60 s, with a final extension at 72 °C for 3 min. The PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Germany) following provider’s recommendation. The purified PCR products were then sequenced using the ABI 3100-Avant Genetic Analyzer (Applied Biosystems, USA).

Data analysis. The COI gene sequences of two subspecies *Tylorrhynchus heterochaetus* (NC 025561.1 and KM111507.1) and one subspecies *Arenicola* (KM042101.1) were used as references. The sequencing results were collected and aligned with reference sequences by ClustalW tool of BioEdit 7.0.0 software [18]. The best model test in MEGA6 6.0 was performed to detect that the Kimura 2-parameter was the best substitution [19]. Then, this parameter and bootstrap 1000 were used to build the maximum likelihood phylogeny tree and count the genetic distance by MEGA 6.0.

Results. Molecular genetic characteristics of the five studied ragworm populations. Our study results recorded some morphological differences in ragworm populations (*T. heterochaetus*?) distributed along the northern coast of Vietnam [15]. These differences are not decisively taxonomical base for separating the five studied ragworm populations of coastal areas of North Vietnam into different species. Therefore, to identify whether they belong to the same species or not, a molecular genetic analysis needs to be performed. The ragworm DNA samples were denoted as R1 – ragworms collected in Hai Phong, R2 – Quang Ninh, R3 – Nam Dinh, R4 –Thai Binh, and R5 – Hai Duong (Table 1a).

The results showed that the sequences of COI gene had three different positions, such as 35 (A-T), 37 (T-C), and 121 (C-T). The genetic distance differences among DNA sequences of the five ragworm populations were relatively small, ranging from 0% to 0.5%. While the DNA sequence distance difference between Hai Phong and Nam Dinh populations were the same, the difference between Hai Phong and Thai Binh populations was the largest. The differences among the rest

T a b l e 1a

The differences in nucleotide positions in COI gene of studied ragworm populations

Ragworm populations	Nucleotide positions				
	The differences in nucleotide positions				
	12	35	37	121	328
R1. Hai Phong (<i>Tylorrhynchus heterochaetus</i> ?)	G	A	C	C	T
R2. Quang Ninh (<i>Tylorrhynchus heterochaetus</i> ?)	.	.	T	.	.
R3. Nam Dinh (<i>Tylorrhynchus heterochaetus</i> ?)
R4. Thai Binh (<i>Tylorrhynchus heterochaetus</i> ?)	.	.	T	T	.
R5. Hai Duong (<i>Tylorrhynchus heterochaetus</i> ?)	.	T	.	.	.
<i>Tylorrhynchus heterochaetus</i> KM111507.1 in China	A	.	.	.	C
<i>Tylorrhynchus heterochaetus</i> NC 025561.1 in China	A	.	.	.	C

T a b l e 1b

Genetic distances between studied ragworm populations and data in Genebank

Ragworm (<i>Tylorrhynchus heterochaetus</i>)	Genetic distance							
	1	2	3	4	5	6	7	8
Hai Phong								
Quang Ninh	0.002							
Nam Dinh	0.000	0.002						
Thai Binh	0.003	0.002	0.003					
Hai Duong	0.002	0.003	0.002	0.005				
KM111507.1 in China (<i>Tylorrhynchus heterochaetus</i>)	0.003	0.005	0.003	0.007	0.005			
NC 025561.1 in China (<i>Tylorrhynchus heterochaetus</i>)	0.003	0.005	0.003	0.007	0.005	0.000		
KM042101.1 Portugal (<i>Arenicola defodiens</i>)	0.271	0.274	0.271	0.271	0.275	0.268	0.268	

of the populations ranged from 0.2 to 0.3%. Among the DNA sequences of the five ragworm populations in Vietnam, two species in China (*T. heterochaetus*), and species *A. defodiens* in Portugal, the sequences of the five populations in Vietnam were 99% similar to the two Chinese species sequences, and 80% similar to the Portuguese sequence (Table 1b) [4].

Phylogenetic analyses of the five studied ragworm populations. We have built a phylogenetic tree for the five ragworm populations in this study and compared them to other populations. The results showed that the five Vietnamese populations and the two Chinese populations were located on the same derivative branch, while the *A. defodiens* KM042101.1 in Portugal was outside the one. In addition, the five Vietnamese sequences were located on the same derivative branch with the two sequences of Chinese *T. heterochaetus*, however, they were separate from bootstrap 67. Among the five Vietnamese sequences, the ragworm populations of Hai Phong, Nam Dinh and Hai Duong were in the same branch, with a certain separation from the ragworm populations of Quang Ninh and Thai Binh (Table 2, Fig. 2).

Thus, the DNA analysis showed the five Vietnamese sequences of Hai Phong (R1), Quang Ninh (R2), Nam Dinh (R3), Thai Binh (R4) and Hai Duong (R5) ragworm populations as the same species (Fig 2). The scientific name of these ragworms should be *T. heterochaetus*.

There were three nucleotide position differences among the five sequences in Vietnamese ragworm populations and two nucleotide position differences of the Vietnamese ragworm populations compared to *T. heterochaetus* originating from China. However, there is not enough scientific evidence to conclude they are new species. On the other hand, due to the difference in geographic location, the ragworm populations in Vietnam had some differences compared to the ragworm populations in China. It has been found that there are some differences within the Vietnamese ragworm populations as discussed above.

T a b l e 2

Genetic differences between ragworm populations collected in Hai Phong province and data in Genebank

Description	Max score	Total score	Query cover	E Value	Indent	Accession
1. <i>Tylorrhynchus heterochaetus</i> mitochondrion, complete genome	1116	1116	100%	0.0	99%	KM111507.1
2. <i>Arenicola</i> sp. 1 AP-2012 isolate avr1 cythochrome oxidize subunit I (COI) gene, partial cds; mitochondrial	466	466	98%	3e-127	81%	JQ950325.1
3. <i>Arenicola defodiens</i> haplotype h03 cythochrome oxidize subunit I (COI) gene, partial cds; mitochondrial	460	460	98%	1e-125	80%	KM042101.1
4. <i>Arenicola defodiens</i> haplotype h01 cythochrome oxidize subunit I (COI) gene, partial cds; mitochondrial	455	455	98%	7e-124	80%	KM042099.1
5. Polychaeta cythochrome oxidize subunit I (COI) gene, partial cds; mitchondrial	451	451	96%	8e-123	81%	KJ736533.1

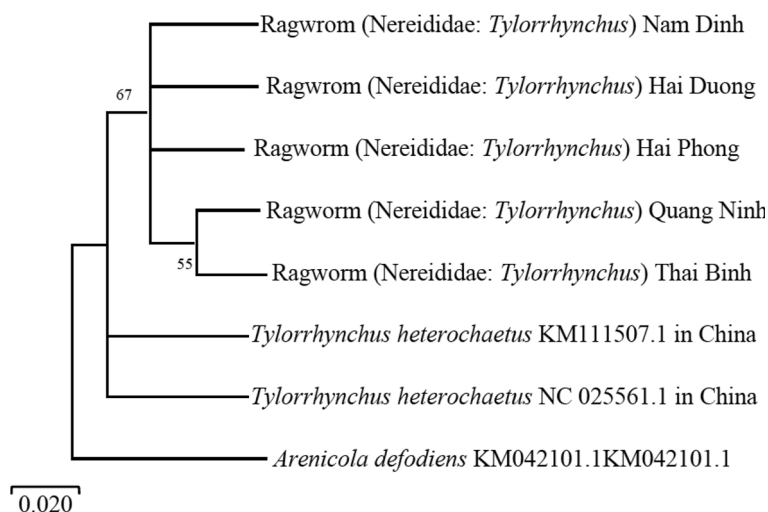


Fig. 2. Phylogenetic tree using Maximum Likelihood method with bootstrap 1000

Thus, DNA analysis showed similarities between ragworm populations in the northern coastal region of Vietnam with other ragworm populations in the World. Although there are certain differences as mentioned above, our study determined that the species status of the five Vietnamese ragworm populations being commonly used as a delicacy in the northern coastal area of Vietnam, is one and the same biological species named *T. heterochaetus* (Quatrefages, 1866). This article is registered in ZooBank under Genebank: R1 – Hai Phong ragworm MN433709,

R2 – Quang Ninh ragworm MN631165, R3 – Nam Dinh ragworm MN631166, R4 – Thai Binh ragworm MN631167, and R5 – Hai Duong ragworm MN631168 (<https://www.ncbi.nlm.nih.gov/Genbank/update.html>).

Discussion. In comparison with morphological descriptions by other authors, we found some morphological differences in ragworm populations (*T. heterochaetus?*) along the northern coast of Vietnam [15]. So, a molecular genetic analysis for DNA sequencing was performed. It is found that, its species name is the same as identified by other authors [14,16,17], and is listed in the Worms taxon detailed [20].

The species status of this ragworm is as follows: Polychaeta > Errantia > Phyllodocida > Nereididae > *Tylorrhynchus* Grube, 1866: *T. heterochaetus* (Quatrefages, 1866). Synonyms: *Nereis heterocheta* Quatrefages (1866) = *Tylorrhynchus chinensis* Grube (1866) = *Tylorrhynchus heterochaetus* [auct. misspelling] = *Ceratocephale osawai* Izuka (1903) = *Chinonereis edestus* Chamberlin (1924) = *Tylorrhynchus sinensis* Dawydoff (1952).

In general, morphological measurements of ragworm populations, namely body length and width, body weight and number of segments have always been greater in Hai Duong than those of Hai Phong, Quang Ninh, Nam Dinh and Thai Binh [15]. This may be one of the most important reasons why the biological productivity of ragworm population in Tu Ky district of Hai Duong province is always among the group of the biggest size in the coastal area of Vietnam. Perhaps that is why, in ancient Hai Duong, the ragworms have been used as a traditional specialty dish and are very famous in the country.

Another issue that needs to be considered is why morphological measurements of the ragworm population in Hai Duong are always among the largest. The reason may be due to the migration and distribution of the reproductive ragworm population here. In the Tu Ky area of Hai Duong province during the reproductive season, ragworm populations can be found migrating inland by river up to 34 km away from the sea. This distance is much greater than those of the distribution areas of the ragworm population along the coastal area in North Vietnam, as in Thai Binh, Quang Ninh, Nam Dinh and Hai Phong, 26 > 20 > 17 > 14.5 km to the sea, respectively (Fig. 1). Thus, it is possible that the inland environmental characteristics and salinity are factors affecting the ragworm populations in Tu Ky area, Hai Duong province. These observations are also supported by other research results [6,14]. Thus, the increased salinity caused by sea level rise inland affects the migration characteristics of ragworm populations. In this way, ragworm populations play a role as a bioindicator of sea level rise along the coastal area of Vietnam. This conclusion was also reached by DEAN [11] and DUROU et al. [12].

Conclusions. The ragworm populations being actively exploited as an exceptional and nutritious dish are widely distributed along the North coast of Vietnam, namely Hai Duong, Thai Binh, Quang Ninh, Nam Dinh and Hai Phong, 34.0 > 26.0 > 20.0 > 17.0 > 14.5 km distance to the sea, respectively. They

all belong to the species *Tylorrhynchus heterochaetus* (Quatrefages, 1866). The ragworm from the Tu Ky area of Hai Duong province, 34.0 km far from the sea, has always been among the groups with the largest size. Salinity and inland environmental conditions are likely factors influencing the morphological development of ragworm populations there. It is the most likely reason ragworms were used as a traditional delicacy in Hai Duong and became very famous throughout the country.

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