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SHORT COMMUNICATION

New insights into the genetic diversity of zooxanthellae in Mediterranean anthozoans

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Abstract Symbiotic dinoflagellates of the genus *Symbiodinium*, also called zooxanthellae, are found in association with a wide diversity of shallow-water anthozoans. The *Symbiodinium* genus includes numerous lineages, also referred to as clades or phylotypes, as well as a wide diversity of genetic sub-clades and sub-phylotypes. There are few studies characterizing the genetic diversity of zooxanthellae in Mediterranean anthozoans. In this study, we included anthozoans from the Western Mediterranean Sea and by means of internal transcriber (ITS) and large sub-unit (LSU) rRNA markers we corroborate what has been previously identified, demonstrating that phylotype "Temperate A" is very common among host Cnidaria in this basin. Our finding of fixed differences in ITS and LSU markers that correspond to different host taxa, indicate that this clade may comprise several closely-related species. Previous studies have reported the occurrence of *Symbiodinium psygmophilum* (formerly sub-clade B2) associated with *Oculina patagonica* and *Cladocora caespitosa* in the Eastern Mediterranean. Here, we identify this association in *O. patagonica* from the Western Mediterranean but not in *C. caespitosa*, suggesting some differences in symbiotic combinations between the Western and Eastern Mediterranean Basins.

Keywords *Symbiodinium* sp. · Genetic characterization · Phylotypes "Temperate A" and B · Western Mediterranean

1 Introduction

Many members of the Class Anthozoa have symbiotic relationships with photosynthetic dinoflagellates belonging to the *Symbiodinium* genus (Trench 1993). These organisms, also known as zooxanthellae, contribute to host nutrition providing fixed carbon, while the host provides inorganic nutrients, a well lit environment environment, and refuge from herbivory (Weis et al. 2001). Most of the anthozoans serving as symbiotic hosts to algal cells show reduced survivorship in the absence of symbiosis (see Furla et al. 2005 for a review).

The genus *Symbiodinium* includes numerous evolutionary lineages, also referred to as phylotypes or clades (A, B, C, D, etc.) (Baker 2003; Coffroth and Santos 2005). Genetic differentiation of these lineages has usually been supported by a variety of genetic markers (Sampayo et al. 2009; Thornhill et al. 2013; Pochon et al. 2014). In general, each clade or phylotype includes a diversity of genetic sub-clades or sub-phylotypes, which exhibit distinctive biogeographical, ecological and host-specific patterns. However, symbiont distributions in scleractinian corals may differ over large geographic ranges (Baker and Rowan 1997; LaJeunesse et al. 2003; LaJeunesse et al. 2010). Some symbiont taxa are widely distributed, both among different hosts and across geographic regions (Loh et al. 2001; Rodriguez-Lanetty and Hoegh-Guldberg 2003), whereas other taxa show high host specificity or appear to be regionally endemic (Baker 1999; Baillie et al. 2000; Santos et al. 2002; LaJeunesse et al. 2003; LaJeunesse et al. 2004; LaJeunesse et al. 2010). However, in some

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temperate regions, such as the Mediterranean Sea, it is difficult to assess the specificity of *Symbiodinium* sp. phylotypes and host taxa, mainly because the study of zooxanthellae diversity in that region is still in the early stages in comparison with studies available from tropical regions. Currently, there are only few studies characterizing the diversity of zooxanthellae from Western Mediterranean anthozoans (Savage et al. 2002; Visram et al. 2006; Forcioli et al. Forcioli et al. 2011), with all of them indicating the "Temperate A" clade as dominant in this basin.

The Mediterranean is considered a biodiversity hotspot with a high level of endemism, as well as an assortment of temperate and subtropical elements (Coll et al. 2010). This is mainly related to its narrow connection with the Atlantic Ocean, to its east–west orientation and its geological history (Boudouresque 2004). Thus, the current biological diversity is due to the interaction between ecological factors, as well as historical processes that shaped the Mediterranean Basin throughout the course of history (Templado 2014).

This study lends new insight on the diversity of zooxanthellae hosted by Western Mediterranean anthozoans using: the large subunit of the ribosomal RNA (LSU) and the Internal Transcriber Spacers (ITS: ITS1-5.8S-ITS2).

2 Material and methods

Samples from two groups of symbiotic anthozoans, Actiniaria (*Anemonia viridis*, *Bunodeopsis strumosa* and *Paranemonia cinerea*) and Scleractinia (*Cladocora caespitosa* and *Oculina patagonica*), were collected at different localities in the Western Mediterranean region (Table 1). Samples were fixed in absolute ethanol and stored until genetic analysis. Sequences from LSU and ITS regions from other zooxanthellate species across different global regions were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Symbiont DNA was isolated from individuals preserved in ethanol following a modified protocol from Coffroth et al. (1992). Molecular variation was detected by polymerase chain reaction (PCR) amplification of the LSU with primers 24D15F1 and 24D2R1 (Baker et al. 1997) and the ITS region using primers ZITSUPM13 and ZITSDNM13 (Santos et al. 2001), and with their corresponding reaction conditions. After PCR amplification, products were purified by ethanol/sodium acetate precipitation or by excising bands. Samples were cycle-sequenced using the ABI Prism BigDye Terminator, and subsequently running them on an ABI 3730 Genetic Analyzer (Applied Biosystems).

2.1 Phylogenetic analysis

DNA sequences were edited using SEQUENCHER 4.6 (Gene Codes), aligned using SeaView 4.4.2. (Gouy et al. 2010) and further revised by eye.

Phylogenetic reconstructions were obtained using the Bayesian inference (BI) and Maximum Likelihood (ML) methods. The evolutionary molecular model that best fit the data sets was selected using jModelTest v3.7 (Posada and Crandall 1998) under Bayesian criterion (BIC). Bayesian analyses were performed using MRBAYES v3.1.2 (Huelsenbeck and Ronquist 2001), with two independent runs of four Metropolis-coupled chains with 5 000 000 generations each, to estimate the posterior probability distribution. Maximum-likelihood (ML) analyses were conducted in PHYML v2.4.4 (Guindon and Gascuel 2003) using the evolutionary model selected by jModelTest. The robustness of the ML-inferred trees was tested by nonparametric bootstrapping

Table 1 Western Mediterranean anthozoan hosts of isolated *Symbiodinium* sp., their correspondent sampling region and LSU rRNA and ITS GenBank sequence numbers

Host species/code	Collection site	Basin	N	GenBank code, LSU rRNA	GenBank code, ITS
Anemonia viridis AvirCP	Cape Palos, Murcia, Spain	Algerian Basin	1	KF886574	KF886572
Anemonia viridis AvirAl	Alicante, Spain	Balearic Sea	1	KF809733	KF809723
Bunodeopsis strumosa BstrMM	Isla del Ciervo, Murcia, Spain	Algerian Basin	2	KF809735 KF809736	KF809725 KF809726
Paranemonia cinerea PcinMM	Mar Menor, Murcia, Spain	Algerian Basin	4	KF809729 KF809730 KF809731 KF809732	KF809719 KF809720 KF809721 KF809722
Paranemonia cinerea PcinDE	Delta el Ebro, Castellón, Spain	Balearic Sea	2	KF809727 KF809728	KF809727 KF809728
Cladocora caespitosa CcCol	Columbretes Islands, Castellón, Spain	Balearic Sea	1	KF886575	KF886573
Cladocora caespitosa CcAm	L'Ametlla, Tarragona, Spain	Balearic Sea	1	KF889734	KF89724
Oculina patagonica Op92	La Azohía, Murcia, Spain	Algerian Basin	1	JQ322775	JQ343057
Oculina patagonica Op160	L'Ametlla, Tarragona, Spain	Balearic Sea	1	JQ322776	JQ343058
Oculina patagonica Op120	Alcossebre, Castellón, Spain	Balearic Sea	1	JQ322777	JQ343059
Oculina patagonica Op100	Cape Palos, Murcia, Spain	Algerian Basin	1	JQ322778	JQ343060

(Bs) (Felsenstein 1985), with 1 000 pseudoreplicates in each case. Bayesian posterior probabilities (BPPs) were used as a measure of the robustness of Bayesian trees.

3 Results and discussion

With the use of ITS and LSU markers, we provide additional information on the genetic diversity of *Symbiodinium* sp. hosted by Mediterranean anthozoans. Both markers show similar levels of resolution (Sampayo et al. 2009), nevertheless, the LSU region has been commonly used to assign *Symbiodinium* isolates to clades or phylotypes and inferring the relationships between them (Coffroth and Santos 2005; Barbrook et al. 2006) and ITS markers are more commonly used to obtain phylogenetic resolution at the sub-phylotype or sub-clade level (LaJeunesse 2001; Rodriguez-Lanetty 2003).

By means of different phylogenetic analyses with ITS and LSU regions, it is determined that the dominant *Symbiodinium* phylotype for our anthozoan species in the Western Mediterranean Sea is "Temperate A" (Fig. 1). This corroborates previous results published by other authors (Savage et al. 2002; Barbrook et al. 2006; Visram et al. 2006). This clade was previously described by Savage et al. (2002) as a phylotype only detected in the NE Atlantic and Western Mediterranean and an ancestral lineage of clade A. Although only moderately supported, our analyses also revealed that the "Temperate A" clade includes some nucleotide sequence diversity, structured in different sub-phylotypes, supported in the ITS phylogeny by a range of 0.80–0.90 B.P. and 70–85 % Bs under the Bayesian and ML analyses respectively (Fig. 2).

From our study, the only Mediterranean species that showed a different *Symbiodinium* sp. phylotype was the scleractinian coral *Oculina patagonica*. The LSU gene phylogeny identifies the clade hosted by this species as clade B with 1 B.P. and 100 % Bs under the Bayesian and ML analyses, respectively (Fig. 1). The ITS analyses defined the sub-phylotype hosted by *O. patagonica* as close to sub-clade B2, with less than 0.90 B.P. under the Bayesian analyses and less than 85 % Bs under the ML analyses (Fig. 2). *Symbiodinium* phylotype B has also been described by

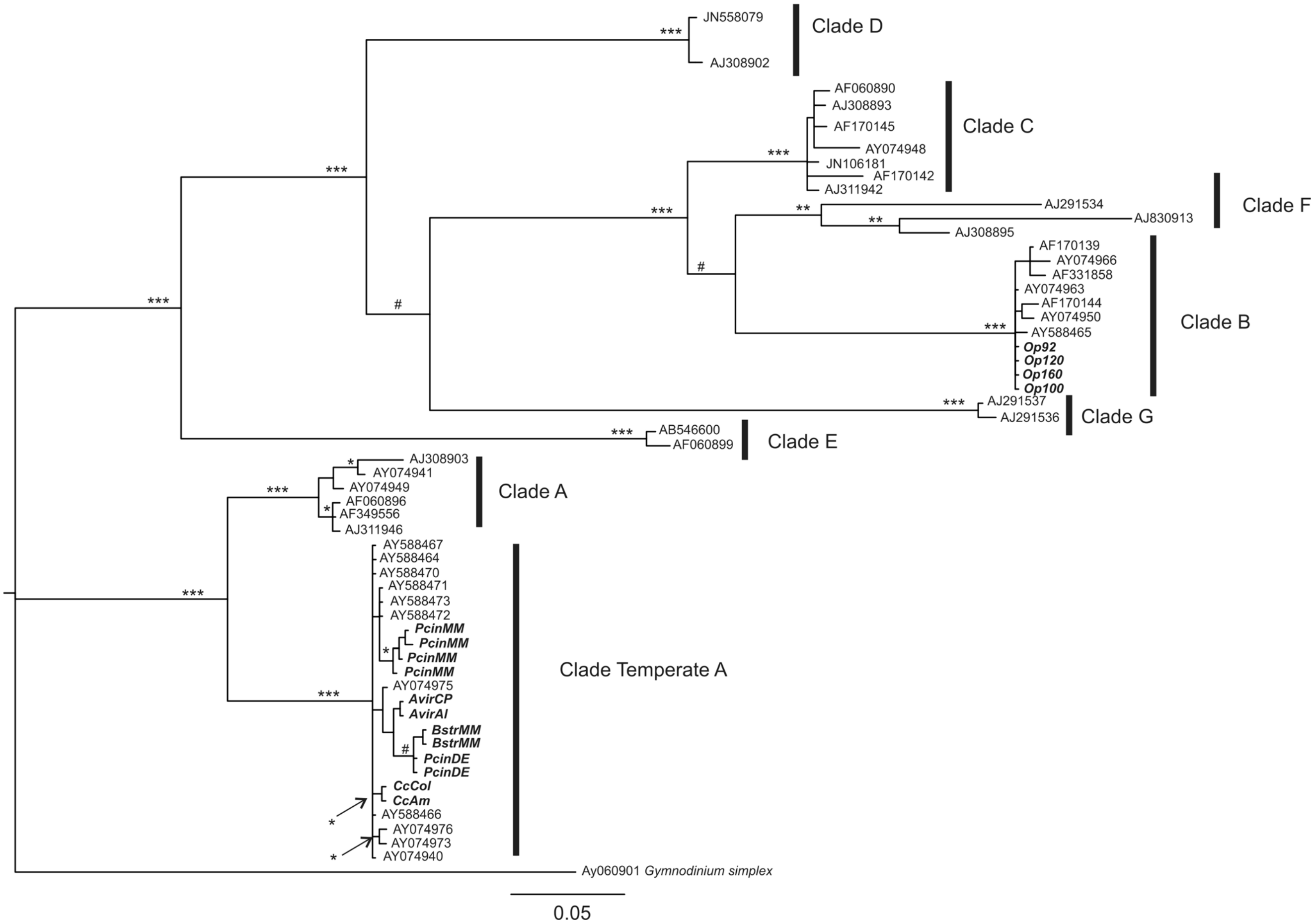

Fig. 1 Phylogenetic tree of *Symbiodinium* LSU rRNA gene sequences. Labels indicate GenBank accession numbers, open keys indicate clade groupings. New sequences from *Symbiodinium* from the Western Mediterranean anthozoans are highlighted on italics and bold. ***BPP=1 and Bs≥95 %; **BPP=1 and Bs<95 %; *BPP≥0.95 and Bs>70 %, #BPP≤0.85 and Bs<60 %

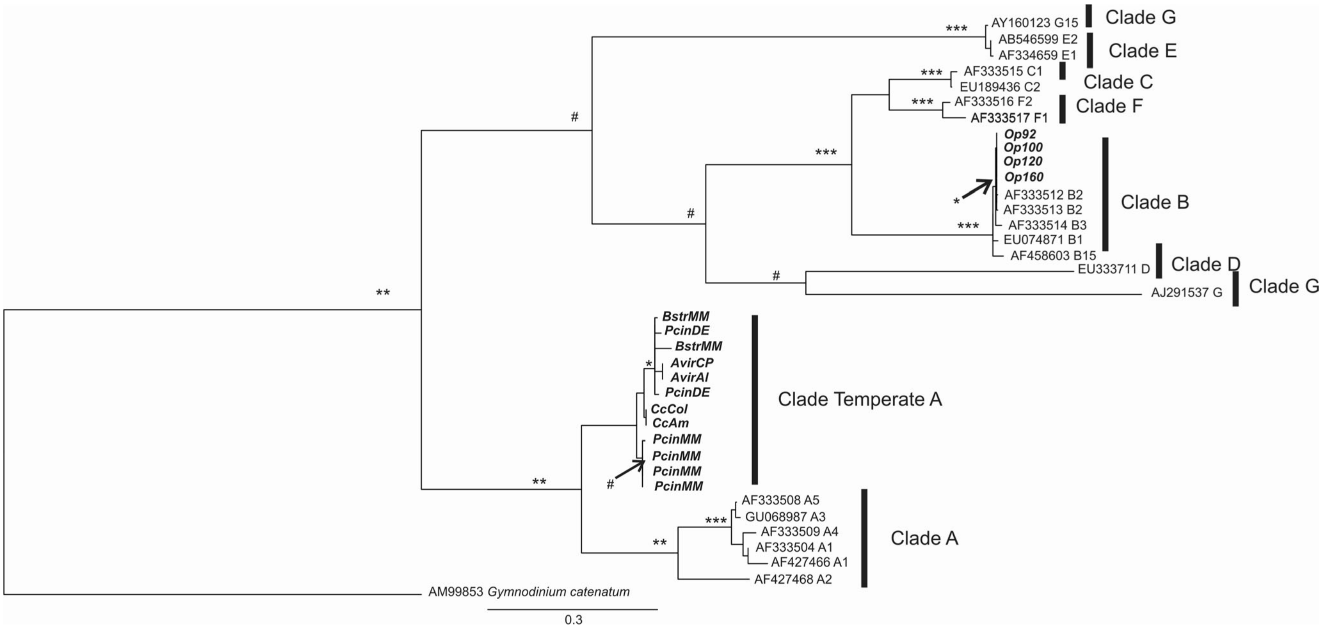

Fig. 2 Phylogenetic tree of *Symbiodinium* ITS gene sequences. Labels indicate GenBank accession numbers, open keys indicate clade groupings. New sequences from *Symbiodinium* of Western Mediterranean anthozoans are highlighted on italics and bold. ***BPP=1 and Bs≥ 95 %; **BPP≥0.90 and Bs≥85 %; *BPP<0.90 and Bs<85 % # BPP≤ 0.80 and Bs≤70

Visram et al. (2006) in the sea anemone *Bunodeopsis strumosa* from the NW Mediterranean (Banyuls, Gulf of Lyon), suggesting that the Western Mediterranean Basin might be richer in terms of *Symbiodinium* diversity than previously established. The *Symbiodinium* ITS sub-phylotype B2, also called *Symbiodinium psygmophilum* (LaJeunesse et al. 2012), has been previously reported only in northerly coral reef habitats in the Western Atlantic, including the Florida Keys and Bermuda (LaJeunesse 2001; Santos et al. 2001; Savage et al. 2002). More recently, LaJeunesse et al. (2012) found phylotype B2 in three species of scleractinians from the coast of Israel in the Eastern Mediterranean Basin: *Oculina patagonica*, *Cladocora caespitosa* and *Madracis pharensis*. In addition, Meron et al. (2012) found this same phylotype in *Balanophyllia europaea* and *C. caespitosa* in the Central Mediterranean (Iscchia Island, Southern Tyrrhenian Sea). However, these authors also found clade "Temperate A" in some individuals of *B. europaea* from the same locality. *Cladocora caespitosa* was also included in our study considering samples from the Western Mediterranean (Castellón and Tarragona, Balearic Sea); however, they harbor the *Symbiodinium* sp. "Temperate A" clade, which differs from that found by LaJeunesse et al. (2012) and Meron et al. (2012). This regional variation in *Symbiodinium* associations within the same host species has been previously reported in the Western Atlantic as well as the Indo-Pacific Oceans, suggesting that those changes might be dependent on regional environmental conditions (Rodriguez-Lanetty et al. 2001; Savage et al. 2002).

Clade B symbionts are particularly common in temperate Western Atlantic regions (Finney et al. 2010), as has been found in anthozoans from temperate regions in the Indo-Pacific (Loh et al. 1998; Rodriguez-Lanetty et al. 2001). However, this clade is not restricted to temperate regions, and has been found in scleractinian corals and other invertebrates in tropical areas (Loh et al. 1998). *Symbiodinium psygmophilum*represents a cold-tolerant lineage able to survive conditions inhospitable to most other *Symbiodinium* species (Thornhill et al. 2008). An experimental study conducted by Thornhill et al. (2008) concluded that *S. psygmophiulm* (formerly sub-phylotype B2) is capable of quickly recovering photosynthetic function upon the return of normal conditions after long periods of cold temperatures. Therefore, assuming the same behavioral pattern in hospite, this symbiont may remain photosynthetically inactive throughout colder periods, persisting without major contribution to the nutrition of the host; while during the warm seasons, the species likely increases its photosynthetic function and therefore would contribute more actively to its host's calcification and growth, thus playing a key role shaping temperate coral communities (Dimond and Carrington 2007).

In this study, we showed that the diversity of zooxanthellae phylotypes in the Western Mediterranean comprises at least two species. Including samples from different Mediterranean anthozoans, we have corroborated the occurrence of *Symbiodinium* sp. phylotype "Temperate A" in the scleractinian coral *Cladocora caespitosa* and the actiniaria *Anemonia viridis*. As well we show the presence of this clade in an anthozoan species that has not been previously study, the actiniarian *Paranemonia cinerea.* It is noteworthy to mention that our study differs from Visram et al. 2006, in that we found "Temperate A" clade in the actiniarian *Bunodeopsis strumosa,* that suggest that the *Symbiodinium* diversity within the same host taxa at the Western Mediterranean Basin might be richer than previously assessed. Moreover, we assessed that the diversity within phylotype "Temperate A" may include two or more closely related species that deserve further genetic and morphologic characterization.. We also verified the occurrence of *Symbiodinium psygmophilum* in the scleractinian coral *Oculina patagonica* in the Western Mediterranean Basin.

Given the importance of zooxanthellae for the survival of host Cnidaria, in deep studies on the characterization and ecology of this species are highly recommended. These kinds of studies are on its beginnings in the Mediterranean Sea. Provided the differences we have found, studies characterizing the genetic diversity of the endemic *Symbiodinium* clade "Temperate A" are needed. As well, the geographical differences we found on symbionts hosted by the scleractinian coral *Cladocora caespitosa*, suggests the need of a complete characterization of the *Symbiodinium* species present in the Mediterranean Basin along latitudinal and longitudinal gradients. All together these studies are of importance to better understand the ecology and evolution of these symbionts and host taxa at the Mediterranean Basin.

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