Identification of *Sphaeroma terebrans* via morphology and the mitochondrial cytochrome *c* oxidase subunit I (COI) gene

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ABSTRACT

Sphaeroma terebrans, a wood-boring isopoda, is distributed worldwide in tropical and subtropical mangroves. The taxonomy of *S. terebrans* is usually based on morphological characteristics, with its molecular identification still poorly understood. The number of teeth on the uropodal exopod and the length of the propodus of the seventh pereopod are considered as the major morphological characteristics in *S. terebrans*, which can cause difficulty in regards to accurate identification. In this study, we identified *S. terebrans* via molecular and morphological data. Furthermore, the validity of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene as a DNA barcode for the identification of genus *Sphaeroma*, including species *S. terebrans*, *S. retrolaeve*, and *S. serratum*, was examined. The mitochondrial COI gene sequences of all specimens were sequenced and analysed. The interspecific Kimura 2-parameter distances were higher than intraspecific distances and no intraspecific-interspecific distance overlaps were observed. In addition, genetic distance and nucleotide diversity (π) exhibited no differences within *S. terebrans*. Our results revealed that the mitochondrial COI gene can serve as a valid DNA barcode for the identification of *S. terebrans*. Furthermore, the number of teeth on the uropodal exopod and the length of the propodus of the seventh pereopod were found to be unreliable taxonomic characteristics for *S. terebrans*.

Keywords: *Sphaeroma terebrans*; DNA barcode; COI gene; Molecular identification

INTRODUCTION

Mangroves are biologically and globally important ecosystems (Giri et al., 2011). Their aerial roots provide an important substrate in which many species of animals live and reproduce (Nagelkerken et al., 2008). *Sphaeroma terebrans*, a wood-boring isopoda, is found worldwide in tropical and subtropical mangroves (Estevez, 1978), where it preferentially burrows into the aerial roots for shelter and reproductive habitat (Harrison & Holdich, 1984; John, 1970). In recent years, substantial *S. terebrans* outbreaks have seriously affected mangrove stands in China, especially in Hainan island (Fan et al., 2014).

The effects of *S. terebrans* on mangroves have been studied by many researchers (Estevez & Simon, 1975; Estevez, 1978; Jones & Icely 1981; Kensley & Schotte, 1999; Perry, 1988; Rehm & Humm, 1973); however, the taxonomic standards of *S. terebrans* remain poorly understood. Due to some minor morphological differences, including the number and arrangement of the tubercles on the pereonite, the structure of the pereopod, and the presence of tubercles furnished with bristle-like hairs on the abdomen, *S. terebrans* was previously named as *S. vastator* (Bate, 1866) and *S. destructor* (Richardson, 1897). Based on morphological identification, Estevez & Simon (1975) concluded that *S. vastator* and *S. destructor* were synonyms of *S. terebrans*.

The classic use of morphological characteristics for species delimitation can result in under- or over-estimation of biodiversity due to factors such as phenotypic plasticity (Knowlton, 1993). DNA barcode, which can supplement taxonomic datasets in the process of species delimitation (Schindel & Miller, 2005), is a practical tool that can be used for the identification of various species within a known taxonomic framework and for linking different biological life stages of the same species (Feng et al., 2011; Puillandre et al., 2009;

Received: 15 July 2016; Accepted: 08 September 2016

Foundation items: This project was funded by the GEF China Wetlands System Project, Science and Technology Foundation of Macao (045/2010/A) and Special Fund for Marine-Scientific Research in the Public Interest (201305021)

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DOI:10.13918/j.issn.2095-8137.2016.5.307

Schindel & Miller, 2005). The mitochondrial COI gene has been proposed as a universal barcode, and has been successfully applied in the identification of Portunidae, fish, bivalve molluscs, and hoverflies (Blair et al., 2006; Hebert et al., 2003; Ma et al., 2012; Persis et al., 2009; Ståhls et al., 2009). The COI gene sequences of *S. terebrans* have been analysed in America and Africa (Baratti et al., 2005, 2011), with results suggesting that cosmopolitan *S. terebrans* is comprised of more than one species. Therefore, its taxonomic status needs to be revaluated.

The aim of the present study was to provide a reliable and valid way to delimit *S. terebrans*. In this study, the validity of the mitochondrial COI gene as a DNA barcode marker for the identification of three species of *Sphaeroma*, namely, *S. terebrans*, *S. retrolaeve*, and *S. serratum*, was examined. To detect if there was any cryptic species in *S. terebrans*, the COI gene sequences of individuals with morphological differences were analysed. Our study should be useful in the identification of the genus *Sphaeroma* and for further research on *S. terebrans*.

MATERIALS AND METHODS

Sampling and scoring of morphological characteristics

The *S. terebrans* specimens were collected from three localities in China (Figure 1). Prior to DNA extraction, all specimens were examined under an anatomical lens and assigned to groups according to comparison with previous morphological descriptions (Harrison & Holdich, 1984). The morphological differences of *S. terebrans* were then photographed by a TM3030Plus tabletop microscope. The *S. terebrans* individuals were sorted according to the following morphological characteristics: the number and arrangement of tubercles on the pereonite, number of teeth on the uropodal exopod, shape of the pleotelson, setae distribution, and length of the second and seventh pereopods. These are considered to be major characteristics for the diagnosis of *S. terebrans* within *Sphaeroma* (Harrison & Holdich, 1984). The *S. retrolaeve* specimens were collected from Hainan and Beihai mangroves. All samples were preserved in 95% alcohol.

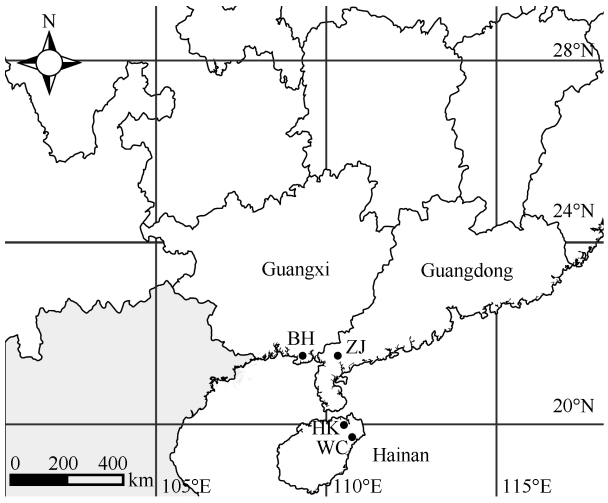

Figure 1 Sample collection sites of *S. terebrans*

HK: Haikou, Hainan, WC: Wenchang, Hainan, BH: Beihai, Guangxi, ZJ: Zhanjiang, Guangdong

DNA extraction, PCR amplification, and sequencing

The genomic DNA of *S. terebrans* and *S. retrolaeve* were obtained from the pereopods. DNA extractions were performed using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 following the manufacturer's protocols. The primers mtd10 5'-TTGATTTTTTGGTCATCCAGAAGT-3' (Roehrdan. 1993) and Florence 5'-CCTAAAAAATGTTGAGGGAA-3' were used for amplification of the mitochondrial COI gene (Baratti et al., 2005). We followed PCR protocols as per Baratti et al. (2005). The PCR products were electrophoresed using 1% agarose gel and sequenced by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

Data analysis

All sequences were aligned using ClustalW (Thompson et al., 1997). Interspecific and intraspecific sequence divergences were calculated using the Kimura 2-parameter (K2P) model with the pairwise deletion option in MEGA 5.0 (Kimura, 1980). Haplotypes were identified and analysed using DNA SP version 4.1 (Librado & Rozas, 2009). Nucleotide diversity (π) and haplotype diversity(h) were calculated according to Nei (1987) using DNA SP version 4.1 (Rozas & Rozas, 1999). Based on the K2P model, neighbor joining (NJ) and maximum likelihood (ML) trees were constructed using MEGA 5.0 (Kimura, 1980; Tamura et al., 2011), with the *Cymodoce fuscina* voucher from the NCBI (GenBank Accession No. KJ410468) used as an outgroup. Node supports for the two approaches (NJ and ML) were inferred with bootstrap analysis (1 000 replicates).

RESULTS

Prior to DNA extraction, we assigned *S. terebrans* specimens into A1-A5 and B morphotypes (Figure 2). The number of teeth on the uropodal exopod and the length of the propodus of the seventh pereopod varied within *S. terebrans*, which were assigned into seven groups (Table 1).

Partially aligned COI sequences 498 bp in length were obtained from 70 *S. terebrans* individuals and 10 *S. retrolaeve* individuals. The COI sequences of *S. serratum* and *C. fuscina* voucher were downloaded from the NCBI. Details of these sequences are shown in Table 2. There were fifteen haplotypes for *S. terebrans* and two for *S. retrolaeve*. Haplotype sequences were deposited in the NCBI under accession numbers KU558703–KU558719.

All haplotype sequences were aligned and edited, and no insertion or deletion sites were found in any of the sequences. The intraspecific distances in *S. terebrans* ranged from 0.001 to 0.013 (Table 3). The maximum interspecific distance (1.394) was between *S. serratum* and *C. fuscina* voucher, while the minimum interspecific distance (0.24) was between *S. serratum* and *S. retrolaeve*. No overlaps between interspecific and intraspecific distances were found, suggesting the existence of a distinct barcoding gap. The NJ phylogenetic tree is shown in

Figure 3. Distinct clusters corresponding to species were found with high bootstrap support.

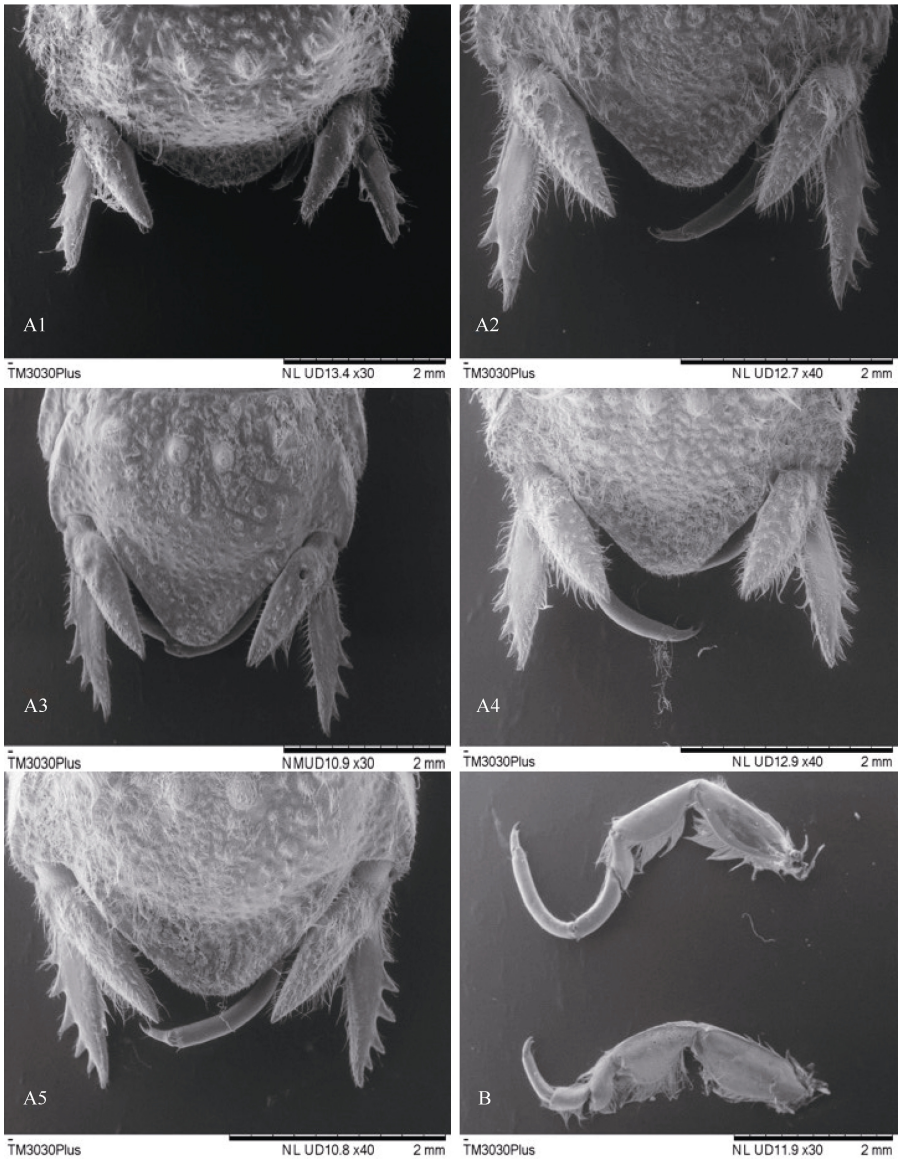

Figure 2 Diagnostic morphological characteristics of *S. terebrans*

A1-A5 are uropodal exopods with different numbers of teeth. B is the seventh pereopod with different propodus length.

The *S. terebrans* individuals were sorted into seven groups according to their morphological traits, and partial COI sequences of *S. terebrans* were aligned and compiled. The intraspecific distances ranged from 0.001 to 0.003 within the SS, SW, WW, WL, and LL groups. The intraspecific distance between PL and PS was 0.001 (Table 4). The mean haplotype diversity (*h*) was 0.555%, and ranged from 0.200% (PL group) to 0.866% (WW group) (Table 5). The highest nucleotide diversity (π) was found in the WW group (0.004), while the lowest was found in the PL group (0.000) (Table 5). Results suggested that there were no mitochondrial genetic variations within *S. terebrans*.

Phylogenetic analysis of genus *Sphaeroma* was performed using NJ and ML methods, which yielded similar results. The NJ tree revealed that the three species of *Sphaeroma* and one species of *Cymodoce* formed monophyletic clusters (Figure 3). The nearest relationship was observed between *S. retrolaeve* and *S. serratum*, while the most distant relationship was found between *S. terebrans* and *C. fuscina* voucher.

DISCUSSION

The rapid and effective identification of closely related wood-borer *Sphaeroma* species is important for the research and

Table 1 List of sampling localities and morphological differences of *S. terebrans*

Codes	Number	Location	Teeth on uropodal exopod		Propodus
			Left	Right	
SS1-5	5	Beihai, Guangxi	3	3	Short
SS6-10	5	Wenchang, Hainan	3	3	Short
SW1-5	5	Zhanjiang, Guangdong	3	4	Short
SW6-10	5	Haikou, Hainan	3	4	Short
WW1-10	10	Haikou, Hainan	4	4	Short
WL1-10	10	Wenchang, Hainan	4	5	Short
LL1-5	5	Beihai, Guangxi	5	5	Short
LL6-10	5	Haikou, Hainan	5	5	Short
PS1-10	10	Wenchang, Hainan	4	4	Short
PL1-10	10	Wenchang, Hainan	4	4	Long

The numbers of teeth on the left and right exopod of *S. terebrans* are 3 and 3, respectively, for SS; 3 and 4 for SW; 4 and 4 for WW; 4 and 5 for WL; and 5 and 5 for LL. PL means that the propodus of the seventh pereopod of *S. terebrans* is long, whereas PS is short. The same in the following table.

Table 2 List of COI sequences, GenBank accession numbers, and geographic sources of samples

Species	*n*	GenBank accession no.	Source
S. terebrans	15	KU558705-KU558719	Hainan, China
S. retrolaeve	2	KU558703, KU558704	Guangxi, China
S. serratum	1	GU130256	Germany
C. fuscina voucher	1	KJ410468	Germany

n indicates the number of sequences analyzed.

Table 3 Pairwise genetic distances (Kimura 2-parameter) of three *Sphaeroma* species and one *Cymodoce* species based on COI sequences

	SR-H1	SR-H2	ST-H1	ST-H2	ST-H3	ST-H4	ST-H5	ST-H6	ST-H7	ST-H8	ST-H9	ST-H10	ST-H11	ST-H12	ST-H13	ST-H14	ST-H15	SS	CF
SR-H1																			
SR-H2	0.004																		
ST-H1	0.254	0.248																	
ST-H2	0.251	0.245	0.006																
ST-H3	0.260	0.254	0.008	0.006															
ST-H4	0.251	0.245	0.008	0.006	0.008														
ST-H5	0.257	0.251	0.006	0.004	0.002	0.006													
ST-H6	0.254	0.248	0.004	0.002	0.004	0.004	0.002												
ST-H7	0.257	0.251	0.011	0.008	0.011	0.011	0.008	0.006											
ST-H8	0.257	0.251	0.006	0.004	0.006	0.006	0.004	0.002	0.008										
ST-H9	0.251	0.245	0.011	0.004	0.006	0.011	0.004	0.006	0.013	0.008									
ST-H10	0.251	0.251	0.006	0.004	0.006	0.006	0.004	0.002	0.008	0.004	0.008								
ST-H11	0.254	0.248	0.006	0.004	0.006	0.006	0.004	0.002	0.008	0.004	0.008	0.004							
ST-H12	0.254	0.248	0.006	0.004	0.006	0.006	0.004	0.002	0.004	0.004	0.008	0.004	0.004						
ST-H13	0.254	0.248	0.004	0.002	0.004	0.004	0.002	0.001	0.006	0.002	0.006	0.002	0.002	0.002					
ST-H14	0.254	0.248	0.004	0.002	0.004	0.004	0.002	0.001	0.006	0.002	0.006	0.002	0.002	0.002	0.001				
ST-H15	0.257	0.251	0.008	0.006	0.008	0.008	0.006	0.004	0.011	0.006	0.011	0.006	0.006	0.006	0.004	0.004			
SS	0.241	0.240	0.243	0.246	0.249	0.243	0.246	0.243	0.252	0.246	0.246	0.246	0.240	0.246	0.243	0.243	0.243		
CF	1.290	1.270	1.099	1.131	1.111	1.135	1.123	1.123	1.135	1.123	1.144	1.135	1.114	1.135	1.123	1.123	1.131	1.394	

H1-H15: Haplotype 1-15, SR: *S. retrolaeve*, ST: *S. terebrans*, SS: *S. serratum*, CF: *C. fuscina* voucher.

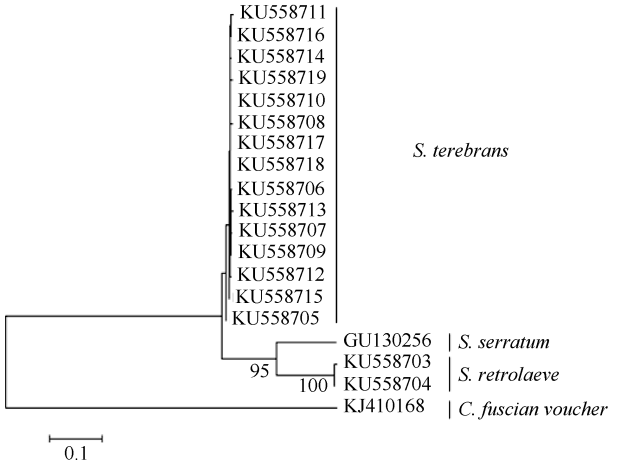

Figure 3 Neighbor-joining phylogenetic tree of individual haplotypes of three species of *Sphaeroma* and one species of *Cymodoce*

Table 4 Intraspecific genetic distances (Kimura 2-parameter) of *S. terebrans* with morphological differences based on COI sequences

	SS	SW	WW	WL	LL	PL	PS
SS							
SW	0.002						
WW	0.003	0.003					
WL	0.002	0.002	0.003				
LL	0.001	0.002	0.003	0.002			
PL	0.001	0.001	0.002	0.002	0.001		
PS	0.001	0.002	0.003	0.002	0.001	0.001	

Table 5 Number of haplotypes, haplotype diversity(*h*), and nucleotide diversity(π) of different groups

Group	No. haplotypes	Haplotype diversity (*h*)	Nucleotide diversity (π)
SS	3	0.377	0.001
SW	5	0.666	0.002
WW	7	0.866	0.004
WL	6	0.777	0.002
LL	3	0.600	0.001
PL	2	0.200	0.000
PS	3	0.416	0.001

restoration of eroded mangroves. Identification of *S. terebrans* based on morphological characteristics alone is weak and, to some extent, ambiguous. Based on morphological characteristics, Some individuals of *S. terebrans* were previously named *S. vastator* (Bate, 1866) and *S. destructor* (Richardson, 1897). In this study, clear evidence was provided for the identification of *S. terebrans* individuals, which exhibited differences in morphological characteristics. The validity of using the mitochondrial COI gene sequence as a DNA barcode for the identification of genus *Sphaeroma* was examined, and included three *Sphaeroma* species, namely, *S. terebrans*, *S. retrolaeve* and *S. serratum*, with *C. fuscina* voucher (Sphaeromatidae) used as an outgroup. A distinct barcoding gap was found between the intraspecific and interspecific distances in each species. The NJ phylogenetic tree consisted of four distinct clusters, each containing individuals from one species only. These results indicate that the partial mitochondrial COI gene is an effective DNA barcode for the identification of the genus *Sphaeroma*.

Individuals of *S. terebrans* had different numbers of teeth on the uropodal exopod and different lengths of the propodus of the seventh pereopod. These individuals were sorted into seven groups, with each group containing 10 individuals. The genetic distance and nucleotide divergence showed no variation among the different groups. Therefore, these results revealed that the COI gene sequences of individuals with morphological differences were almost no difference. Although Harrison & Holdich (1984) determined that the propodus of the seventh pereopod of subadult males is relatively short, Our investigations showed that the length of the pereopodal propodus in *S. terebrans* was not necessarily linked with gender. Previous research concluded that cosmopolitan *S. terebrans* was comprised of more than one species (Baratti et al., 2011, 2005), but morphological taxonomic details of *S. terebrans* were not mentioned. In our research, specimens in China were carefully checked according to morphological characteristics and were assigned into different groups, with molecular methods used for further identification. This combination of morphological taxonomy and molecular divergence should provide results of greater reliable.

CONCLUSIONS

In this study, the mitochondrial COI gene was found to be an effective DNA barcode for the identification of *Sphaeroma* species, whereas the number of teeth on the uropodal exopod and the length of the propodus of the seventh pereopod were found to be invalid taxonomic characteristics. The phylogenetic relationships determined in this study will be of use for studying the species composition of *Sphaeroma* in eroded mangroves in China and for establishing a good foundation for the restoration of mangrove ecosystems.

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