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Molecular Survey on *Symbiodinium* of Some Scleractinean Coral Spp. and a Fire Coral sp. along the Red Sea of Egypt

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Abstract

The present study introduce an overview on the cladal structure of *Symbiodinium* population associated with some species of scleractinean corals and fire coral in the Egyptian Red Sea coast and discuss the possible consequences of recent climate changes on coral reefs. Cladal structure of *Symbiodinium* populations associated with eight keystone species of scleractinean corals and one species of fire coral that collected along Egyptian Red Sea coast, during 2012-2013, had been resolved based on 18S nrDNA and ITS2 genetic markers. Only *Symbiodinium* subclades C1 and A1 were identified from all examined species. *Symbiodinium* C1 was the dominant subclade that associated with 61% of coral samples. Results revealed that the studied pocilloporid corals were associated with *Symbiodinium* C1 and/or A1 while acroporids were only associated with *Symbiodinium* C1. The present data also indicated that *Symbiodinium* C1 occurred at high densities than A1 or A1+C1 combination. Because of the relative thermal susceptibility of clades C and A, the current study addresses that the recent climate changes may derive dramatic changes on community structure of coral reefs at the Red Sea.

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Introduction

Corals are known to be the typical hosts for symbiotic dinoflagellates of the genus *Symbiodinium* which are functionally diverse [1]. This diversity reflects the functional ecology of different *Symbiodinium* types that establish different coral-symbiont partnerships in habitats of different conditions. Accordingly, genetic diversity of endosymbionts associated with corals is considered critical for coral life. Many studies had revealed that *Symbiodinium* clades have different thermal tolerance in which *Symbiodinium* D is the most thermotolerant and C is the most thermosensitive [2]. Though, under environmental stressors, members within the same clade do not necessarily acquire similar physiological responses [3]. At finer levels, *Symbiodinium* types within each clade possess differential secondary thermal tolerances [1, 4]. As a consequence, bleaching events are severe at reefs dominated by thermosensitive types [5, 6, 7]. Yet, determining the definite thermal tolerance that outlines the susceptibility of corals to bleaching, of many types is still in its infancy [3]. However, inspecting genetic composition of coral's endosymbiotic systems is considered crucial for understanding susceptibility of corals to environmental stressors and predicting the consequences of increasing temperature on coral reefs.

Red Sea coral reefs represent the upper northern limits for coral reefs distribution. Although Red Sea coral reefs are considered unique reefs, they had received little concern about their endosymbiotic systems [8]. Only few studies on the genetic diversity of *Symbiodinium* at the northern tip of the Gulf of Aqaba and at the eastern coasts had been published [9, 10, 11]. Despite that the Egyptian coast of the Red Sea extends approximately 1800 km [12], genetic structure of endosymbiotic systems along this western part had not resolved yet, especially in scleractinian corals which are considered the main reef builders. Here, we assess *Symbiodinium* diversity on the reef building corals host species and one species of fire coral that exist along Egyptian coast of the Red Sea. Coral samples were collected from shallow (1-5) and mid-depth (5-10) habitats. We address geographical differences between coral populations of western Egyptian coast and eastern Saudi Arabian coast of Red Sea in terms of phylogenetic diversity of their dinoflagellate symbionts.

Materials and Methods

Study Sites and Sample Collection

Six sampled locations were chosen along the western Red Sea coast off Egypt. Three locations were tropical in the proper Red Sea (Marsa Samadai: 25° 00′ 50″ N, 34° 55′ 37″ E; Zabargad: 23° 35′ 51″ N, 36° 12′ 31″ E; Rocky Islands: 23° 33′ 47″ N, 36° 14′ 33″ E), or subtropical-temperate in the northern Res Sea (Fanous Reef: 27° 15′ 57″ N, 33° 53′ 30″ E) and Gulf of Aqaba (Marsa Ghozlani: 27° 49′ 20″ N, 34° 15′ 58″ E; Solomon reef: 28° 29′ 10″ N, 34° 30′ 52″ E) (see Fig. 1, Table 1). Eight species of scleractinian corals (*Acropora digitifera*, *Acropora humilis*, *Acropora pharaonis*, *Pocillopora verrucosa*, *Stylophora pistillata*, *Stylophora wellsi*, *Porites* sp., and *Favia* sp.), and one species of hydrozoan fire coral (*Millepora dichotoma*) were selected for the present study. Fragments of mixed samples of coral skeleton and resident symbiont communities were collected by SCUBA during 2012-2013.

Samples were collected using a hammer and hollow steel punch (<1 cm^2 diameter) penetrating the coral skeleton deeply enough to capture all tissue layers (~3 to 6 mm deep). To minimize the likelihood of repeatedly sampling corals that were the same genetic individual, only conspecifics separated by >10 m were sampled. Samples were taken only from the tops of healthy colonies and identified according to a key reference [13]. Immediately, collected tissue was preserved for DNA analysis in saline DMSO buffer [20% (v/v) DMSO, 250 mM EDTA, saturated NaCl] [14].

Symbiodinium Phylotyping

DNA of *Symbiodinium* was extracted using phenol/chloroform methodology [15]. Accordingly, 600 µL of 2x CTAB extraction buffer [1.4M NaCl, 0.02M EDTA (pH=7.8), 0.1M Tris-HCl (pH=8.0), 2% CTAB (w/v), and 0.2% β-mercaptoethanol (v/v)] were added to *Symbiodinium* pellets. After homogenization, 10 µL of proteinase K (20 mg/ml) were immediately added. Well shacked suspension was then incubated at 65°C for 2 hours with frequent shacking. After that, 600 µL of chloroform isoamyl alcohol [24:1 (v/v)] were added twice with an intermediate addition of phenol chloroform

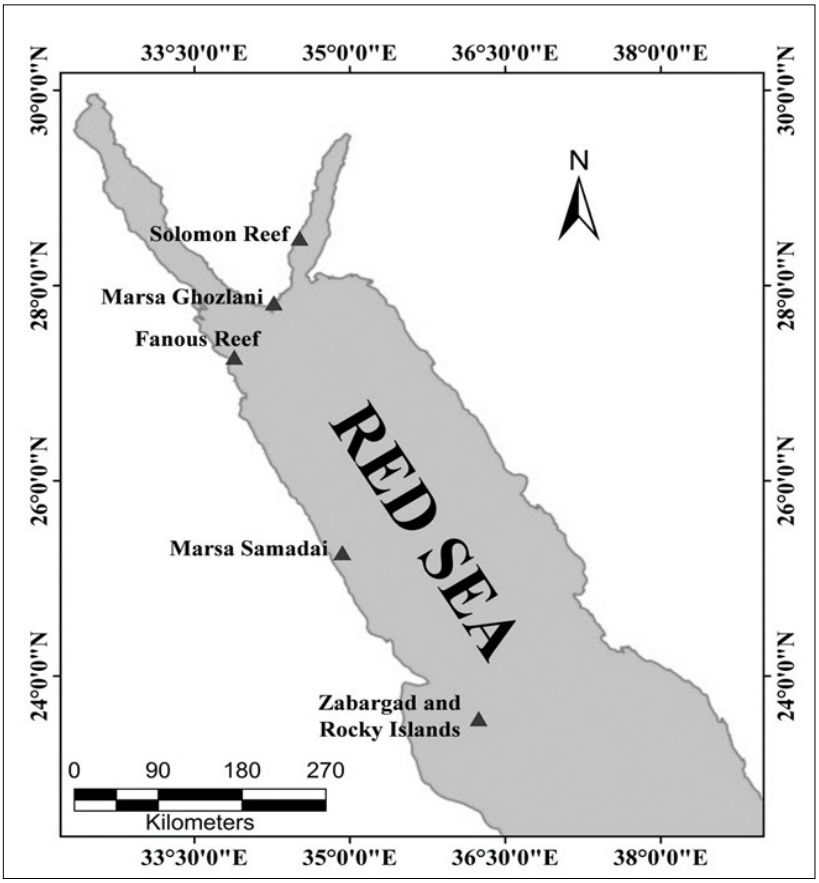

Figure 1. Sampling sites along the Egyptian Red Sea.

Table 1. *Symbiodinium* types isolated from different coral species along with corresponding NCBI accession numbers.

Host	Site	Symbiodinium Subclade	Accession number
A. digitifera	Marsa Ghozlani	C1	KM066980
A. digitifera	Marsa Samadai	C1	KM066985
A. humilis	Solomon Reef	C1	KM066982
A. humilis	Marsa Ghozlani	C1	KM066981
A. humilis	Fanous Reef	C1	KM066984
A. humilis	Zabargad Island	C1	KM066987
A. pharaonis	Solomon Reef	C1	KM066983
A. pharaonis	Marsa Ghozlani	C1	KM066979
A. pharaonis	Marsa Samadai	C1	KM066986
Favia sp.	Marsa Ghozlani	C1	KM066978
P. verrucosa	Marsa Samadai	A1	KM066991
S. pistillata	Marsa Ghozlani	A1	KM066988
S. pistillata	Marsa Samadai	A1	KM066990
S. wellsi	Marsa Ghozlani	A1	KM066989

isoamyl alcohol [25:24:1 (v/v)] to DNA extract (1:1). Prior to each extraction step, solution was shaken for 5 min then centrifuged for 15 min at 13,000 rpm.

DNA was precipitated by adding 1ml of 95% cold ethyl alcohol to the aqueous layer and incubated overnight at -20°C. Samples were then centrifuged for 30 min at 13,000 rpm. DNA pellets were washed twice with 500 µL of 70% cold ethyl alcohol and then centrifuged at 13,000 rpm for 5 min. Pellets of DNA were dried at room temperature and finally dissolved in 100 µL double distilled sterilized water.

The two genetic markers, 18S nrDNA and ITS2, were amplified. The first one, 18S nrDNA, was used to identify *Symbiodinium* clades in all collected samples (*n*=98), while ITS2 of a randomly selected subsamples (*n*=14) was used to identify *Symbiodinium* subclades.

Each reaction was composed of 1x PCR buffer, 1 mM dNTPs, 3.5 mM $MgCl_2$, 0.2 µM of each forward and reverse primer (18S nrDNA: ss5 and ss3z [16]; ITS2: ITS2intfor and ITS2clamp but without GC clamp [17]), 0.05U/µL *Taq* polymerase, 30 ng/µL DNA template, and PCR grade water to final volume of 25µL for 18S nrDNA or 50µL for ITS2. The two markers were amplified under the following conditions: hot start at 94°C for 2min; followed by 35 cycles of 94°C for 1min, 54°C for 1min, and 72°C for 2min; final extension at 72°C for 8min. For *Symbiodinium* phylotyping, however, 18S nrDNAs were digested by *Taq I* restriction enzyme. The reaction was composed of 1x PCR buffer, 0.2U/µL *Taq I* restriction enzyme and 5µL PCR product in a final volume of 25µL with PCR grade water. Isothermal restriction enzyme digestion was performed in thermocycler at 65°C for 30 min. RFLP digests were run on 1.5% agarose gel along with 3kb Mid-Range DNA ladder [Jena Bioscience (#M-203)]. Gel bands produced by RFLP analysis were analyzed by Gel-Pro Analyzer software v4.0 to estimate molecular size of each band. RFLP profiles in the current study were compared with profiles previously published on *Symbiodinium* clades using the same primers set and restriction enzyme.

To confirm RFLP analysis results and to identify *Symbiodinium* subclades upon which phylogenetic relationships would be constructed, ITS2 was sequenced. Part of successfully amplified samples (*n*= 5) were initially sequenced in the forward and reverse directions by Macrogen (Macrogen Ltd., Korea) while the remainder samples (*n*= 9) were sequenced by Biotechnology Research Center (Suez Canal University) at forward direction only.

Phylogenetic Analysis

After editing, ITS2 sequences were blasted using MEGABLAST on NCBI GenBank under default algorithm parameters. All the edited ITS2 sequences had been submitted and archived in NCBI GenBank (KM066978.1 – KM066991.1).

ITS2 sequences in the current study and that downloaded from NCBI GenBank were aligned together using ClustalW. Phylogenetic relationships between 55 sequences, among which 14 were resolved by the current study, of *Symbiodinium* harbored by different taxa of invertebrates along the Red Sea were inferred using p-distance Neighbor-Joining method for 1000 bootstrap replication value [18]. Gaps were treated by pairwise deletion. *Protodinium simplex*, *Pelagodinium beii*, and *Polarella glacialis* (JN558105, JN558107, and JN558108, respectively) were used for tree rooting. All phylogenetic analyses were conducted using MEGA v6.0 [19].

One way ANOVA was performed upon original or transformed data. Data of abnormal distribution, even after transformation, were subjected to Kruskal-Wallis test.

Data Accessibility

All ITS2 sequences in this study were archived at the NCBI GenBank with accession numbers KM066978.1-KM066991.1.

Results

Based on 18S nrDNAs, three different banding patterns were produced by *Symbiodinium* harbored studied corals. The first banding pattern was characterized by two bands of 1173 and 800 pbs, while the second pattern consisted of three bands of 800, 678, and 288 pbs (Fig. 2). The third was consisted of a combination of the first and second banding patterns (Fig. 3). The first and second banding patterns are corresponding to previously published RFLP profiles for *Symbiodinium* clades C and A, respectively, while the third one is a combination of clades A and C (A+C).

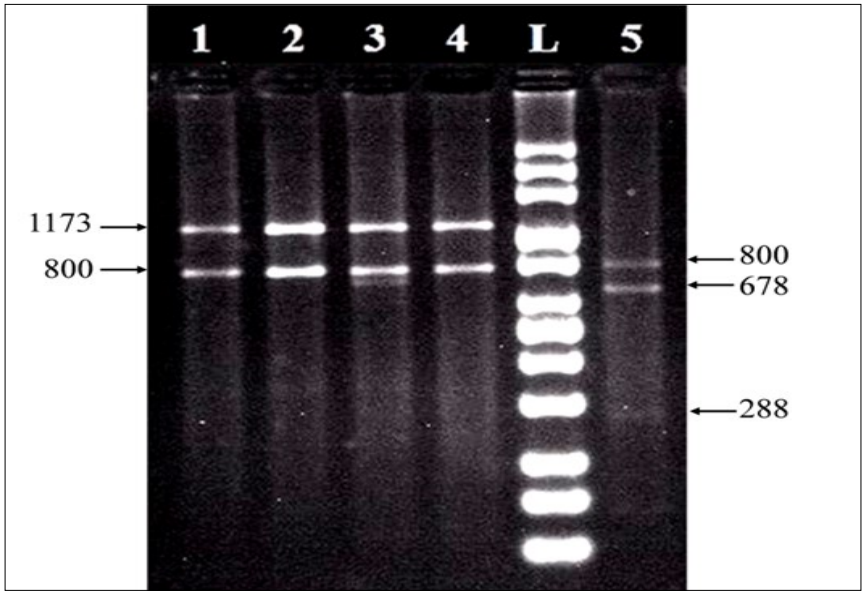

Figure 2. RFLP profiles of 18S nrDNAs digested with *Taq I*. Clade C profiles (lanes 1-4) was identified from *Acropora digitifera* , *Acropora humilis*, *Acropora pharaonis*, and *Stylophora pistillata*, while clade A (lane 5) had been identified from *Pocillopora verrucosa*.

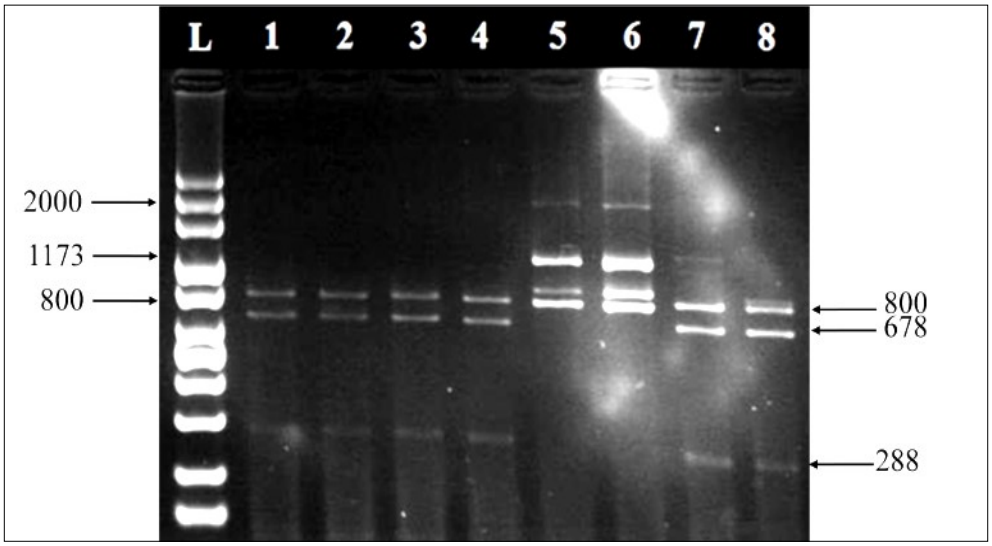

Figure 3. RFLP profiles of 18S nrDNAs digested with *Taq I*. Clade A isolated from *Stylophora pistillata* (lanes 1 and 2), *Stylophora wellsi* (lane 3), *Pocillopora verrucosa* (lane 4), and *Millepora dichotoma* (lane 8) while clade C had been isolated from *Acropora pharaonis* and *Acropora humilis* (lanes 5 and 6, respectively). A+C combination (lane 7) was isolated from *Pocillopora verrucosa*.

MEGABLAST search on NCBI GenBank for ITS2 sequences of all *Symbiodinium* isolated from different coral species confirmed that they relate either to clade A or C. Further sequence data showed that each *Symbiodinium* clade represented by only one subclade (Fig. 4). For clade A, all ITS2 sequences were highly similar to *Symbiodinium* A1 type (*Tridacna maxima*, Egypt, Red Sea, GU069005) with a maximum identity of 99%, E value of 3e-120, and query cover of 100%. On the other hand, for clade C, 10 sequences were highly similar to *Symbiodinium* C1 type (*Polycyathus* sp., Taiwan, JQ180021) with a maximum identity of 99%, E value of 2e-136, and query cover of 100%. Sequences of isolated *Symbiodinium* from studied corals were archived in the NCBI GenBank with accession numbers showed in Table 1.

Of nine examined species (n=98), the current genetic survey revealed that 61% of coral samples hosted *Symbiodinium* C, 33% associated with *Symbiodinium* A, and only 6% formed symbiosis with A+C combination. *Symbiodinium* C1 was recorded in acroporid, poritid, faviid, and pocilloporid species, while clade A1 was only detected in the three studied pocilloporid species as well as a fire coral *M. dichotoma* (Fig. 5).

Phylogenetic analysis of *Symbiodinium* sp. associated with scleractinian corals along wide geographic localities in the Red Sea revealed low diversification in endosymbionts (Fig. 6). Nevertheless, eastern coast of the Red Sea showed higher *Symbiodinium* diversity than western side. Regarding host flexibility, pocilloporid corals hosted more phylogenetically diverse *Symbiodinium* types. For instance, *P. verrucosa* harbored *Symbiodinium* A1, D1a, B1, C15, or C85; while *S. pistillata* harbored *Symbiodinium* closely related to A1, F2, C72, or C3. In contrast, *Acropora* spp. were associated mainly with *Symbiodinium* C1.

Discussion

In spite of the great concern paid for coral reef ecosystem, *Symbiodinium* genetic diversity along the Red Sea had been overlooked. So, the current study introduced an overview on *Symbiodinium* genetic diversity in seven species of scleractinian corals and one species of fire coral along the Egyptian Red Sea coast.

The present study revealed that *Symbiodinium* population associated with Red Sea corals were primarily composed of C and A clades. In general, these two clades were recorded to have a global distribution [20]. Among different types of *Symbiodinium* under each clade, only C1 and A1 types were recorded with the dominance of C1 type comprising 61% of the recorded symbionts in all studied corals. C1 was detected to be the most dominant and widely distributed type because it has the ability to associate with a wide array of host species and a great adaptation with different environmental conditions [21, 22].

Dominance of *Symbiodinium* C1 was explained by two arguments with the same perception. The first supposed that *Symbiodinium* C1 type has a generalist nature along wide geographic scales [22]. The second proposed that coral-*Symbiodinium* symbiosis is directed toward specialist-types preference along evolutionary history of coral reefs [21]. One of studies suggested that dominance of C1 type in west Pacific had been transferred to western Indian Ocean, and eventually to the Red Sea, before biogeographic partitioning between the two oceans [23]. Because northern Red Sea coral reefs are considered recent reefs [24], far from major natural stressors, dominance of C1 type associated with scleractinian corals may be explained on the basis of recent low-specialist symbiosis.

Symbiodinium population at Yanbu was dominated by clade C (69%) followed by clade A (27.3%) while clade D formed only 3.7% of symbionts [11]. The current results emphasized the previous mentioned dominance order of clades C and A, and added the endosymbiotic system composed of a combination from the two clades C and A which could be considered as a unique system in Red Sea corals as reported by [20]. This is in contrast with corals of the Arabian Gulf where *Symbiodinium* D represents the dominant clade [11, 25]. Predominance of such thermotolerant clade at the Arabian Gulf is associated with thermal tolerance of >33°C [2]. However, *Symbiodinium* D and B that are known to associate with scleractinian corals were not detected.

Compared with high host diversity along the Red Sea, symbiont diversity was low. The same pattern was previously recorded along other reefs at Great Barrier

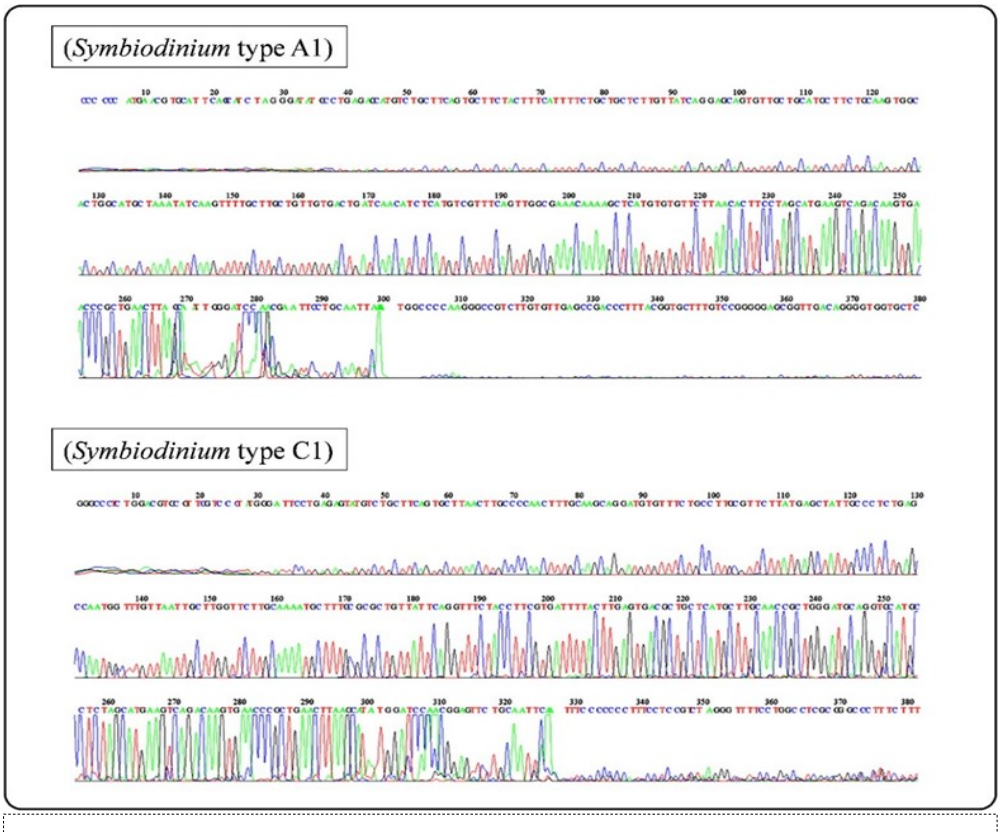

Figure 4. ITS2 electropherogram of *Symbiodinium* A1 and C1 isolated from *Stylophora pistillata* and *Acropora pharaonis*, respectively.

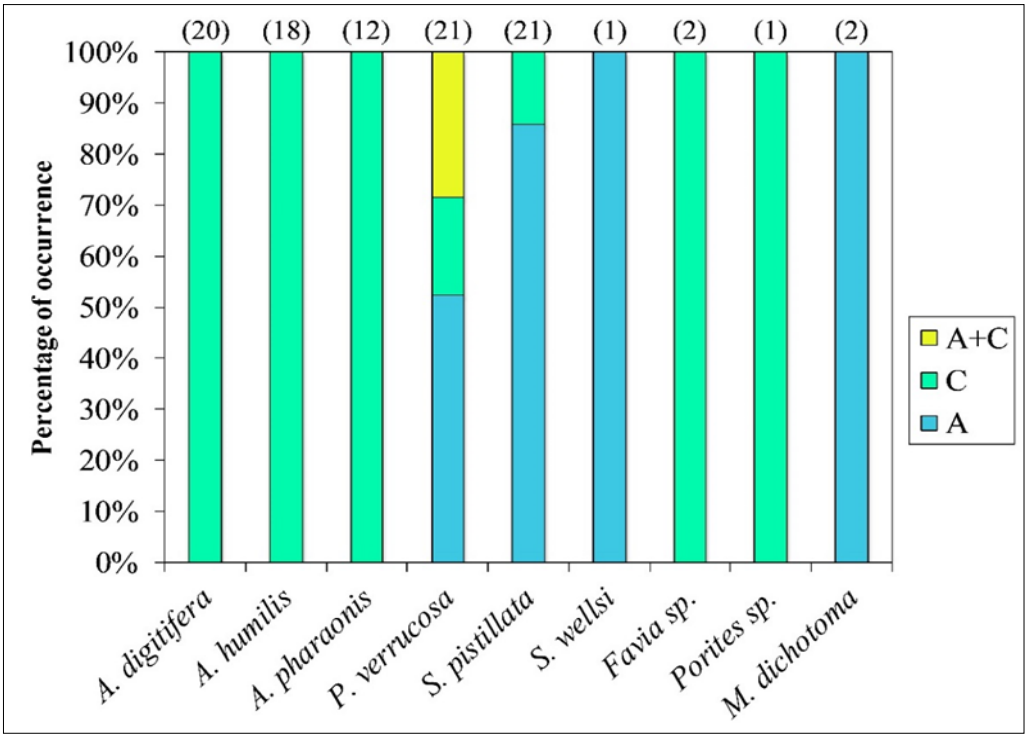

Figure 5. Cladal composition of *Symbiodinium* population in the collected coral species. Top numerals in parentheses indicate numbers of collected fragments in each species.

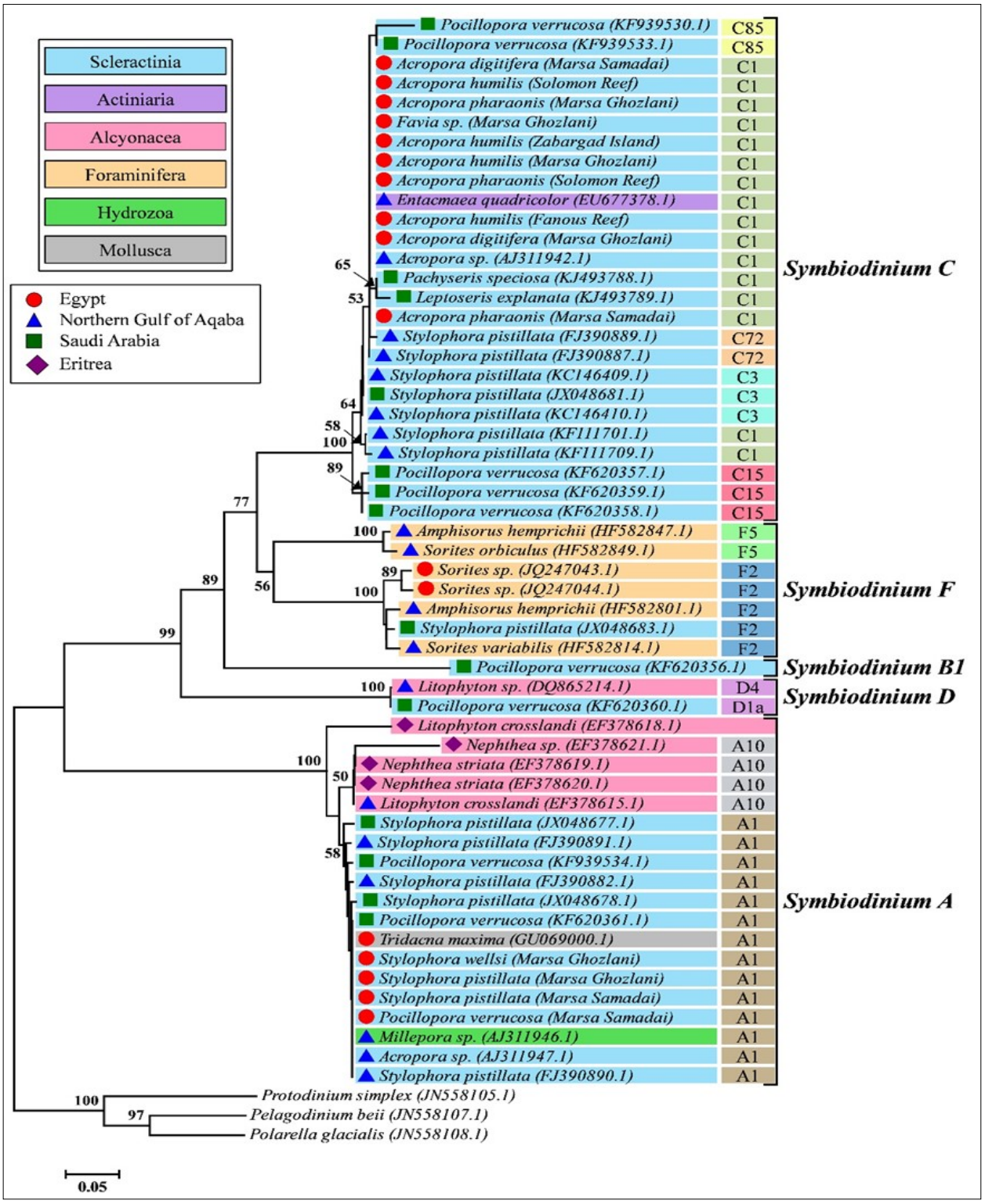

Figure 6. Reconstruction of ITS2 phylogenetic tree between members of *Symbiodinium* harbored by different taxa of invertebrates in the Red Sea. The phylogenetic distance was inferred by Neighbor-Joining method. Only bootstrap values >50% are shown.

Reef and Indian Ocean [26, 27]. The flexibility of *P. verrucosa* for harboring *Symbiodinium* A and C was coincident with that recorded in the central Red Sea [28]. On the other hand, divergence within phylotype C may explain the dominance of this phylotype in coral reefs of the Red Sea.

Based on coral reproductive strategy, *Acropora*., *Favia* and *Porites* are mainly broadcast spawners and tend to utilize horizontal transmission strategy for *Symbiodinium* acquisition from surrounding water [10, 29]. In contrast, *S. pistillata*, *S. wellsi*, and *M. dichotoma* are principally brooder species and consequently associated with *Symbiodinium* A by vertical mode of transmission [10]. Though, *S. pistillata* in the Red Sea has the ability to associate with *Symbiodinium* C [11, 30]. Previous studies indicated that colonies of *S. pistillata* collected from the Red Sea are able to harbor clades A, C, or A+C combination. They stated that 13% of *S. pistillata* collected from Gulf of Aqaba (17m depth) harbored clade C, while 87% of the colonies (5 and 17m depth) associated with clade A [31, 32]. Comparatively, our results revealed that 14% of *S. pistillata* colonies (mainly collected from surface water at Fanous Reef during winter) were populated with clade C.

It is worth mentioning that the first stages of corals are more flexible than adult stages [33]. Simultaneous or individual occurrence of clades A and C in colonies of *S. pistillata* and *P.verrucosa* may be interpreted upon assumption that acquisition of *Symbiodinium* C by colonies that already harbor clade A, may occur by horizontal transmission at later stages during coral ontogeny. A or C clades could be transmitted vertically from parent to juveniles that may maintain cladal structure along life cycle or switch to other clade by horizontal transmission in later stages [34].

At central Red Sea, coral reefs were affected by mass bleaching event at Thuwal during summer 2010 [35]. They reported that, at some sites, 80-100% of Oculinidae, Agariciidae, and Fungiidae were severely impacted by this event, while bleaching cover among Pocilloporidae, Acroporidae, and Faviidae was 19, 33, and 40%, respectively. More recently, sea surface temperature was raised up to 34°C at the Egyptian coasts of the Red Sea during August 2012. This high temperature anomaly resulted in mass bleaching event in some reef building coral species (Hanafy and Ahmed, unpublished data). Among these, *Stylophora*, *Montipora*, *Porites*, and *Millepora* were the most affected genera by thermal stress. *Symbiodinium* A or C harbored by Red Sea corals were considered thermosensitive clades [36]. The dominance of these clades observed in the present study may indicate that coral reefs at the Red Sea are endangered by the thermal stress. Accordingly, patterns of temperature anomalies may have dramatic effects on coral reefs diversities and structures at the Red Sea as suggested by [35]. However, with little knowledge on *Symbiodinium* genetic diversity at these levels, we could not generalize our investigation on Red Sea corals. Accordingly, intensive molecular survey with deep resolution on *Symbiodinium* populations may contribute in understanding of bleaching patterns along the Red Sea. In parallel, future experimental studies are required to derive comprehensive perception about thermal tolerances of these clades and/or types.

Conclusively, the current study emphasizes the need for resolving *Symbiodinium* genetic diversity along Egyptian coasts of the Red Sea. The level at which this diversity will be resolved is critical for identifying threats to coral reefs at the Red Sea. Identifying *Symbiodinium* types harbored by different hosts may contribute largely in understanding *Symbiodinium* dynamics and responses to environmental changes at these reefs.

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Conflict of interest statement

We declare that we have no conflict of interest.

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