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High level of genetic differentiation in the marine isopod *Sphaeroma terebrans* (Crustacea Isopoda Sphaeromatidae) as inferred by mitochondrial DNA analysis

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Abstract

Sphaeroma terebrans Bate 1866 is a marine isopod belonging to the large family Sphaeromatidae, which normally colonises the aerial roots of the mangrove genus *Rhizophora* in tropical and subtropical areas. *S. terebrans* is part of a group of species whose complete life cycle occurs within the same mangrove wood. In this paper, we provide clear evidence of significant genetic differentiation among geographic populations of the taxon *S. terebrans.* The consistently low internal variation and the large interpopulation distances indicate that almost all the mitochondrial variation (cytochrome oxidase I) in *S. terebrans* is apportioned among populations rather than within them. The mean haplotype diversity (h) is 0.71%, and the mean nucleotide diversity (π) is 0.34%. The Minimum Spanning Tree (MST) reveals a complex pattern: three principal haplotype groups corresponding to the geographic locations investigated are distributed in a network. This suggests an ancient evolutionary history and very restricted gene flow between populations. The large genetic distances between the populations of *S. terebrans* could suggest that this taxon is not a single species but a species complex whose taxonomic status must be revaluated.

Keywords: *Sphaeroma terebrans*; Isopods; Mangrove ecosystems; Cytochrome oxidase I; Genetic structure

1. Introduction

Isopoda is an order of Crustacea Malacostraca comprising various families which have colonised all possible environments, both terrestrial and aquatic. The latter include entire families of marine species.

Some biological aspects of this group differentiate it from the other Malacostraca and have important effects on the population structure (Wilson, 1991). Unlike most crustaceans, which present pelagic larval stages, the females of Isopoda retain the fertilised eggs

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in a marsupium composed of sternal processes of the abdominal segments, the oostegites, in which the eggs develop to maturation.

Sphaeroma terebrans Bate 1866 is a marine isopod belonging to the large family Sphaeromatidae, which normally colonises the aerial roots of the mangrove genus *Rhizophora* in tropical and subtropical areas (Harrison and Holdich, 1984; Villalobos et al., 1985).

The mangrove forests inhabited by *S. terebrans* are dispersed along tropical and subtropical coasts (Fig. 1). They appear as "islands" of different shape, separated from each other by sandy or rocky shores of various extension. The discontinuous distribution of mangroves represents an interesting natural model, in which the dispersal capabilities of the species inhabiting this ecosystem can be investigated in relation to their reproductive strategies.

S. terebrans lives in burrows bored in the wood, which serve as refuge areas where mating and the reproductive cycle take place. Males leave the burrows after copulation, while females remain inside

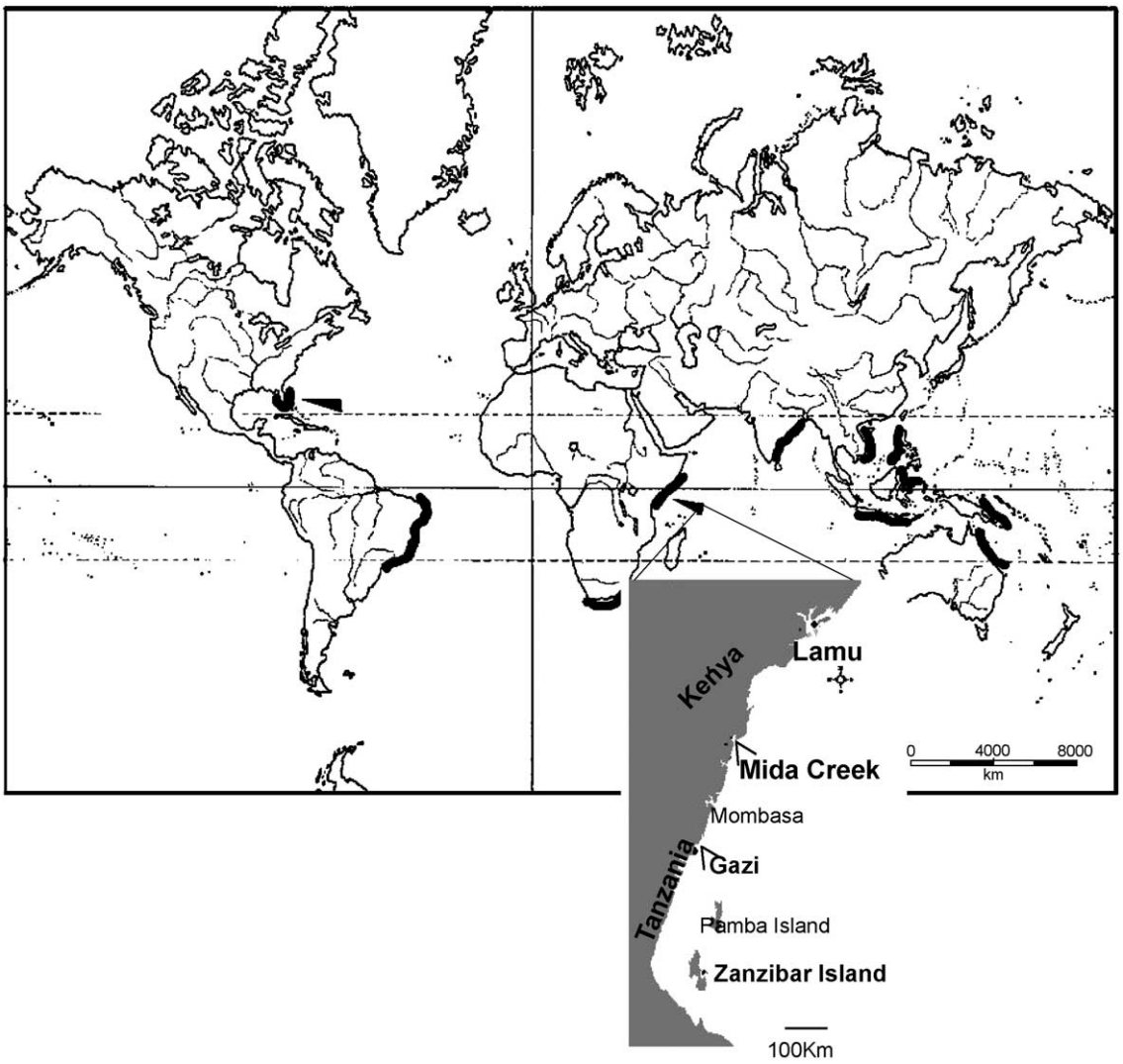

Fig. 1. Distribution of the taxon *S. terebrans* represented by solid black lines. Arrows indicate the collecting sites.

the burrow even after the offspring are released from the brood pouch. The mother spends most of the time in the hole, blocking the entrance with her telson and creating a flow of water with her pleopods to oxygenate the environment and provide a food supply. The protective behaviour of the female is therefore crucial for survival of the offspring (Messana et al., 1994; Thiel, 1999, 2001).

An important goal of many microevolutionary studies is to quantify the relationships between the dispersal capabilities and the spatial scale over which populations differ genetically (Bohonak, 1999). Marine populations often present low levels of genetic structure as a result of high dispersal abilities and the absence of obvious physical barriers (Bohonak, 1999). In sedentary or sessile marine animals, the population structure depends on the dispersal capability of the larval stages. Species with a short larval phase usually have a higher genetic structure than species with a longer larval stage. Several authors have demonstrated that adult populations of sessile species lacking planktonic larval stages show a significant genetic structure (Hedgecock, 1986; Arndt and Smith, 1998; Kyle and Boulding, 2000). However, this issue is still highly controversial, and there are contrasting results. For instance, a study of four species of the marine gastropod *Littorina*, two with planktonic larvae and two direct developers, showed a significant genetic structure in one of the taxa with planktonic larvae and a lack of structure in one of the direct developer species (Kyle and Boulding, 2000).

The reproductive strategy of Isopoda and in particular *S. terebrans* suggest that gene flow would be rather limited in this species, even between populations only a few tens of kilometres apart. However, the cosmopolitan distribution of the taxon raises some questions about its evolutionary history. Indeed, preliminary morphometric and molecular studies of *S. terebrans* have led to doubts about the existence of a single species for this taxon (Baratti et al., 2000, unpublished data).

Therefore, the aim of this study is to investigate the genetic structure of *S. terebrans* on a large geographic scale to elucidate the mechanisms driving the evolution of this species in relation to its peculiar reproductive strategy.

2. Materials and methods

2.1. Material studied

Specimens of *S. terebrans* were collected in four African and one American mangrove ecosystem [Gazi Bay, Dabaso and Sita (both in Mida Creek) in Kenya; Zanzibar Island, Tanzania; Indian River Lagoon, FL]. The total number of specimens was 88: 21 from Dabaso, 16 from Sita, 16 from Gazi Bay, 20 from Zanzibar and 15 from Florida. They were preserved in absolute ethanol until DNA extraction, which was carried out from thoracic legs (pereopods).

DNA extractions were performed with the QIAamp tissue kit (Qiagen, USA) following the manufacturers protocol, with minor changes.

2.2. Amplification

We amplified a 500-bp fragment of the mitochondrial cytochrome oxidase gene using the mtd10 universal primer 5′ -T TGA TTT TTT GGT CAT CCA GAA GT-3′ of Roehrdanz (1993) and a primer specifically designed by the authors for *S. terebrans* named Florence 5′ -C CTA AAA AAT GTT GAG GGA A-3′ obtained by modification of a primer reported by Gopurenko et al. (1999). PCR was carried out in a Perkin Elmer Gene-Amp® PCR System 9700 thermocycler with the following thermocycle profile: 35 cycles, denaturation at 94 °C for 30 s; annealing at 48 °C for 30 s; extension at 72 °C for 1 min, followed by 10 min at 72 °C. PCR products were electrophoresed and purified using the ExoSAP-IT kit (Amersham Biosciences, Uppsala, Sweden). Purified DNA was quantified with a concentration marker, sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing v. 2.0-ABI PRISM, Applied Biosystems, Foster City, U.S.A.) and then analysed with an ABI Prism 310 automated sequencer. Sequences used in this study have been deposited in Genbank under accession numbers reported in Table 1.

Electrophenograms were visualised with CHROMAS software version. 1.45 (Technelysium), and the sequences (manually corrected) were analysed with ProSeq v 2.9 Beta (http://helios.bto.ed.ac.uk/evolgen/filatov/proseq.html) and aligned using CLUSTALX version 1.81 (Thompson et al., 1997). Nucleotide

Table 1
Haplotypes from 1 to 35 with relative Genbank accession numbers and their distribution in each investigated population

Haplotype no.	Genbank accession no.	Dabaso	Sita	Zanzibar	Gazi Bay	Florida
1	AF453223	6	2			
2	AF447859	1				
3	AF453224	1				
4	AF453225	1				
5	AF453228	2				
6	AF453226	1				
7	AF453227	1				
8	AF453229	5	5			
9	AF453230	1				
10	AF453233	1				
11	AF453231	1	3			
12	AF453232			6		
13	AF453234		1			
14	AF447835		3			
15	AF453236		1			
16	AF453237		1			
17	AF453238			2		
18	AF453239			1		
19	AF453241			1		
20	AF453240			3		
21	AF453242			1		
22	AY247973			1		
23	AY247974			1		
24	AY247975			2		
25	AY247976			1		
26	AY247977			1		
27	AY247978					11
28	AY247979					1
29	AY247980					2
30	AY247981					1
31	AY247982				1	
32	AY247983				12	
33	AY247984				1	
34	AY247985				1	
35	AY247786				1	
	Total	11	7	11	5	4

diversity (π) and haplotype diversity were calculated according to Nei (1987) using the program DnaSP 3.0 (Rozas and Rozas, 1999).

Nucleotide composition, TS/TV rates, synonymous–nonsynonymous mutations and the number of haplotypes were calculated with ARLEQUIN software version 2.0 (Schneider et al., 1999). The level of genetic differentiation was investigated through a molecular variance analysis [analysis of molecular variance (AMOVA)] as implemented in Arlequin version 2000 (Schneider et al., 1999). The AMOVA was carried out at three hierarchic levels: among geographic regions (FCT), among populations of the same region (FSC) and within populations (FIS). The FST values (Wright, 1951) and their significance levels (10.000 permutations) were computed using both haplotype frequencies and nucleotide divergences (Kimura two parameters, Kimura, 1980) for each population pair.

The genealogical relationships between haplotypes were represented by a Minimum Spanning Tree (MST) obtained using the TCS software package (TCS 1.13 software, Clement et al., 2000), which employs the method of Templeton et al. (1992). It calculates the number of mutational steps by which pairwise haplotypes differ and computes the probability of parsimony (Templeton et al., 1992) for pairwise differences until the probability exceeds 0.95.

The correlation between geographic distances and genetic distances was tested with the Mantel test (Mantel, 1967) using NTSYS v. 2.2 (Rohlf, 2000).

3. Results

Using the Florence-mtd10 primer pair, we generated a 500-bp fragment of unambiguous sequence from each of the 88 individuals from the five populations. There are 133 variable sites, of which 17 are parsimony informative. Substitutions (n=125) are silent with the exception of eight mutations that are not synonymous and determine an amino acid change (all the amino acid changes are in the Florida population).

There is a bias towards A-T in all the populations (data not shown). These 88 individuals carry 35 unique haplotypes. Sequences from specimen collected at the same site appear closely related. Three haplotypes are shared by the Dabaso and Sita populations, while the other 32 haplotypes are restricted to single populations.

Table 2

Località	N	Na	h	π
Dabaso	21	11	0.889	0.6
Zanzibar	20	11	0.895	0.09
Gazi Bay	16	5	0.450	0.2
Sita	16	7	0.883	0.5
Florida	15	4	0.467	0.2

N: number of individuals; Na: number of haplotypes; h: haplotypic diversity; π: nucleotidic diversity.

The mean haplotype diversity (h) is 0.716%, with a range from 0.895% (Zanzibar) to 0.450% (Gazi Bay) (Table 2). The highest nucleotide diversity (π) is found in Dabaso (0.6), while the lowest in Zanzibar (0.09) (Table 2).

The relationships between haplotypes was provided by the Minimum Spanning Tree (MST) (Fig. 2), revealing three groups of haplotypes distributed in the network (GaziBay+Zanzibar; Mida Creek+-Zanzibar and Florida), suggesting an ancient evolutionary history. The haplogroups correspond to the geographic locations, with the exception of Zanzibar individuals, whose haplotypes are distributed in two haplogroups, that of Gazi Bay and Mida Creek. These three subnetworks are very homogeneous, and the genealogical relationships within them are well reconstructed, with the exception of some reticulations probably explained by migration events. The

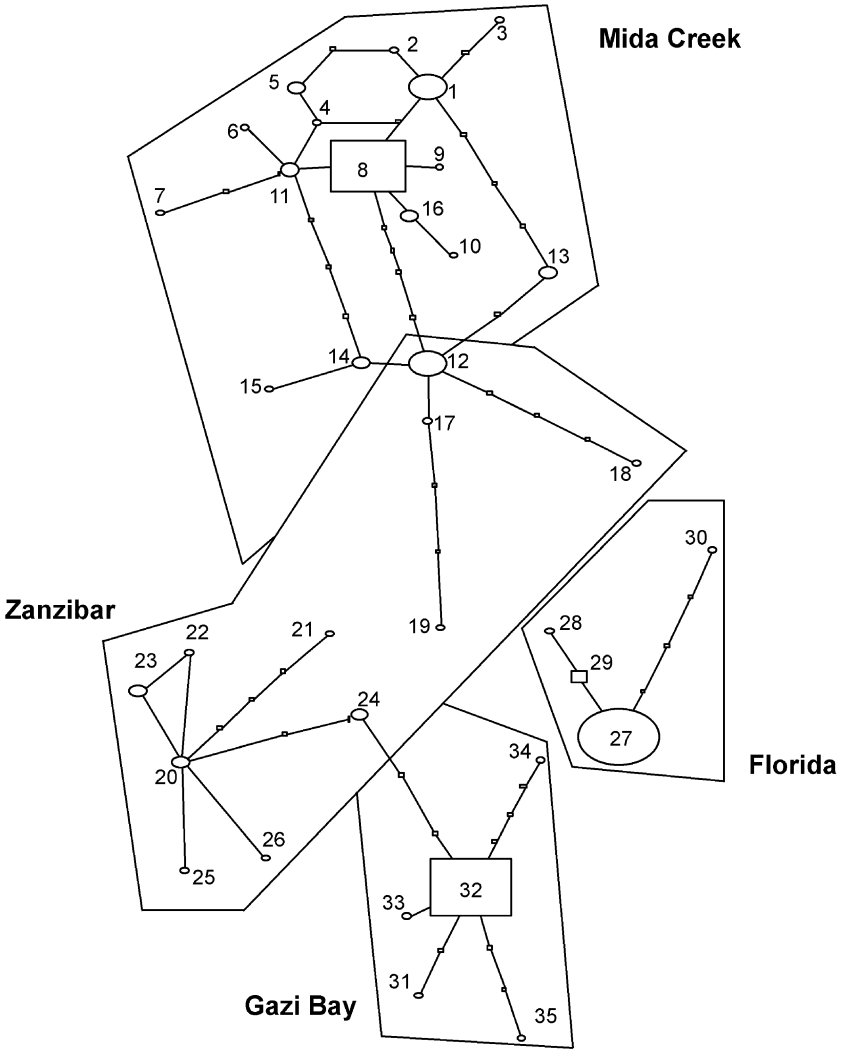

Fig. 2. Minimum spanning network (MST) among haplotypes: each oval represents a haplotype, and the squares represents the haplotype with the highest outgroup probability. The size of the square or oval corresponds to the haplotype frequency. The numbers correspond to the haplotypes as reported in Table 1. Solid lines delimit haplogroups in relation to each collecting site.

linear branches sustain steps varying from one until three mutations, while the three principal haplogroups are very differentiated from each other, and the great distances (over 100 bp) often exceed the saturation limit (number maximum of mutational connections between pairs of sequences justified by the "parsimony" criterion=9 bp), obstructing the connection between them.

Division of the molecular variance into three components allows estimation of the relative levels of genetic divergence. The AMOVA carried out at three levels (within populations, among populations and among continents) provided the following percentage values of molecular variation, respectively, based on the nucleotide divergence (Table 3a) and on the haplotype frequency (Table 3b): within population variation component (14.39% and 66.58%), variability between populations (49% and 18.44%) and variability between continents, America vs. Africa (36.62% and 14.98%). The nucleotide divergence pattern of low internal variation and large interpopulation distances indicates that essentially all mitochondrial genetic variation in *S. terebrans* is apportioned among populations rather than within them.

Table 3
Amova results based on (a) nucleotide divergences and (b) haplotypic frequencies

IIIa	Freedom degrees	Variance components	Percentage of variation
Variation			
Among groups	1	26.87213 Va	36.62
Among populations within groups	3	35.95813 Vb	49.00
Within populations	83	10.56076 Vc	14.39
Total	87	73.39102	
	FSC:	0.77298*	
	FST:	0.8561*	
	FCT:	0.36615*	

IIIb	Freedom degrees	Variance components	Percentage of variation
Variation			
Among groups	1	0.08223 Va	14.98
Among populations within groups	3	0.10124 Vb	18.44
Within populations	83	0.36556 Vc	66.58
Total	87	0.54902	
	FSC:	0.21688*	
	FST:	0.26299*	
	FCT:	0.14978	

* Significant value at $p<0.05$.

Table 4
Pairwise values of Fst among populations of *S. terebrans*

Fst	Sita	Dabaso	Gazi Bay	Zanzibar
Sita				
Dabaso	0.05			
Gazi Bay	0.97*	0.98*		
Zanzibar	0.52*	0.50*	0.44*	
Florida	0.97*	0.95*	0.98*	0.68*

When Dabaso and Sita were considered together, were named Mida Creek.

* Significant value $p<0.05$.

The global Fst based on the Kimura two parameters distances shows a highly significant value (0.85); in the paired comparisons, the highest value is between the Gazi Bay and Florida populations (0.98), while the lowest not significant value is between the Sita and Dabaso populations (0.05), both belonging to the same creek (Mida) and only 2 km far apart (Table 4).

The global Fst estimation based on haplotype frequencies (not shown) gives a total value of 0.33, with the lowest value for the Sita–Dabaso comparison (0.01) and the highest for Florida–Gazi Bay populations (0.54).

The Mantel test applied to Fst and geographic distances was not significant ($r^2=0.51$, $P=0.07$). Therefore, an isolation by distance model is not supported statistically.

4. Discussion

The present study provides clear evidence of significant genetic differentiation among geographic populations of the taxon *S. terebrans*.

In the past, morphological differences between populations of *S. terebrans* have been attributed to phenotypic geographic variation of the species (Calman, 1921; Pillai, 1955; John, 1968; Harrison and Holdich, 1984; Jacobs, 1987). However, preliminary morphological and morphometric data on populations from Kenya, the Philippines and Florida showed significant differences between the Philippines and Kenya, while the differences between Kenya and Florida were less evident,

and they were almost absent between populations of the same geographic area (Baratti et al., 2000, unpublished). Unfortunately, the only observations that could be done with the Philippine populations where those related to morphological characters. In fact, no specimen was available for genetic studies, being incorrectly preserved. Moreover, it was impossible to obtain fresh material neither from colleagues travelling in the region nor from local correspondents.

These findings, together with the large genetic distances between the populations of *S. terebrans* in this study, could suggest that the taxon is not a single species, whose valid taxonomic characters used until now should be revaluated.

The genetic distances between the populations of *S. terebrans* can be placed in context when compared with the values for other isopod taxa obtained with the same molecular marker (Wetzer, 2001; Rivera et al., 2002; Taiti et al., 2003). The genetic distances (15–18%) between most of the *S. terebrans* populations match those for different isopod species belonging to the same genus (Table 5).

The genetic differentiation between the American and African populations appears more substantial when genetic distances based only on the first and second positions are considered (Table 4). This high genetic differentiation supported also by the fact the nonsynonymous changes were observed only between African and American populations is paralleled by morphological analysis (Messana, G., personal observation).

The analysis of the African populations is more complicated: the morphological data do not support a separation of the different populations of the African coasts, although the high genetic differentiation between the Mida Creek and Gazi Bay populations suggests two distinct gene pools.

Table 5
Genetic p divergences considering the three codon positions below diagonal and the 1st and 2nd codon positions only above diagonal

	Gazi Bay	Sita	Dabaso	Zanzibar	Florida
Gazi Bay	**	0.040	0.040	0.024	0.034
Sita	0.19	**	0.000	0.034	0.056
Dabaso	0.20	0.006	**	0.034	0.056
Zanzibar	0.10	0.10	0.10	**	0.038
Florida	0.14	0.18	0.18	0.15	**

In spite of the low vagility of *S. terebrans*, mechanisms of passive dispersal, probably through floating mangrove woods, could be responsible for the worldwide distribution of the taxon, which is until now considered cosmopolitan.

The mangrove fauna and flora often exhibit strong ecological specialization and are highly differentiated with respect to organisms inhabiting the wide coastal tracts separating one mangrove area from another. These sandy or rocky shores are exposed to the forceful action of the sea, whereas the thick mud layer constituting the ground of the mangrove ecosystem testifies to the mild mechanical action of waves. However, even species with low dispersal abilities are affected by coastal currents since larvae, propagules and mangrove fragments with animals on board appear at the entrance of the creek.

The effects of sea currents on the genetic structure of species and the separation between them have been demonstrated, although some authors maintain that the action of currents cannot explain the gene flow in a very predictable manner (Kyle and Boulding, 2000).

In East Africa, the action of the Somali Current (SC), flowing south–north in summer and north–south in winter (Fig. 3), could explain the clustering of Zanzibar haplotypes with the two African haplogroups, Mida Creek (comprising Dabaso and Sita) and Gazi Bay. During the ebb tide, the very strong outlet currents at the creek mouth could eject floating pieces of mangrove into the Somali Current (SC) which could then transport the wood for several kilometres. Zanzibar Island is situated almost exactly where, in winter, the SC current converges with the East African Coastal Current (EACC) and deviates eastward to become the South East Counter Current (SECC) (Schott and McCreary, 2001). While it is very difficult for floating wood to exactly centre in the mouth of a creek when the tide rises and water enters the creek, the catching capacity of an island at the crossroads of two opposite currents might be much higher.

S. terebrans belongs to a group of species whose complete life cycle occurs within the same mangrove wood. The females develop larvae in a ventral brood pouch and take care of the young in their holes for

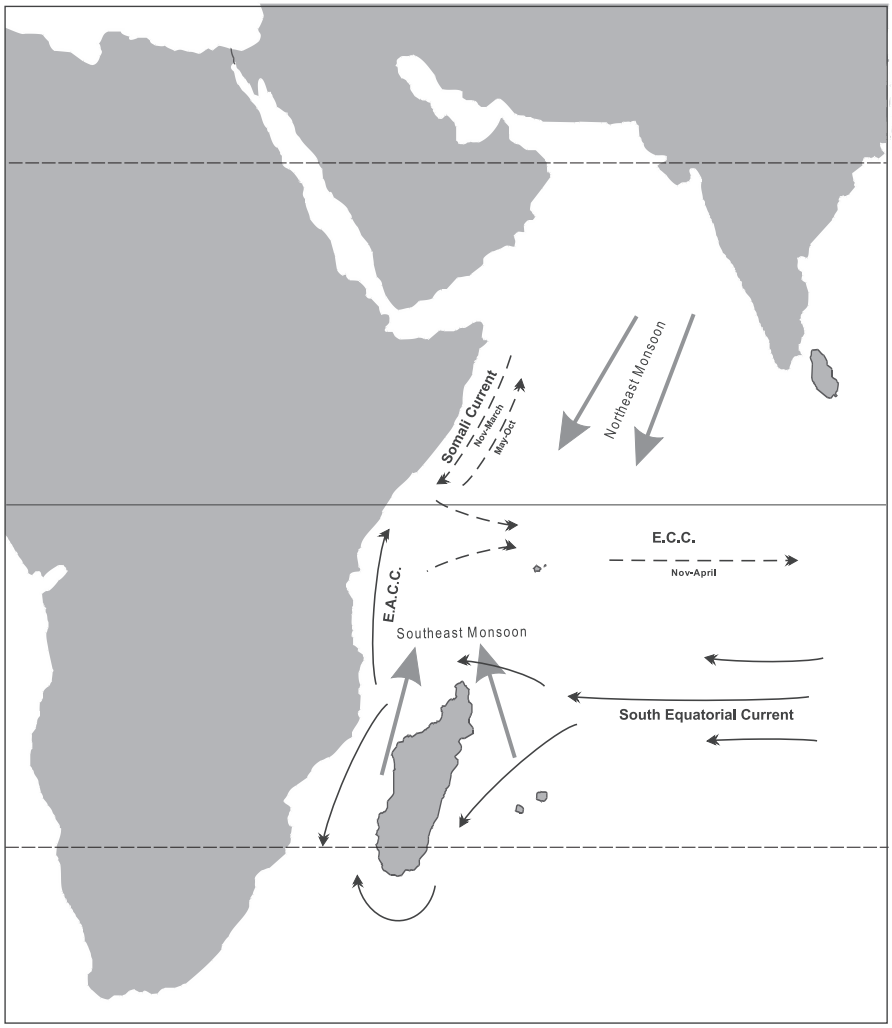

Fig. 3. Sea currents in the western Indian Ocean. E.C.C.: Equatorial Counter Current; E.A.C.C.: East African Coastal Current.

about 40 days. The young can dig their own galleries without leaving the root where they were born, remaining there for their entire life. This could greatly influence the population biology and intraspecific interactions. In fact, the animals found on the same root probably have family links (Thiel, 2001).

Nevertheless, this reproductive strategy is not sufficient to produce the high level of reproductive isolation between *S. terebrans* populations since passive dispersal through floating mangrove wood transported by currents could maintain a certain degree of gene flow between populations.

It is possible that the ancestor of *S. terebrans*, a taxon widely distributed throughout the world, adopted ecological and reproductive strategies that resulted in its populations becoming isolated and slowly giving rise to different gene pools. The high Fst values (calculated on the basis of nucleotide divergence) between the Kenyan populations of *Sphaeroma* suggest a long-term process of differentiation. Moreover, the divergence between the Kenya and Florida populations is easily explained by the geographic isolation of populations colonising continents that shifted apart millions of years ago.

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