Mitochondrial DNA Analysis Suggests Invasion of Thailand Coast by a Single Species of Dreissenidae, *Mytilopsis sallei*

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ABSTRACT

In 2001, a species of false mussel was recorded in coastal areas in southern Thailand. In 2008, established populations were reported in several estuaries and lagoons. Based on shell morphology, the bivalve was identified as a non-native species belonging to Dreissenidae. To date, populations have been observed in five locations in southern Thailand on both the Andaman and Gulf of Thailand coasts. The variation in shell morphology within and between these locations led us to question whether specimens in different locations belonged to different species. We used morphometric techniques to investigate variation in shell characteristics among the five locations and mtDNA (COI) analysis to investigate genetic variation. Shell characteristics varied among locations; the shell morphology of specimens from some locations on the Andaman coast appeared to be similar to that of specimens from the Gulf coast. Most specimens had long, prominent apophyses, but in some specimens they were rounded and not prominent. In spite of the high variation in shell morphology, molecular analysis indicated that all COI gene sequences of the specimens examined are nearly identical and belong to a single species, *Mytilopsis sallei*. Phylogenetic analyses also revealed the monophyly of 25 sequences between our specimens and other *M. sallei* sequences from GenBank, with high bootstrap support. Our recent findings suggest that the name *M. sallei* should be applied to the false mussel in southern Thailand. However, this merits confirmation with analysis using other molecular markers, and more specimens should be included.

Keywords: Alien invasive species, COI gene, False mussels, *Mytilopsis*, Thailand

INTRODUCTION

Dreissenid mussels have invaded almost every continent and become pests in fresh and brackish waters (e.g., Hebert *et al*., 1989; Willan *et al*., 2000; Therriault *et al*., 2004; Verween *et al*., 2010; Wong *et al*., 2011; Fernandes *et al*., 2018). In the Indo-Pacific region, the most widespread and successful dreissenid is arguably the black-striped mussel, *Mytilopsis sallei* (Récluz, 1849), which was first described from Guatemala, Central America (Tan and Tay, 2018; Lutaenko *et al*., 2019). All the false dreissenid mussels reported from Asia were identified by morphology and/or molecular genetic analyses as *M. sallei* (Morton, 1981; Wong *et al*., 2011; He *et al*., 2016; Lutaenko *et al*., 2019). In Thailand, the observation of a dreissenid was recorded for the first time by Swennen *et al*. (2001). In southern Thailand, populations of the false mussel are currently established in estuaries, lagoons, and brackish man-made water bodies at locations on the coast of the Andaman Sea and the Gulf of Thailand (Personal observation).

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For brackish-water species, oceans and freshwater could act as barriers to their dispersal (Van der Gaag *et al*., 2016; Wells, 2019). The Thai peninsula is another geographical barrier that prevents propagule exchange between marine populations from the Andaman Sea and the Gulf of Thailand (Pongparadon *et al*., 2015). The false mussel is thought to be restricted to brackish waters of these systems (Wangkulangkul, 2018), as the survival rate of planktonic larvae in high salinity conditions (>25 psu) is poor (Raju *et al*., 1975; Sa-Nguansil and Wangkulangkul, 2020). Dreissenid populations in Thailand are thought to derive from multiple anthropogenic introduction events rather than natural dispersal processes. It is also likely that more than one species of dreissenids have been introduced to Thailand (see Fernandes *et al*., 2018, for the case in Brazil), but this hypothesis has yet to be examined.

Variation in shell shape and size of false mussels has been reported in several studies (Pathy and Mackie, 1993; Lajtner *et al*., 2004; Beggel *et al*., 2015). Those works also evaluated whether morphological characteristics and morphometric data were useful for identification of the specimens. Some characteristics of the shells, such as the presence of an apophysis (Marelli and Gray, 1983; Pathy and Mackie, 1993) could be used to distinguish several species of dreissenids.

This study aims to report variations in shell morphology among locations and investigate whether dreissenid mussels from five locations in southern Thailand belong to the same species. The possible taxonomic identities of these false mussels are also evaluated. We used morphometric analysis to examine morphological variation in false mussel shells and mtDNA analysis to examine genetic variation.

MATERIALS AND METHODS

Study sites and sampling

Adult false mussels (shell length >1 cm; according to Tan and Tay, 2018) were collected from each of the five locations shown in Figure 1 and preserved in 70% ethanol. Descriptions of locations and numbers of specimens collected are given in Table 1. False mussels were randomly collected from different aggregates when possible.

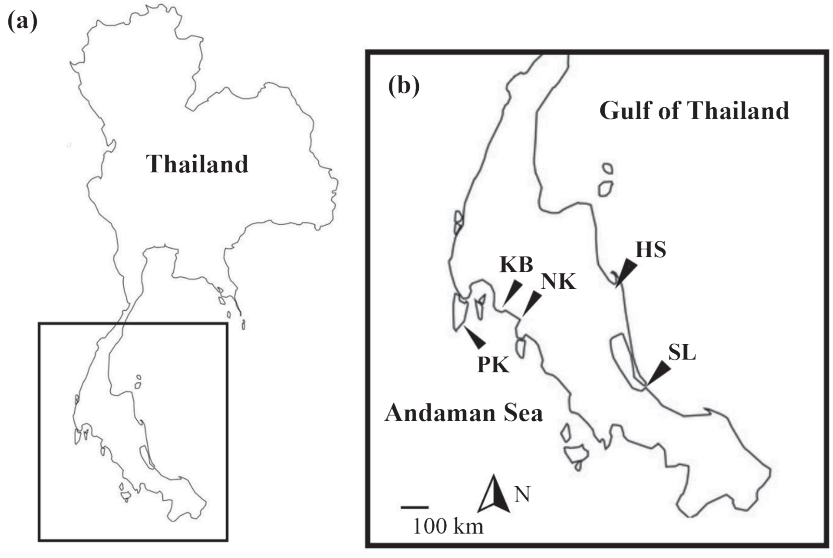

Figure 1. (a) Delimitation of southern Thailand (square); (b) Locations where *Mytilopsis* populations were observed and specimens were collected (indicated by black arrowheads): Phuket (PK), Krabi (KB) and Nuea Klong (NK) on the Andaman coast; and Hua Sai (HS) and Songkhla Lake (SL) on the Gulf of Thailand coast. Asterisks indicate locations of major international deep-water ports.

Table 1. Descriptions of locations where specimens were collected.

Location name	Coordinates	Description	No. of specimens
Andaman coast			
Phuket (PK)	7°52'08.8"N 98°23'46.5"E	Artificial concrete pond; False mussel clumps were on concrete poles and a small floating wind turbine. salinity = 15 psu	23
Krabi (KB)	8°01'27.5"N 98°52'33.4"E	Man-made earthen pond; False mussels formed dense patches on the bottom and on dry tree branches. salinity = 23 psu	21
Nuea Klong (NK)	7°56'47.8"N 98°58'12.5"E	Man-made canal; A few false mussel aggregates were found on the bottom. salinity = 5 psu	18
Gulf of Thailand coast			
Hua Sai (HS)	8°06'36.9"N 100°16'10.8"E	River; False mussels formed dense patches on the bottom. salinity = 25 psu	29
Songkhla Lake (SL)	7°06'52.3"N 100°33'23.6"E	Tidal creek; False mussels formed dense patches on the bottom and on hard man-made structures. salinity = 22 psu	27

Morphometric analysis

Morphometric analysis was based on a sample of 18-29 specimens per location. Shell elongation, shell inflation and shell angle were quantified following the method of Beggel *et al*. (2015). Length, height, and width (Figure 2) were measured to the nearest 1 mm. Shell angle was measured where a line connecting the posterior and anterior parts of the shell met a line tangent to the point of dorsal curvature (Figure 2). Shell elongation was calculated as shell length divided by shell height. Shell inflation was calculated as shell width divided by (shell length + shell height)/ 2. One of the main shell characteristics used to distinguish dreissenid species is the shape of the apophysis (Marelli and Gray, 1983; Fernandes *et al*., 2018). Here, the apophysis of each specimen's left valve was categorized as either a long or short type. The long type is pointed, prominent, sometimes hook- shaped, while the short type is rounded and not prominent (Figure 3).

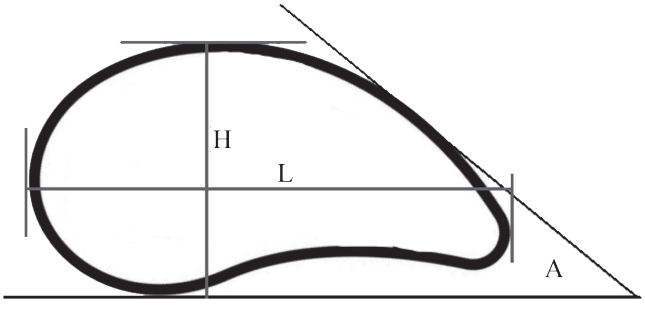

Figure 2. Morphometric measurements, based on Beggel *et al*. (2015). A = shell angle, H = shell height, L = shell length.

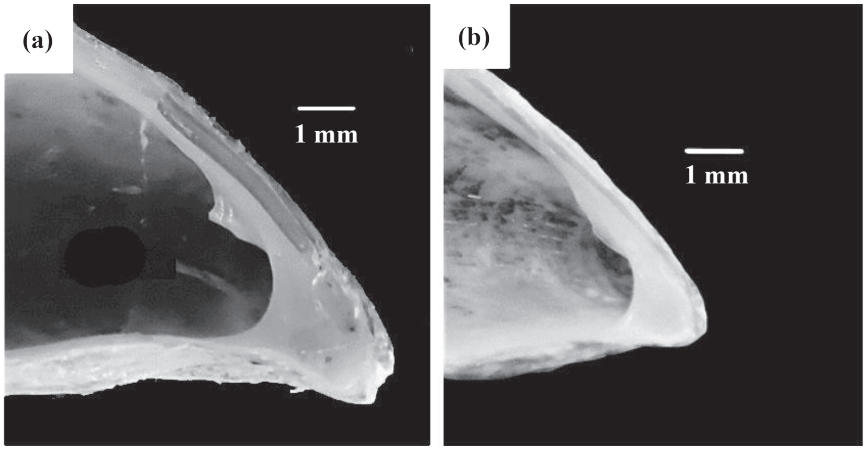

Figure 3. Left valves of *Mytilopsis* exhibiting the long-type apophysis (a) and the short-type apophysis (b).

The morphometric data were normalised. Morphometric shell characteristics among locations were compared by permutational multivariate analysis of variance (PERMANOVA) based on a Euclidean distance resemblance matrix with 999 unrestricted permutations of raw data. PERMANOVA was used rather than the conventional ANOVA because the sample size, and therefore degree of freedom, differed among locations. Pairwise analysis was also carried out. Principal component analysis (PCA) was used to reveal patterns of shell morphological variation among locations. PCA based on a correlation matrix of the morphometric variables of shell elongation, shell inflation and shell angle was performed to determine their loadings on the principal components. To demonstrate relationships among locations, hierarchical clustering using group-average linking was performed based on a resemblance matrix built from Euclidean distances. Multivariate analyses (PERMANOVA, PCA and Hierarchical clustering) were performed in the PRIMER v7 add-on package PERMANOVA+ (Anderson *et al*., 2008).

mtDNA analysis

Five specimens from each location were randomly taken for genotyping analysis. Mantle tissue was collected from samples preserved in 70% ethanol. Genomic DNA was extracted from 100 mg of tissue using the phenol-chloroform method modified from Sambrook *et al*. (1989). A 492-bp fragment of COI gene was amplified by the polymerase chain reaction (PCR) with a pair of primers, Myt_COI-F (5'-GGAGCTTAGTGCT CCTGGA-3') and Myt_COI-R (5'-AAGCATTGT CAGCCCACCA-3') (Wong *et al*., 2011). DNA amplifications were performed in 20-µL volumes containing 10 µL of 2X MyTaq HS Mix (Bioline, USA), 2 µL of 10 mM forward and reverse primer and 3 µL of genomic DNA template. The cycling conditions were 94° C for 5 min, 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR product was purified and sequenced by Gibthai Co., Ltd. (Thailand). The obtained sequences were edited using MEGAX (Kumar *et al*., 2018) and the BioEdit software (Hall, 1999). The similarity of sample sequences was determined through a BLAST search (Altschul *et al*., 1997), using the option 'Megablast' for nucleotides. Sample sequences were identified to species level against GenBank sequences showing top query coverage and high percent identity in the Nucleotide BLAST result. After the sequence similarity search, all sequences were submitted to GenBank.

The phylogenetic relationships among 25 samples in this study and other dreissenids were analysed. Multiple sequence alignment was performed with the ClustalW model using MEGA X (Kumar *et al*., 2018). The optimal nucleotide substitution model was selected by jModelTest

(Posada, 2008) according to Akaike information criterion corrected for small sample sizes (AICc). The best-fitting model for phylogenetic construction was HKY+G+I. A phylogenetic tree based on the COI gene was constructed using the maximum likelihood method (ML) in MEGA X. Various samples of dreissenid species from many locations were used to analyse relationships with our false mussel (Table 2). The outgroup was *Corbula erythrodon* Lamarck, 1818, a bivalve species belonging to the same order as the dreissenids. The tree was drawn to scale with branch lengths measured in numbers of substitutions per site.

Table 2. Sources of COI sequence data in this study.

Family	Genus	Species	Location	Accession No.	Reference
Dreissenidae	*Congeria*	*Congeria kusceri*	Croatia	JX099430	(Bilandzija *et al*., 2013)
		Congeria jalzici	Slovenia	JX099421	(Bilandzija *et al*., 2013)
		Congeria mulaomerovici	Bosnia and Herzegovina	JX099418	(Bilandzija *et al*., 2013)
	Dreissena	*Dreissena presbensis*	Greece	EF414478	(Albrecht *et al*., 2007)
		Dreissena stankovici	Macedonia	DQ840108	(Gelembiuk *et al*., 2006)
		Dreissena blanci	Greece	EF414481	(Albrecht *et al*., 2007)
		Dreissena bugensis	Ukraine	DQ840132	(Gelembiuk *et al*., 2006)
		Dreissena polymorpha	Turkey	EF414493	(Albrecht *et al*., 2007)
		Dreissena caputlacus	Turkey	DQ840106	(Gelembiuk *et al*., 2006)
	Mytilopsis	*Mytilopsis sallei*	Asia	DQ078487	(Wong *et al*., 2011)
			China	JX099435	(Bilandzija *et al*., 2013)
			Brazil	MF139876	(Fernandes *et al*., 2018)
			Thailand	MN720716 - MN720740	This study
		Mytilopsis leucophaeata	Brazil	MF139865	(Fernandes *et al*., 2018)
			Iran	HM100262	(Heiler *et al*., 2010)
			USA	KU906056	(Lodeiros *et al*., 2019)
Corbulidae	*Corbula*	*Corbula erythrodon*	Japan	AB740137	(Owada *et al*., 2013)

RESULTS AND DISCUSSION

Variation in shell outlines was observed both within and among locations (Figure 4). A nearly straight ventral margin was observed in some specimens collected from Phuket (Figure 4a). A convex dorsal margin and rounded posterior margin were features shared among locations (with a few exceptions), in which the posterior and ventral margins joined at an abrupt but rounded angle (Figure 4b). Morphometric analyses suggested that the false mussels exhibit significant variation in shell characteristics among locations (Figure 4, 5, 7; Table 3-5). Shell angles are smaller in specimens from HS (Gulf coast) than in others (Figure 5a; Table 3). Shells from KB (Andaman coast) are elongate, while shells from NK (Andaman) and SL (Gulf) are more inflated than others (Figure 5b-5c; Table 3). Most specimens had long, prominent apophyses but among specimens from NK (Andaman), the ratio of short to long types was 50/50 (Figure 6). Pairwise comparisons

of multivariate analyses showed that the shell characteristics of specimens from most pairs of locations were different (Table 5). However, specimens from NK and KB on the Andaman coast had similar morphs to specimens from SL and HS on the Gulf of Thailand coast (Table 5). The existence of these similarities was supported by the results of hierarchical clustering (Figure 7b); however, PCA did not present a clear separation among locations nor between coasts (Figure 7a). The PCA plot indicated that the variation in shell characteristics among specimens from Songkhla Lake was greater than within-location variation at other sampling sites.

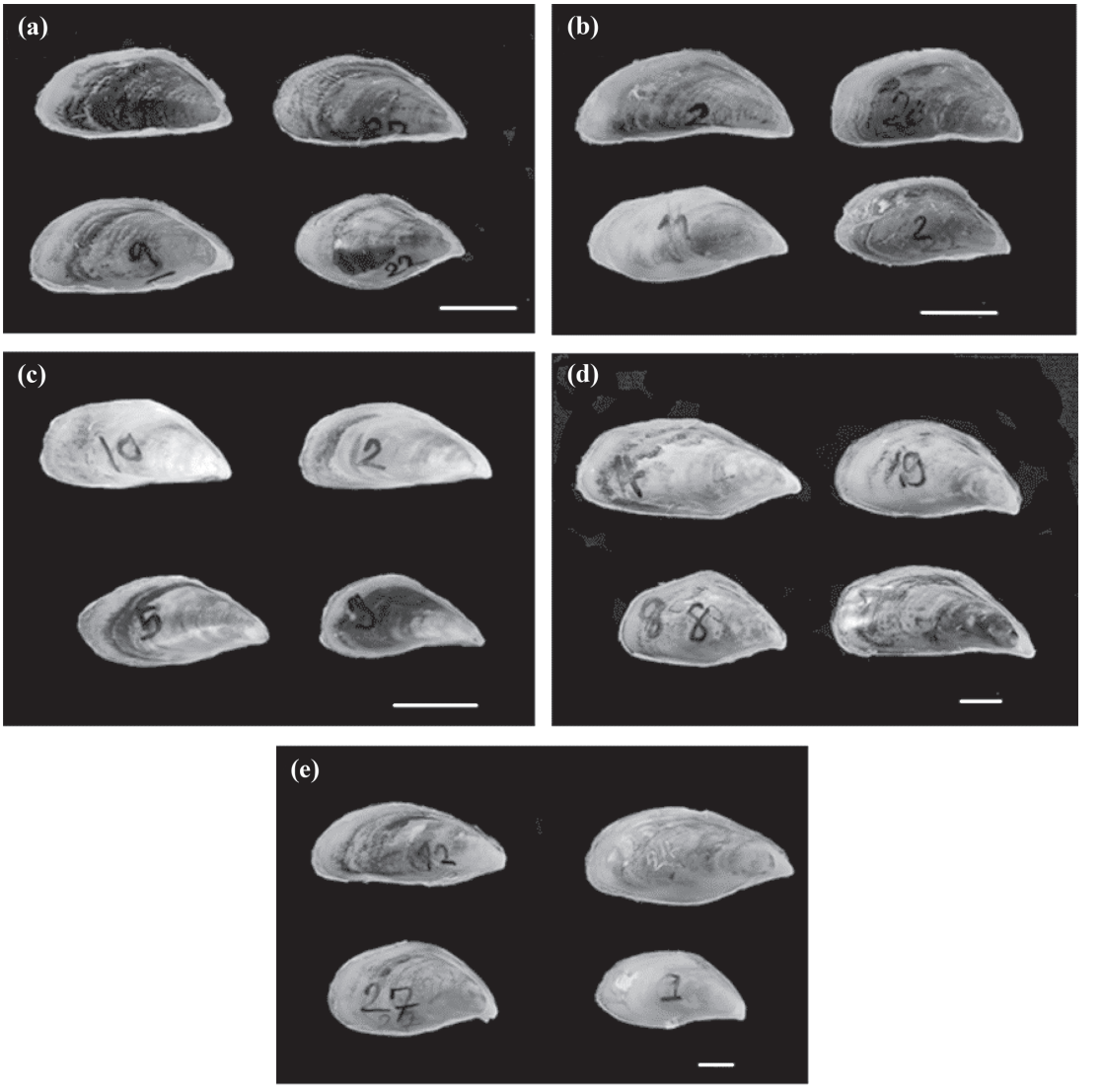

Figure 4. Left valves of *Mytilopsis* specimens collected from (a) Phuket (PK), (b) Krabi (KB), and (c) Nuea Klong (NK) on the Andaman coast; and from (d) Hua Sai (HS) and (e) Songkhla Lake (SL) on the Gulf of Thailand coast. Scale bar = 1 cm.

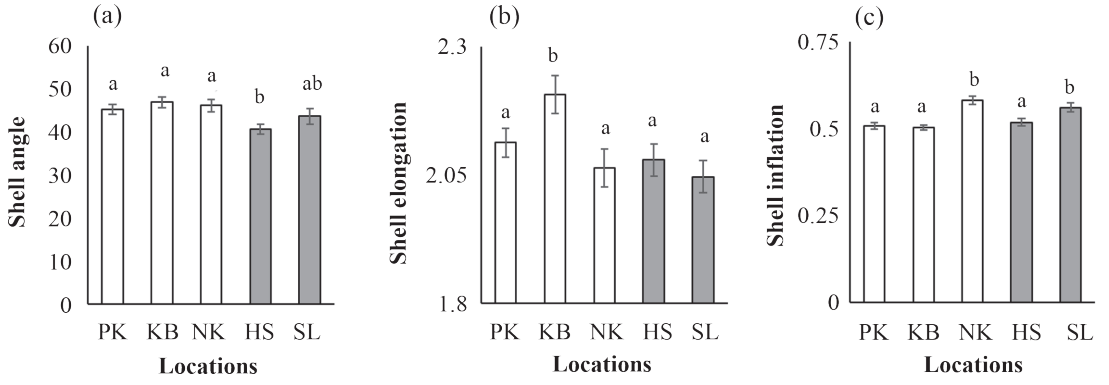

Figure 5. Results of morphometric analysis of *Mytilopsis* by collection site: (a) Shell angle, (b) Shell elongation, and (c) Shell inflation; White bars = Andaman coast; Gray bars = Gulf of Thailand coast. PK = Phuket, KB = Krabi, NK = Nuea Klong, HS = Hua Sai, and SL = Songkhla Lake; Different letters above bars indicate significant differences between means.

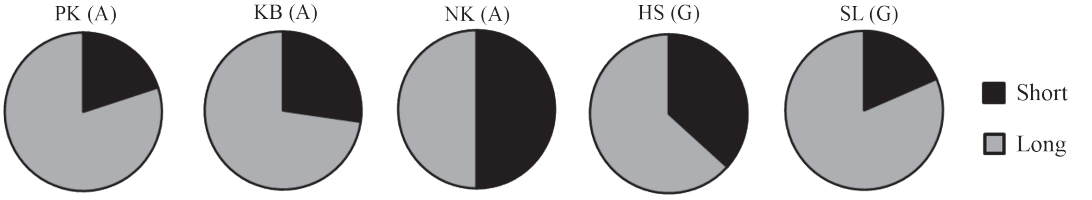

Figure 6. Ratios of short:long apophysis type; PK = Phuket, KB = Krabi, NK = Nuea Klong, HS = Hua Sai, and SL = Songkhla Lake; (A) = Andaman coast and (G) = Gulf of Thailand coast.

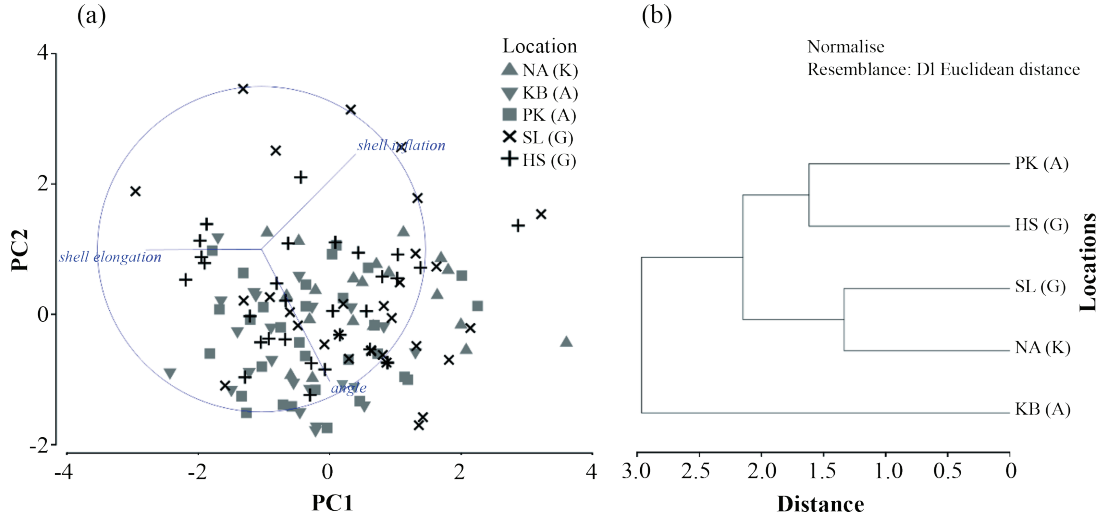

Figure 7. (a) Principal component analysis (PCA) correlation biplot, (b) Dendrogram for hierarchical clustering (group-average linking) of shell characteristics; PK = Phuket, KB = Krabi, NK = Nuea Klong, HS = Hua Sai, and SL = Songkhla Lake; (A) = Andaman coast and (G) = Gulf of Thailand coast.

Table 3. Univariate PERMANOVA testing for variation in shell characteristics among locations.

Source	Df	SS	MS	Pseudo-F	p
Shell angle					
Location	4	665.8	166.45	3.03	0.03
Residuals	128	7023.4	54.87		
Total	132	7689.3			
Shell elongation					
Location	4	0.39	0.09	3.49	0.01
Residuals	128	3.58	0.02		
Total	132	3.98			
Shell inflation					
Location	4	0.117	0.3	9.23	0.001
Residuals	128	0.405	0.01		
Total	132	0.522			

Table 4. Multivariate PERMANOVA testing for variation in shell characteristics among locations.

Source	Df	SS	MS	Pseudo-F	p
Location	4	53.98	13.49	5.05	0.001
Residuals	128	342.02	2.67		
Total	132	396			

Table 5. Pairwise comparison of differences in shell characteristics between locations.

Groups	t	p
NK (A) vs KB (A)	3.40	0.001
NK (A) vs PK (A)	2.83	0.001
NK (A) vs SL (G)	0.90	0.48
NK (A) vs HS (G)	2.77	0.001
KB (A) vs PK (A)	1.48	0.1
KB (A) vs SL (G)	2.78	0.001
KB (A) vs HS (G)	2.68	0.002
PK (A) vs SL (G)	2.07	0.004
PK (A) vs HS (G)	1.64	0.05
SL (G) vs HS (G)	1.73	0.041

Note: (A) = Andaman coast; (G) = Gulf of Thailand coast

BLAST results enabled us to identify 25 samples at species level based on 428-bp COI gene fragments. Twenty sequences were identified as being from *Mytilopsis sallei* with 100 % similarity at 100 % sequence coverage with GenBank sequences. Five other sequences from NK were designated to *M. sallei* with 99.5 % similarity, which is high enough to be possibly classified to this species. The investigation of 25 sequences in this study strongly supported monophyly with two *M. sallei* sequences from Asia and China by a bootstrap value of 100 %; meanwhile, *M. sallei* from Brazil was a sister group (Figure 8). Genotyping assessments classified all samples from our five sampling locations to *M. sallei.*

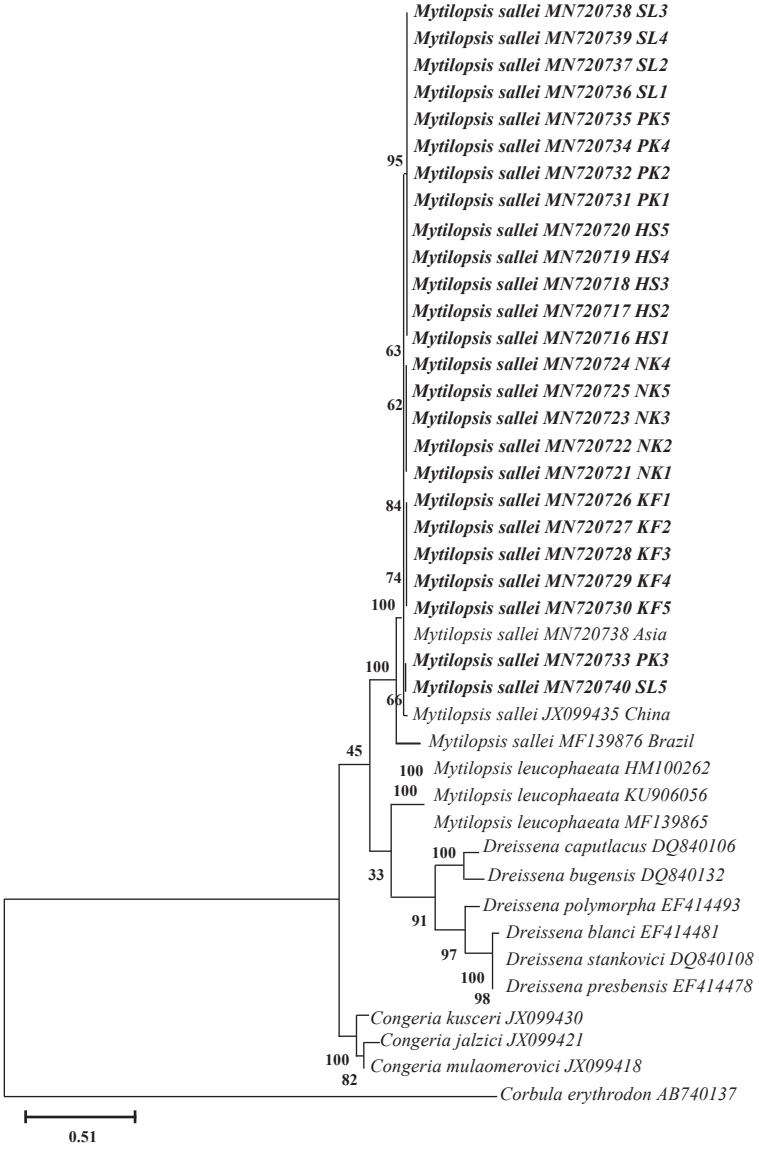

Figure 8. Phylogenetic relationships derived from maximum likelihood analyses among samples from five collection sites (Andaman coast and Gulf of Thailand coast) and other dreissenids (Log likelihood = -2423.85). PK = Phuket, KB = Krabi, NK = Nuea Klong, HS = Hua Sai, and SL = Songkhla Lake. Sequences obtained in the present study are in bold letters.

Morphological variation in dreissenids has been addressed in several studies (Lajtner *et al*., 2004; Peyer *et al*., 2010; Pavlova, 2012). Identifying false mussel species solely from shell morphology is difficult because growth patterns are enormously influenced by environmental variables such as temperature, calcium level, pH, dissolved oxygen, and depth, as well as the density of the aggregate (Pathy and Mackie, 1993; Lajtner *et al*., 2004; Trichkova *et al*., 2008; Peyer *et al*., 2010; Beggel *et al*., 2015). Among dreissenids, phenotypic plasticity is relatively high and variation in shell morphology may not reflect differences in their genotypic background (Pavlova, 2012; Beggel *et al*., 2015).

The false mussel specimens in this study were collected from aggregates in shallow waters (<1 m deep). However, differences in the general characteristics of habitats were observed: some locations were man-made reservoirs, and some were natural streams. Therefore, physical and chemical environmental properties could be distinct. At Krabi, false mussels were found in a man-made shallow earthen pond with no regular freshwater supply. The greater shell elongation of false mussels collected from this location could be a result of living in a dense aggregate, which reduced food availability and therefore restricted growth (Alunno-Bruscia *et al*., 2001; Lajtner *et al*., 2004). Specimens from the same locations showed variations in the shape of the apophysis. At most locations, shells with pointed and prominent apophyses predominated. Although our aim was not to identify specimens based on shell morphology, it is important to note that this shape matches the description of *Mytilopsis sallei* in Marelli and Gray (1983) and Fernandes *et al*. (2018).

It is also important to note that reports from other Southeast and East Asian countries similarly assigned their false mussels to *M. sallei* (Mohan and Prakash, 1998; Wong *et al*., 2011; He *et al*., 2016; Lutaenko *et al*., 2019). Previous works in Thailand (Swennen *et al*., 2001; Wangkulangkul and Lheknim, 2008; Wangkulangkul, 2018) identified their specimens as *M. adamsi*. It is possible that the identifications were made in error. However, the application of the name *M. sallei* has been questioned by some authors (Willan *et al*., 2000; Fernandes *et al*., 2018). There remains the possibility that more than one Mytilopsis species exist in the same native range (Fernandes *et al*., 2018).

CONCLUSION

Notwithstanding the high morphological variation in shell characteristics, mtDNA analysis revealed that false mussels from all locations belonged to the same species. Based on comparison of 25 COI sequences with sequences obtained from false mussels from China, Asia and Brazil, our specimens from southern Thailand were identified as *Mytilopsis sallei*. The clade containing *M. sallei* from Thailand, China and Asia was separated from the clade of *M. sallei* from Brazil. The taxonomy of *Mytilopsis* needs urgent revision, including the examination of type materials and an expanded analysis of genetic material from its native ranges.

We propose that the use of the name *M. sallei* should be implemented in future publications with a caution that taxonomic revision of the genus *Mytilopsis* is still required. Moreover, future studies should involve higher numbers of samples and analysis using different molecular markers.

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