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DNA barcoding identification of the macroalgal flora of Tunisia

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Doctoral Thesis
2018
S.S.D. BIO/01

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Programme emmag
Erasmus Mundus

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Acknowledgements

First and above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully. Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the assistance and support that I received from many people.

Foremost, I would like to express my sincere gratitude to my Tunisian supervisor, ***Prof.Slim Abdelkafi*** *who has been a tremendous mentor for me. I'm grateful to him for accepting me into his group, for his constant support, motivation and allowing me to grow as a research scientist. I have been extremely lucky to have a supervisor who care so much about my work and I would never have been possible for me to take this work to completion without his support and encouragement.*

Special and heartily thanks to my Italian supervisor ***Prof. Marina Morabito*** *for accepting me into her group, for her patience, motivation, enthusiasm and immense knowledge in DNA barcoding approach. It is with her supervision that this work came into existence. Her guidance helped me in all the time of research and correction of this thesis. As my supervisor, she has constantly forced me to remain focused on achieving my goal.*

I'm greatly indebted to all the persons at both the University of Sfax and the University of Messina who agreed to conclude the co-tutorship agreement that allowed me to compete for a joint PhD degree especially to ***Prof. Salvatore Cuzzocrea****, Coordinator of the PhD in Applied Biology and Experimental Medicine at Messina.*

Furthermore, I am thankful to the funding sources ***EMMAG*** *organization that allowed me to pursue my studies in Messina.*

Dr. Angela Garozzo *at the foreign PhD Secretary and* ***Dr. Francesca Pollicino*** *at the Erasmus office were very helpful and remarkably kind with me during my entire stay in Messina.*

My sincere thanks also go to ***Dr. Antonio Manghisi*** *for his advice and for acting as a mentor to me. I'm deeply grateful to him who gave me the opportunity to serve as a Teaching Assistant in his courses during my thesis. I gained a lot from his vast Biology knowledge and scientific curiosity.*

I would also like to thanks ***Prof. Giusi Genovese*** *for her guidance, encouragement, friendship and support during the whole period of the study. I never forget the opportunity that she gave to me to work on evaluation of antibacterial activity of algae against pathogens relevant in aquaculture, to improve my personal skills.*

In my daily work, I have been blessed with a friendly and cheerful group of Professors, Technicians and fellow students in Messina and Tunisia. They made a cooperative atmosphere at work and also useful feedback and insightful comments on my work.

I am especially grateful to ***Dr. Fakher Kamoun*** *and* ***Dr. Mohamed Ali Ayadi*** *for their incredible support and encouragement.*

I would also like to thank my external referees for their interest in my work and for their very valuable comments and suggestions on this thesis.

Finally, last but not the least, I would like to thank my family: the most special thanks go to my ***Mom****, who took the lead to heaven before the completion of this work. Words cannot express how grateful I am to her for encouraging me to follow my dreams. Her prayer for me was what sustained me thus far. Also, this thesis is heartily dedicated to the memory of my father* ***Sleim****. I miss him every day, but I'm glad to know he saw this process through to its completion. I never forget the sacrifices that he made on my behalf.*

I warmly thank and appreciate my brother ***Najib*** *for his material and spiritual support throughout my years of study. Also, I'm grateful to my brother* ***Ahmed*** *and my two lovely sisters* ***Ola*** *and* ***Mejda*** *for their encouragement and assistance in numerous ways.*

I can just say thanks for my two best friends of my life ***Heni*** *and* ***Houcine*** *who helped me in algae collection and may Allah give them all the best in return.*

Thank you.

Ramzi MILADI

Abstract

Tunisia has a key position in the Mediterranean and constitutes a transition area with a rich habitat diversity between eastern and western basins.

The latest inventory of marine macrophytes dates back to 1987, updated in 1995.

The target of this thesis was to carry on a molecular-assisted alpha taxonomy (MAAT) survey of macroalgae, which allowed to reveal cryptic species, allochthonous introductions and to identify problematic taxa.

Twenty-six genetic species of Florideophyceae were resolved, including five new reports, two of which considered as doubtful for the Mediterranean, and two others as alien species. Of the remaining 21, one was an alien, showing cryptic diversity among the Mediterranean reports of this taxon, two revealed cryptic diversity in other geographical regions, five required a taxonomic update and three a nomenclature update.

Nine genetic species groups were resolved within the genus *Ulva* (Chlorophyta), including the non indigenous species *Ulva ohnoi*, newly reported for Tunisia.

The actual picture of the taxonomy of *Ulva* spp. in the Mediterranean as a whole is far to be clarified and the present data on Tunisian collections aim to be a step towards its clarification.

This is the first DNA barcoding study on macroalgae in Tunisia. This paper is useful to add records to the BOLD system catalogue, amplifying the biodiversity knowledge linked to geographical information, and making them freely available for the scientific community.

An effective monitoring of the biodiversity changes by means of a quick and accurate tool, such as DNA barcoding, is essential to provide the basis for a correct environmental management.

Keywords: Alien species, COI-5', cryptic species, DNA barcoding, Florideophyceae, molecular assisted alpha-taxonomy (MAAT), non indigenous species (NIS), *tuf*A, Tunisian algal flora.

1. Introduction

1.1. Species concept in algae

Species is considered as the currency for global biodiversity assessments (Caldecott *et al*., 1996) and important to conservation biology (Agapow *et al*., 2004). It is used as a fundamental tool of analysis in several areas of biogeography, ecology, and macroevolution (Brown *et al*., 1996; Blackburn & Gaston, 1998; Brooks & McLennan, 1999; Barraclough & Nee, 2001). The debate on the right species concept is certainly old and ongoing. In the post-Darwinian time, more than 24 different species concepts were proposed with different criteria for species delineation (Mayden, 1997).

The typological Species Concept characterizes species as a set of individuals that are distinct from others by peculiar diagnostic characters. It is based on collecting and describing a "type" specimen for a given species (Linnaeus, 1751).The problem with this concept is the difficulty to find the good (diagnostic) characters, the polymorphism within populations, the geographic variation among populations and sibling or cryptic species (sibling species are reproductively isolated groups (different genetic entities) that are morphologically indistinguishable).

Biological species concept defines species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr, 1942). Nonetheless, this is not applicable to asexually reproducing entities, such as many protist lineages.

Phylogenetic species concept is based on genetic indicators and defines a species as the smallest monophyletic group of populations (Cracraft, 1989). The drawbacks are that a large quantity of data are used, variable levels of evolution occur between markers, lineage sorting, lateral transfer and hybridization also occur. It is applicable to allopatric and sympatric populations.

Ecological species concept defines species as a lineage (or a closely related set of lineages) occupying an adaptive zone slightly different from that of any other lineage within its range and evolving separately from all lineages outside its range (Van Valen, 1976).This concept is problematic because it is based on very large datasets from different ecosystems.

Genetic species concept defines species as a homogenous group forming a genotypic cluster (Mallet, 1995). The problem is the variable levels of evolution between molecular markers for the same studied taxa.

Despite there is diversification in contemporary species concepts, all biologists share the fundamental idea that species are segments of lineages at the population level of biological organization, which differ in the secondary properties (e.g., intrinsic reproductive isolation, monophyly, diagnosability).

De Queiroz (1998; 2005; 2007) proposed a **unified species concept** according to which species are seen as inheriting character lines by vertical descent, which evolve under the influence of the same constraints and the same strength evolving in a spatio-temporal space data. This author suggested that a consensus is possible between the conflicting boundaries of species. When divergences accumulate between two lineages (genetic divergence at different loci, genetic isolation, etc.) two distinct lineages appear. Eventually, all the criteria set by the different concepts of species should be acquired to consider definitely two distinct lineages. The speciation phenomenon is progressive and for recently separated species there is a gray area where different concepts of species may conflict. It is essential to address the delimitation of species through integrative approaches (eg. Barrett & Freudenstein, 2011; Chesters *et al*., 2012; Roe & Sperling, 2007) in view of the large number of available methods (Sites & Marshall, 2004).

1.2. What are Algae?

Algae are the organisms that are capable of oxygenic photosynthesis (or belong to phylogenetic groups that are capable of oxygenic photosynthesis) to the exception of Embryophytes. The photosynthetic organelle of algae and plants (the plastid) traces its origin to a primary endosymbiotic event in which a previously non-photosynthetic protist engulfed and enslaved a cyanobacterium. This eukaryote then gave rise to the red, green and glaucophyte algae. The other algal lineages have a more complicated evolutionary history involving secondary endosymbiotic events, in which a protist engulfed an existing eukaryotic alga, tertiary endosymbioses (engulfment of an alga bearing secondary plastid) and even quaternary endosymbioses (Bhattacharya *et al*., 2004).

Algae constitute a large group of plants primarily aquatic and represent 18% of the plant kingdom (Ramade, 2009). During their evolution, they formed diverse groups colonizing a large number of habitats and presenting, *inter alia*, a variety of sizes, cell structures and life cycle (De Reviers, 2003). They play very important roles in many marine communities. They are the nutritional base of aquatic food webs and provide a three-dimensional space where animals shelter, breed, and deposit eggs.

Algae can be microscopic, unicellular microalgae or macroscopic, multicellular macroalgae. Some seaweeds are organized in holdfast, stipe and frond or blade, but many others lack one or more of these structures, due to morphological modification and adaptation. According to their thallus construction, the internal structures of seaweeds are composed of similar cells with simple differentiation. In fact, most of them are filamentous or are built up of united or corticated filaments. Parenchymatous development is found only in kelps, fucoids, *Ulvales*, *Dictyotales* and some others (Lobban & Harrison, 1994).

Macroalgal systematics is very complex and in continuous evolution. Lamouroux (1813) was the first to use colour to separate algal taxa. Since Harvey (1836) seaweeds are divided into three main groups (red, green and brown) based on their pigmentation. This was the first use of a biochemical criterion in plant systematic. Numerous taxonomic changes were made in the following decades, but only with the relatively recent advent of ultrastructural and molecular systematic data a more detailed

classification of the multitude of species belonging to this group was started. Feldmann (1963) wrote "algae actually constitute an extensive and heterogeneous branches set very distinct of each other and having between them only a little common characters." This polyphyletic assembly includes at least seven distinct phylogenetic lineages (Lewis & McCourt, 2004).

1.2.1. Chlorophyta

Green algae have the same photosynthetic system of vascular plants, which is dominated by the chlorophylls a and b, usually in association with a pyrenoid. The chlorophylls are the pigments that give this group of algae its green coloration. These pigments absorb red light, which is available in shallow waters, but absent in deeper water. The cell walls usually contain cellulose, and they store carbohydrate in the form of starch located in chloroplasts (Judd *et al*., 2002). Only about 10% of green algae are marine species (mainly macroalgae), most live in freshwater (mostly microalgae). Thus, green seaweeds live most commonly in the shallow intertidal zone. There are more species of green algae in warm tropical oceans than in cooler temperate seas. Many of them are single-celled, living in freshwater, marine or terestres environment. Some are filaments, branched or not, blades, tubes, cords or crusts. Some others have siphonous structure i.e very large cells without partition (e.g., *Bryopsis*, *Codium*).

Reproductive cycles of green macroalgae present alternating sporophytes and gametophytes either similar such as *Ulva* and *Cladophora*, or completely different as in *Monostroma* and *Ulothrix*. Most gametes and spores are flagellated. They usually live with two or four apical flagella, or, in rare cases, a crown of flagella.

The Chlorophyta comprise an old and taxonomically complex lineage including also land plants (Lewis & McCourt, 2004; McCourt *et al.,* 2004; Hall & Delwiche, 2007; Pröschold & Leliaert, 2007; Becker & Marin, 2009). Their classification has been based mostly on ultrastructure (Norton *et al.,* 1996; Pröschold & Leliaert, 2007) until the introduction of the molecular phylogenetic approach, which allowed to infer their evolutionary history (Leliaert *et al.*, 2012). It is currently recognized the occurrence of two early divergent lineages: the Chlorophyta and Streptophyta (Picket-Heaps & Marchant, 1972; Bremer, 1985; Lemieux *et al.*, 2007). The former includes the majority

of green algal species, while the latter comprises Charophytes, a paraphyletic assemblage of freshwater algae, and the land plants.

1.2.2. Rhodophyta

The red algae are a distinct monophyletic lineage characterized by a two-membraned plastid that contains the photosynthetic pigments (phycocyanin, phycoerythrin and allophycocyanin), which are organized in phycobilisomes within unstacked thylakoids (Gabrielson *et al.*, 1985; Gabrielson & Garbary, 1986; Gabrielson *et al.*, 1990). They can grow in the intertidal zone and in deeper waters than the other algae thanks to these accessory pigments. Their carbon storage polysaccharide is named "rhodamylon" and it is stocked outside of the plastid. Red algae have peculiar cell-juctions, named pit connections or pit-plugs (Wetherbee & Quirk, 1982; Gabrielson *et al.*, 1985; Gabrielson *et al.*, 1990; Saunders & Hommersand, 2004). Two relevant polysaccharides are found in their cell walls: agar and carrageenan, which are gelling compounds used as an ingredient in food products and scientific applications in microbiology and biotechnology. The red algae are found in a variety of morphologies, including simple and branched filaments, fleshy plants, and sheets, most constituted by simple or complex filamentous aggregations. Some red algae are single celled, while others can reach lengths of 2 or 3 meters.

The division Rhodophyta includes the oldest taxonomically defined eukaryote organism, dated at 1.2 billion years old (Butterfield, 2000) and this marks the onset of a major evolutionary radiation of eukaryotes. The majority of species thrive in marine or brackish environments with a few species found in freshwater (Lobban & Harrison, 1994). More recent studies have divided this phylum into seven classes including: the Rhodellophyceae, Cyanidiophyceae, Compsopogonophyceae, Stylonematophyceae, Porphyridiophyceae, Bangiophyceae and Florideophyceae (Saunders & Hommersand, 2004; Yoo *et al*., 2006).

The majority of red algae belong to the last class, including marines species exhibiting a complex life history that involves the alternation of three generations, two of which with independent life, gametophytes and tetrasporophytes, and a third, carposporophyte, that can be interpreted as a vegetative multiplication of the zygote. Sporophytes and gametophytes can be morphologically similar (recognizable by their

reproductive organs) or completely dissimilar. In the latter case, these generations may have received not only separate names but have been classes in different families, before anyone noticing that they were part of the cycle of a single species.

In all red algae reproductive cells always lack flagella and most show amoeboid movements.

1.3. Classification of algae

The classification of algae is traditionally based on morpho-anatomical characters. This method may cause often frustration in identification of mostly macroalgal species because of two main problems: 1) it fails for the many asexual lineages of algae, as well as for species for which life history patterns are not known; 2) it leads to misidentifications in the case of cryptic species (i.e. with morphologies identical or similar also if they represent different taxa), or of environmentally influenced morphological characters. In particular, the presence of several morphotypes belonging to the same taxon can lead to two possible errors: assignment of different morphotypes to distinct species or misidentification of species actually different. These features are a great hindrance for the development of a more universal, rather than more restrictive, taxonomy of algae. Even for the experienced systematist, accurate seaweeds identification can remain elusive due to their simply morphologies, phenotypic plasticity and convergent evolution (Saunders, 2005). It is thus not surprising that algal systematists have come to rely heavily on molecular approach to obtain informations about the evolutionary history of species independent from the phenotypic characters.

1.4. Species delimitation by a DNA barcoding approach

DNA barcoding is a taxonomic method for the quick identification of any species based on extracting a DNA sequence from a tiny tissue sample of any organism. It differs from molecular phylogeny in that it aims to recognise an unknown sample basing on a pre-existing classification rather than to resolve relationship (Kress *et al*., 2005). A DNA barcode is a standardized short sequence of DNA (400 – 800 bp) that can be easily amplified and sequenced for (ideally) all species on earth (Hebert *et al*.,2003; Savolainen *et al*., 2005). It was first proposed by Paul Hebert and colleagues and initially applied to animals (Hebert *et al*., 2003, 2004). The ideal barcode is a highly variable region, useful for species discrimination, flanked by highly conserved regions in order to design PCR primers. Species identification is successful when isolates from a single species group cluster together in distance analyses and the largest intraspecific divergence is less than the smallest interspecific one, this difference named the "barcoding gap" (Hebert *et al*., 2003a; Meier *et al*., 2008). Furthermore, the DNA barcode should be short enough (<700 bp) to be sequenced in a single read (Hollingsworth *et al*., 2009b).

The mitochondrial COI gene encoding the cytochrome c oxidase 1 has been selected for animal phyla (Herbert *et al*., 2003, 2004), due to uniparental transmission, a high rate of evolution, the absence of introns, large numbers of copies in each cell, and a limited recombination (Galtier *et al*., 2009). However, the selection of standard DNA barcode loci for other groups of eukaryotes has been more complex.

The barcode of life project seeks the establishment of a reference database useful to assign a species name to unidentified specimens while promoting the discovery of new species (Schindel & Miller, 2005). To coordinate scientific projects, the international initiative CBOL (Consortium for the Barcode life) was created in 2004 to promote and coordinate the project internationally. The cornerstone of this project, the BOLD database (Barcode of Life database) (Ratnasingha & Herbert, 2007) was established with the intent to maintain a strong link between specimens (georeferencing, biological data and conservation site in an accessible collection) and sequences.

1.4.1. Algae identification using DNA barcoding

The DNA barcoding method has become increasingly common to identify species of marine algae. Several DNA markers of different origin (nuclear, plastid and mitochondrial) have been applied to study phylogenetic relationships at various taxonomic levels. The mitochondrial marker COI initially proposed for the animals was tested in algae to conform to theoretical universality of the barcode marker. In fact, a 650 base pair (bp) segment of the 5' region of the mitochondrial COI gene is currently used for cataloguing red algal biodiversity, examining intraspecific variation, and resolving differences between closely related species (Saunders, 2005; Sherwood *et al*., 2010; Le Gall & Saunders, 2010). Moreover, this marker has proven to identify or delimit species in brown algae as well (Lane *et al*., 2007; Kucera & Saunders, 2008; Mcdevit & Saunders, 2009, 2010; Macaya & Zuccarello, 2010; Mattio & Payri, 2010).

The idea of a universal barcode with a standard gene, applicable to all biodiversity proved to be not feasible, particularly for algae, which belong to several major lines of the tree of eukaryotic life (Hampl *et al*., 2009). In fact, the COI-5' does not appear as a good candidate in green algae because of the inability to sequence the gene. Indeed, in this group, this gene can contain multiple introns (e.g., five in *Chaetosphaeridium,* Turmel *etal*., 2002) and the position and number of them in the gene are not known for most species.

Several markers have been selected to investigate as potential DNA barcode markers for the marine green macroalgae such as the nuclear internal transcribed spacer region of the ribosomal cistron (ITS), extensively used for investigations of phylogeny, molecular ecology and evolution of marine green macroalgae (Hayden & Waaland, 2004). Besides, the plastid *rbc*L gene (ribulose-1,5-bisphosphate carboxylase/oxygenase) was employed extensively to resolve taxonomic issues in green algae, for instance in the genus *Ulva* (e.g., Hayden & Waaland, 2002; Hayden *et al*., 2003; Hayden & Waaland, 2004). Unfortunately, also *rbc*L is affected by the presence of introns in some green algae (Hanyuda *et al.*, 2000). A study by Saunders & Kucera (2010) evaluated new markers for barcode approaches in green algae and showed that the plastid elongation factor gene (*tuf*A) was the best candidate for chlorophytes, except for Cladophoraceae where the amplification and sequencing of different markers tested

remain problematic. Since then, *tuf*A gene has been used largely to discriminate among green algal species (Famà *et al.,* 2002; Zuccarello *et al.,* 2009; Händeler *et al.,* 2010).

1.4.2. DNA Barcoding applications

The case of marine macroalgae is an excellent example of the value and strength of DNA barcoding. Therefore, in a context of impoverishment of taxonomic experts, it proves to be a fast and reliable identification tool within the reach of non-specialists. As a research tool for taxonomists, DNA barcoding assists in identification by expanding the ability to diagnose species by including all life history stages of an organism (e.g., seedlings, juveniles, mature individuals both fertile and sterile), unisexual species, damaged specimens, gut contents, scats and fecal samples. In addition, DNA barcoding has proven as phenomenal tool to aid in the delimitation of cryptic species (organisms morphologically identical but with significant genetic divergence; Bickford *et al*., 2007) and pseudo-cryptic (organisms with significant genetic divergence but with subtile morphological distinctions), likewise to explore new records for red and brown seaweeds (Saunders, 2005; Robba *et al*., 2006; Kucera & Saunders, 2008; Saunders, 2008; McDevit & Saunders, 2009; Saunders, 2009; Walker *et al*., 2009; Le Gall & Saunders, 2010). Furthermore, DNA barcoding can be used to quickly and accurately identify non indigenous species, especially invasive aliens and prompt preventive measures with subsequent regulatory control can be initiated. Finally, besides the fundamental study of biodiversity, DNA barcoding can be used as an important tool for identification, authentication and safety assessment of sea food, particularly for processed products.

However, comparison with type specimens (on which species names are based) or other relevant historical material, typically stored in museum collections, is important. For example, this approach can be fundamental to understand if a new reported alien taxon is the result of a recent introduction or was already present in the environment, but erroneously classified because of its cryptic morphology, or it can be useful to solve some current systematic problems, such as the great number of superfluous and synonymised species names recorded and the use of distinct names for the same species sampled from different regions. Moreover, the comparison between fresh collected and historical samples can be important to compare seaweed biodiversity

patterns and to summarize the occurrence and the distribution of introduced species (Provan *et al.,* 2008; Lister *et al.,* 2010). The use of these markers also for phylogenetic reconstruction of a particular algal group can be useful to determine which morphological characters are congruent with the molecular data within a specific clade and which not, so that only the ones having a genetic basis will be used for correct species or genus identifications in future studies.

1.4.3. Limitations of barcoding

The identification of vast numbers of unknown organisms using DNA sequences becomes more and more important in ecological and biodiversity studies. In this framework, limits of the DNA barcoding method can result from its single-locus identification system.

In addition, DNA barcoding depends on the assumption that speciation (whatever the species concept in use) is generally accompanied by divergence in the sequence of the barcode marker. However, sequence divergence is stochastic rather than steady and so some closely related species will not be resolved in cases of incomplete lineage sorting (Verbruggen*et al*., 2009).

Furthermore, the major drawback is the current lacks of universal amplification primers. For examples, the original red-algal primers (Saunders, 2005) are successful in some groups, but show mixed results in other lineages, which may be due to heterogeneity within a species at positions near the 3' end of the primers (Saunders, 2008). New primers have been developed and the search for the most widely effective ones is still in progress (Saunders & McDevitt, 2012).

Another statistical difficulty is caused by the low number of individuals analyzed per species, which does not allow a precise estimation of the intraspecific variation compared with interspecific variation (Matz & Nielsen, 2005; Nielsen & Matz, 2006). However, the constant increase of data in the BOLD archive is going to correct this last problem.

Another limitation related to many potential DNA barcoding applications that can only be based on degraded DNA. This is the case for most environmental samples where the target is DNA from damaged specimens. It is usually difficult to amplify DNA fragments longer than 150 bp from such samples (Deagle *et al*., 2006). As a

consequence, shorter barcoding markers are under evaluation (Hajibabaei *et al*., 2006; Meusnier *et al*., 2008; Erickson *et al.*, 2017).

1.5. Specific biodiversity of marine flora in Tunisia

1.5.1. Geographical position

Tunisia, occupying a central place in the Mediterranean, opens up widely onto the sea, mainly on its eastern and western shores. It has more than 1300 km of coastline that constitute transition area and habitat diversity between the two basins of the Mediterranean. The topography of the Tunisian coast is very varied: rocky or sandy coast, deep gulf, sometimes protruding capes, numerous islands and islets and shoals belt are the most characteristic elements (Azouz, 1966; Ben Mustapha,1966; Poizat, 1970, Azouz, 1973; Ben Othman, 1973; Blanpied*, et al*., 1979; Pergent & Kempf, 1993). All these specificities allow the existence of a particularly interesting marine and coastal biodiversity especially concerning algal biodiversity. The flora of the northern coast of Tunisia (including the Gulf of Tunis) has some affinities with that of the Western Mediterranean with its rich algal flora (Ben Maiz &Boudouresque, 1986). Conversely, the eastern and southern sides have the properties of the flora of the eastern Mediterranean which considered poor in algal biodiversity compared to that of the countries of the western basin. This reflects the fact that this region, located in the western basin has a very diverse topography.

1.5.2. Inventory

The first records of Tunisian marine algae were published by Piccone (1879, 1884), who reported a limited number of taxa from the northern coasts, while the first inventory was compiled by Debray (1897). After, a few studies were carried out until the valuable works of Ben Maiz (Ben Maiz *et al.,* 1987, Ben Maiz, 1995), which represent the latest inventory available of marine macrophytes from this country.

Subsequently, only papers on specific taxa (Djellouli, 2000, Ben Said & Ksouri, 2002, Ksouri *et al*., 2002, Langar *et al*., 2002, Chebil-Ajjabi *et al*., 2005, Bouafif *et al*., 2014, Bouafif *et al*., 2016), on a limited area (Ben Maiz & Shili, 2007, Ben Mustapha & Afli, 2007, Shili *et al*., 2007), with an ecological aim (Ben Mustapha & Afli, 2007, El Ati-Hellal *et al*., 2007), dealing with non indigenous species (NIS) (Sghaier *et al*., 2016) or papers dealing with applicative purposes in which macroalgae are exploited (Ksouri

et al., 2007, Azaza *et al*., 2008, Yaich *et al*., 2011, Kolsi *et al*., 2015), have been published

However, floristic and taxonomic studies on Tunisian marine flora have been carried on only by means of a classic morpho-anatomic identification approach.

1.5.3. Alien seaweed introduction

Algal biodiversity can be impacted upon negatively (overall reduction and/or shift in composition) by factors such as global warming, increased environmental stress arising from fisheries and aquaculture activities and by accidental introductions of invasive species. The term invasive species typically refers to non indigenous species (NIS) that have a negative impact on the environment or on human activities. In fact, macroalgae represent not only a large component of the globally introduced biota (e.g., Ribera & Boudouresque, 1995; Lewis, 1999; Ribera Siguan, 2002; Schaffelke *et al.,* 2006), but also significant economic and environmental risks for which control and management options are limited (e.g., Ribera & Boudouresque, 1995; Thresher, 1999; McEnnulty *et al.,* 2001; Anderson, 2007; Schaffelke & Hewitt, 2007). In consequence to the recent climate change and temperature increase, they spread in wider regions finding numerous habitats suitable for their growth. Climate-driven invasions could lead to completely transformed ecosystems where alien species dominate for function or richness or both, leading to reduced diversity of native species (Mack *et al.,* 2000; Gritti *et al.,* 2006). We distinguished three categories of invasive species according to Zenetos *et al*. (2010): cryptogenic species, with no definite evidence of their native or introduced status; established species, known from more than two localities or records; questionable species, with insufficient information. A total of 27 NIS macrophytes were recorded from Tunisia, which is low compared to the 129 species listed for the Mediterranean Sea (Verlaque *et al*., 2015). However, compared to nearby Mediterranean regions, this number is close to that reported in the Straits of Sicily (18) (Occhipinti-Ambrogi *et al*., 2011) and Algeria (17) (Verlaque *et al*., 2015), and higher than those recorded in Malta (12) (Sciberras & Schembri, 2007; Evans *et al*., 2015a, b; Schembri *et al*., 2015), and Libya (14) (Bazairi *et al*., 2013). This number has greatly increased since the late 20th century, with sixteen new NIS reported after 1960.

1.5.4. Introduction pathways of alien seaweed

1.5.4.1. Natural introduction.

The Mediterranean Sea, transition zone between the Atlantic Ocean (through the Strait of Gibraltar) and the Indian Ocean (via the Suez Canal and the Red Sea), had at the end of the nineteenth and in the twentieth centuries several disturbances of which opening of the Suez Canal. In consequent, composition of flora and fauna has greatly changed. The Tunisian coast, particularly the Gulf of Gabes region, suffer the consequences of this disturbance that has led to modifications in the composition of flora and fauna. Indeed, several animal and plant species have arrived in Tunisia from either the Atlantic or the Indo-Pacific. These species are mainly from the Red Sea (Lessepsian species) and secondarily from the Atlantic. For example, the entrance of invasive species may unbalance the environment and affect native communities, such as the case of *Caulerpa taxifolia,* which supplants *Posidonia oceanica,* and the Lessepsian shrimp *Trachypenaeus curvirostris,* which replaces the royal shrimp *Penaeus kerathurus* (Bradai, 2000). The identity and abundance of NIS in Tunisian waters could be explained by its southern geographical position located at the crossroads between the eastern and western basins of the Mediterranean Sea.

1.5.4.2. Anthropogenic introduction

Some Tunisian localities such as Bizerte, El Kantaoui, Cap Monastir and Tunis displayed a high number of recorded aliens compared to the average, confirming that hotspots for marine species introduction are coastal lagoons and harbours where human activities such as shipping and fishing activities, recreational marinas and aquaculture affect the health of ecosystems and facilitate the introduction and the secondary dispersal of alien species (Cohen & Carlton, 1998; Verlaque, 2001; Occhipinti-Ambrogi & Savini, 2003; Rilov & Crooks, 2009; Hoffman *et al*., 2011; Occhipinti-Ambrogi *et al*., 2011).

Aquaculture represents a source of pollution rarely taken into account, still poorly assessed, but which seems considerable. Non-indigenous species introduced by aquaculture are of two kinds: species introduced intentionally to implement new aquaculture industries, and species introduced accidentally when for example they are fixed to the species deliberately introduced. At present seaweed introductions belong to the latter kind. An example of the first kind of introduction is the case of the oyster

Crassostrea gigas, from the Northwest Pacific Ocean, introduced in the Mediterranean due to the high mortality of the local species, the Portuguese oyster, *C. angulata.* The importation of *C. gigas* allowed the introduction of another species of gastropod mollusc, *Crepidula fornicata*, now becomes quite intrusive, and of several algal species, such as *Undaria pinnatifida*. Oyster farming is an important vector of introduction because of the many exchanges occurring between oyster farms. In the Thau lagoon (France, Mediterranean), there are at least 9 species of algae whose introduction is linked to the introduction of oyster spat of *Crassostrea gigas* (Perez *et al*.,1981; Riouall, 1985; Riouall *et al*., 1985; Ben Maiez, *et al*., 1987; Verlaque & Riouall, 1989). Recently, Manghisi *et al*. (2010) demonstrated that the red alga *Agardhiella subulata*, endemic to the Atlantic coast of North America, was introduced to Sicily from the Netherlands as a plantlet growing on a *C. Gigas* shell. *P. morrowii* has been reported as an intertidal species and it has been found on a large variety of substrata including rocks, wooden piles, ropes, mussels, crabs and shells, as well as other large algae, such as *S. muticum* and *U. pinnatifida* (Kim*et al*., 1994; Kudo & Masuda, 1992).

Aquaculture in Tunisia is a very old activity dating back to Roman times as evidenced by some mosaics of the Bardo Museum in Tunis. Fisheries and aquaculture play an important role in socio-economic terms and as a source of food. The recent Tunisian aquaculture experience began in the 1960s. Initiated by the private sector, this activity began with growing the Mediterranean mussel *Mytilus galloprovincialis* and the Pacific cupped oyster *Crassostrea gigas* on intertidal trestles in Bizerte. The supply of mussels spat is provided locally by capture in the Bizerte Lagoon while the oysters spat was imported from abroad (France, Italy). The main marine aquaculture projects and farms are located, for the moment, within the area situated off the east coast, from the gulf of Hammamet to the beginning of the golf of Gabes. Four shellfish farming projects were established in the Bizerte Lagoon to the north of Pats, and four other Bluefin tuna fish fattening projects on the east coast: two of them in the Sousse Governorate, and two others in the Mahdia Governorate. Another fish farm has just been opened to breed Nile tilapia in Southern Tunisia. Only two species of fish were farmed in the past – European seabass and gilthead seabream –but during the last decade, freshwater fish species (42%), shell-fish (5%), bluefin tuna (16%) and marine fishes (37%) have also been farmed.

The beautiful green alga *Caulerpa taxifolia*is frequently used to decorate aquariums. It is through aquariology that this species has been introduced in the Mediterranean. His accidental discharge into the sea following the cleaning of aquariums has been responsible for its introduction and expansion in many localities very fast. On the other hand, other introduced species do have an impact on the indigenous species or communities: this is referred to as biological pollution (Sindermann *et al*., 1992). The threatening of native species may give rise to alterations in the functioning of the ecosystem.

2. Aim of the thesis

Algae exhibit simple morphologies, with high phenotypic plasticity and convergence, and in some groups heteromorphic generations alternating in life histories, which are all features making species identification a very challenging task.

Algal taxonomists have solved such difficulties using "*the more objective technique of MAAT* [molecular assisted alpha-taxonomy]*, which uses differences in gene sequence data to delineate genetic species groups that are subsequently analysed morphologically and assigned to existing species or established as novel taxa"* (Filloramo & Saunders, 2016).

As detailed above, different barcodes have been selected as markers for the different branches of the tree of life, including red and green macroalgae lineages (Saunders & McDevit, 2012). Since its beginning, MAAT using the mitochondrial COI-5' region showed to be effective and reliable for defining red algal species limits (Saunders, 2005, Manghisi *et al*., 2010, Le Gall *et al*., 2015). Differently, for Chlorophyta, the plastid *tuf*A gene resulted as a viable marker with a higher resolution power at species level in comparison to the other molecular markers (Saunders & Kucera, 2010).

To achieve a comprehensive knowledge of the macroalgal flora of a specific area, as well as of a specific taxonomic group, an integrative approach is essential, including morpho-anatomical observations, ecological and biogeographic data together with phylogenetic analyses. However, this approach is time consuming and requires trained specialists. In the aim of carrying on a rapid and effective survey of the macroalgal diversity in a specific area, which allows to uncover cryptic species, very common in macroalgal taxa, to monitor allochthonous introductions and to identify problematic taxa (Manghisi *et al*., 2010, Kress *et al*., 2015, Machín-Sánchez *et al*., 2016, Kogame *et al*., 2017), DNA barcoding should be the preferred choice.

The aim of the present thesis is to provide a molecular-assisted alpha taxonomy (MAAT) survey of florideophycean algae and *Ulva* spp. along Tunisian coasts for the compilation of a DNA barcode inventory.

At the best of our knowledge, this is the first study on Tunisian macroalgal flora using DNA barcoding methods, since as already mentioned, up to the present, previous floristic and taxonomic studies have been carried on only by means of a classic morpho-anatomic identification approach.

3. Materials and Methods

3.1. Specimen collection and preservation

3.1.1. Collecting Locations on the Tunisian Coasts

The Tunisian coastline is approximately 1300 km long. The northern boundary (08°45'00" N lat., 36°57'00" E long.) is 5 km west of the city of Tabarka and the southern boundary (11°34'00" N lat., 33° 10' 00" E long.) is 6 km south of Lake El Bibane. Sixteen sampling sites distributed along the Tunisian coastline were investigated, from February 2014 to March 2016 (**Figure 3**, **Table 1**).

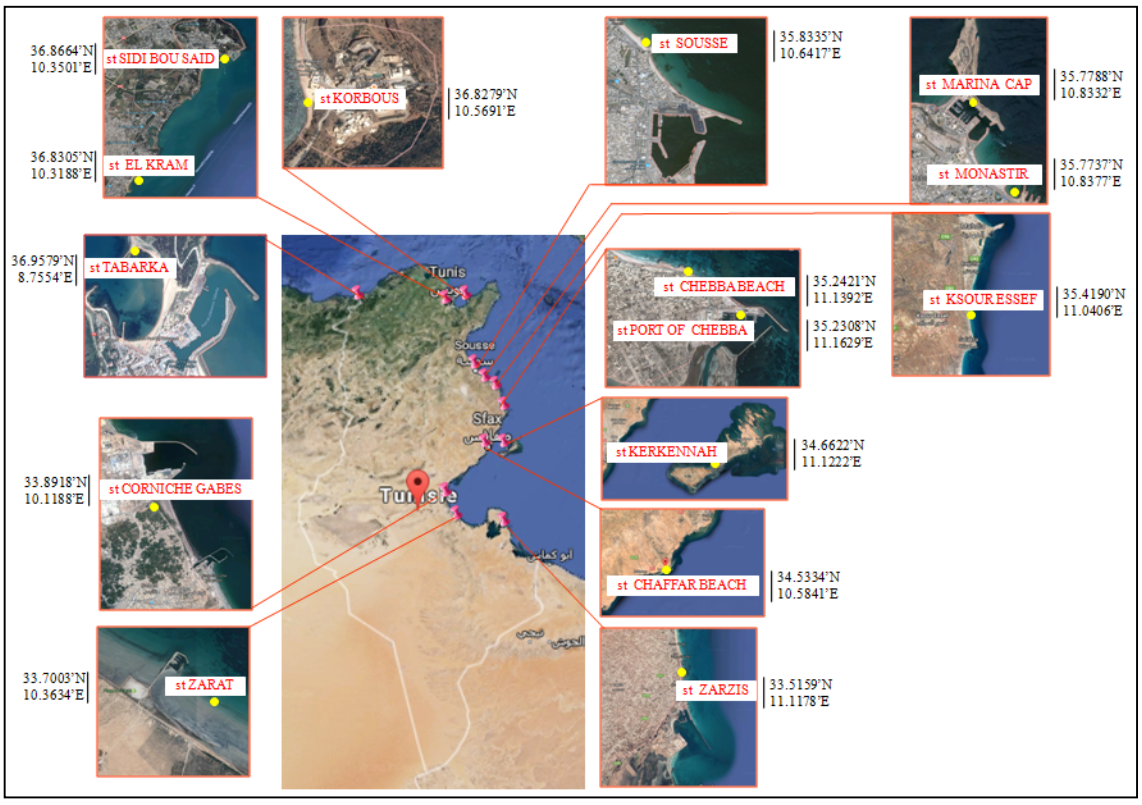

Fig. 3. Location of sampling sites in Tunisia. The characteristics of site numbers are given in the Table 1.

Macroalgae were hand collected between the seashore and 6 m of depth. List of specimens with collection information is reported in **Appendix 1**. Collection were made from three different types of environment (i) regions within ports and harbors where invasive or exotic species would be most expected to be present due to decades (even

centuries) of international shipping, (ii) open-coast regions not on shipping routes where truly native species might be expected to have persisted up to the present, and (iii) low-salinity or freshwater habitats in sites unconnected to navigable river systems and where no routes of invasion are apparent.

Tab. 1: Sampling sites in Tunisia.

	Locality (North to South Tunisia)	Coordinates (Lat. N - Long. E)	Date	Depth (m)	Substrate
1	Tabarka	36.9579-8.7554	30/07/2014 04/04/2015	1-3	rock/sand
2	Sidi Bou Saïd	36.8664-10.3501	25/04/2014	3-4	rock/sand
3	El Kram/Tunis	36.8305-10.3188	01/03/2014	0-1	rock
4	Korbous	36.8279-10.5691	08/04/2015	0-1	rock
5	Sousse	35.8335-10.6417	01/08/2014 03/09/2014 12/04/2015 13/08/2015 26/03/2016	0-3	rock/sand
6	Marina Cap Monastir	35.7788-10.8332	02/08/2014	0-1	rock
7	Monastir	35.7737-10.8377	03/08/2014 10/04/2015	1-3	rock/sand
8	Ksour Essef/Mahdia	35.4190-11.0406	05/02/2014	0-1	muddy sand
9	Chebba Beach	35.2421-11.1392	17/03/2014 12/04/2014 22/06/2014 04/08/2014	1-6	rock/sand
10	Port Of Chebba	35.2308-11.1629	27/04/2014		
11	El Kantra/ Kerkennah	34.6622-11.1222	20/04/2014 01/06/2014 15/08/2015	1-4	rock/sand
12	Chaffar Beach/Sfax	34.5334-10.5841	28/06/2014	0-1	muddy sand
13	Corniche Gabes	33.8918-10.1188	10/08/2014 10/08/2015	0-1	muddy sand
14	Port Of Zarat/Gabes	33.7003-10.3634	15/08/2014	0-1	rock/sand
15	Zarat Beach/Gabes	33.6901-10.3819	12/03/2014	0-1	muddy sand
16	Sidi Kbir Beach/ Zarzis	33.5159-11.1178	30/08/2014	0-1	rock/sand

For each specimen, notes were taken about collection details, such as where the algae was growing (e.g., location in the intertidal, low to high...), what it was growing on (e.g., rock, other algae, etc.), and any other distinguishing features that may be lost when the specimen is removed from the field (e.g., iridescence).

3.1.2. Preservation

Freshly collected material was placed in plastic bags filled with seawater and transported to the laboratory, where it was rinsed in clean seawater to remove debris and most epiphytes. For each sample, a voucher specimen was prepared by pressing a single individual on an herbarium sheet with a subsample dried in silica gel for molecular analyses. Vouchers are housed in the Phycological Lab Herbarium (PhL) of the University of Messina, Italy (http://grbio.org/institution/phycological-lab-herbarium-university-messina).

3.2. Molecular techniques

3.2.1. Genomic extraction for Macroalgae

DNA was isolated from silica dried thalli as detailed in Manghisi et al. (2010) from red algal samples, and from green algal samples using a standard CTAB-extraction method (Doyle & Doyle, 1987), with few modifications: 2-mercaptoethanol was excluded from the extraction buffer, while 1% PVP and 0.02% of proteinase K were added; lysis was performed at room temperature for 2 hours on a rotary shaker.

3.2.1.1. DNA extraction protocol for red algae (Wizard ® DNA clean up System, Promega)[1]

- Defrost 20 mg/ml Proteinase K (stored in the freezer) and keep it on ice.

1. Mark a sets of 2.0 ml microcentrifuge tubes according to the number of samples. Add to each tube 600 µl of DNA extraction buffer (Reds), 60 ul of 10% Tween 20 (both should be at room temperature when used), 6 ul of 20 mg/ml Proteinase K[2].
2. Grind small amount (50-100 mg dry weight) of clean tissue in liquid nitrogen. Transfer it in the prepared microcentrifuge tube (or grind directly in the tube and then add the buffer).
3. Mix constantly for 60 (120) min at room temperature (e.g. on rotary shaker).
4. Incubate on ice 10 (20) min.
5. Spin for 10min @ top speed (17000 rpm) at 4°C.

All of the following steps should be done under the hood and wide bore tips should be used for all of transfers until precipitation.

- Move to Wizard ® DNA clean up System (Promega):

6. Move supernatant in a 2,0 ml tube.
7. Mix the resin before use. Add 1ml of Wizard® DNA resin to each tube and and mix by gently inverting several times (invert tubes for 20 seconds; allow to stand for 20 seconds; invert for 5 seconds; allow to stand for 20 seconds).
8. Prepare column by removing the plunger and then screwing a Wizard® minicolumn onto the end of a new syringe. Pipette sample into the top of the syringe.

[1] *See manufacter instructions for further details.*

[2] *Proteinase K is heat sensitive and should be handled with care. Store it in the freezer. Before use, thaw it, flick and spin it and keep on ice while using. Store it back in the freezer as soon as possible.*

9. Replace plunger and pump solution through at a rate of about 1 drop per second (into a waste vessel).
10. Remove minicolumn from the syringe and suck up 2ml of newly made 80% isopropanol.
11. Replace minicolumn and pump the isopropanol through the minicolumn at a rate of 1 drop per second (into a waste vessel).
12. Remove minicolumn from the syringe and transfer it to a capless 1.5ml microcentrifuge tube and spin at 14000 rpm for 2 minutes to completely dry the column.
13. Move the minicolumn to a new microtube. Allow to stand for 3 minutes to allow excess isopropanol to evaporate.
14. Add 50 (100) µl of warm (70^0C) (or at room temperature) ddH_2O to the minicolumn. Allow to stand for 90 seconds. Spin at 14000 rpm for 1 minute.

    DNA templates can be stored at –20ºC.

    DNA extraction recipes **Appendix 2**.

3.2.1.2. DNA extraction protocol for green algae (CTAB)[3]

- Defrost 20 mg/ml Proteinase K (stored in the freezer) and keep it on ice. Add 1/10 volume of 10% PVP to CTAB buffer[4].

1. Mark a sets of 2.0 ml microcentrifuge tubes according to the number of samples. Add to each tube 600 µl of DNA extraction buffer (2X CTAB, should be at room temperature when used), 6ul of 20mg/ml Proteinase K[5].
2. Grind small amount (50-100 mg dry weight) of clean tissue in liquid nitrogen. Transfer it in the prepared microcentrifuge tube (or grind directly in the tube and then add the buffer).
3. Mix constantly for 60 (120) min at room temperature (e.g. on rotary shaker).
4. Spin for 10min @ top speed (17000 rpm).

   All of the following steps should be done under the hood and wide bore tips should be used for all of transfers until precipitation.

[3]Doyle J.J. & Doyle J.L., 1987 - A rapid isolation procedure for small quantities of fresh leaf tissue. *Phytochemical bulletin* 19(1): 11-15.

[4]*Add 1% polyvinylpyrrolidone (PVP) to an aliquot of CTAB buffer just prior to starting extraction. Once it has been added the shelf life of the buffer is only 2-3 days.*

[5]*Proteinase K is heat sensitive and should be handled with care. Store it in the freezer. Before use, thaw it, flick and spin it and keep on ice while using. Store it back in the freezer as soon as possible.*

5. Move supernatant in a tube containing 500 µl of chloroform-isoamyl alcohol (24:1) and mix by inverting for 5 min. Solution should form a milky emulsion. Spin for 5 min @ 17000 rpm. Recover supernatant and move to a new tube.[6]
6. Repeat last step once or more times if supernatant is not clear.
7. Add 1x the sample volume of ice cold isopropanol (stored in the freezer). Mix gently and incubate for 1h+ (or leave overnight) at 4°C (or on ice).[7]
8. Spin for 30min @ top speed at 4 °C.
9. Decant supernatant and wash pellet 3 times in 150ul 70% ethanol (stored in the freezer; pellet should go slightly white).
10. Air dry pellet until fully dry (should go pale/translucent).
11. Redissolve in 50 (100) µl of sterile ddH_2O.

DNA templates can be stored at –20ºC.

3.2.2. PCR Profiles and Primers

The mithochondrial COI-5' region was PCR amplified as described in Saunders and McDevitt (2012), as a DNA barcode for red algae. The plastidial *tuf*A gene was PCR amplified as described in Saunders and Kucera (2010), as a DNA barcode for green algae (**Table 2)**.

Tab. 2: List of used PCR primers (from Saunders and McDevit 2012).

Region	Primer name	Direction	Sequence	Taxonomic group
COI-5'	GWSFa	forward	CAAAYCAYAARGATATYGGAAC	Halymeniales, Rhodophyta
	GwsFn	forward	TCAACAAAYCAYAAAGATATYGG	Rhodophyta
	GazF1	forward	TCAACAAATCATAAAGATATTGG	Rhodophyta
	GazR1	reverse	ACTTCTGGATGTCCAAAAAAYCA	Rhodophyta
	GWSRi	reverse	GGRTGICCRAARAAYCARAA	Rhodophyta
	GWSRx	reverse	ACTTCTGGRTGICCRAARAAYCA	Rhodophyta
***tuf*A**	tufAR	reverse	CCTTCNCGAATMGCRAAWCGC	Chlorophyta
	tufAGF4	forward	GGNGCNGCNCAAATGGAYGG	Chlorophyta

[6] *Following centrifugation, you should have three layers: top, aqueous phase; middle, debris and proteins; bottom, chloroform*

[7] *For low DNA concentrations: higher final concentrations of alcohol, longer precipitations (1 hr to overnight), lower temperatures (-20°C to -70°C) and longer centrifugation times.*

3.2.2.1. Polymerase Chain Reaction

Always wear gloves while working with PCR reagents.

Work on ice bath.

- Wipe down all working surfaces with H_2O and then denatured ethanol.

On the pre-PCR bench top:

- Retrieve DNA templates from freezer and let them defrost. DO NOT work with DNA templates on the PCR bench-top. Prepare DNA dilution if needed.
- Change gloves before working with PCR reagents.

On the PCR bench top:

- Mark 0.2 ml tubes according to the number of samples plus one for control and a (0.5-1.5-2.0 ml) centrifuge tube for PCR mix.
- Retrieve the 5X PCR Buffer, $MgCl_2$, dNTPs (working aliquots), PCR primers (working aliquots) and PCR-H_2O (sterile ddH_2O) from the freezer. Thaw all tubes, flick and spin them down and then place on ice.
- Prepare PCR mix according to **Table 3**; retrieve Taq DNA polymerase from freezer just before use and put it back immediately (note that it does not freeze at -20 °C).

Tab. 3: PCR master mix (Promega).

Reaction: 20 ul			
	ul	**Final concentration**	
Sterile H_2O	12.10		*x number of samples*
5X Buffer	4.00		
25 mM $MgCl_2$	2.00	2.500 mM	
2.5 mM each dNTPs	0.40	0.050 mM	
10 uM Primer forward	0.20	0.100 uM	
10 uM Primer reverse	0.20	0.100 uM	
5 U ul^{-1} Taq polymerase	0.10	0.024 U ul^{-1}	
Optional (if used subtract volume from water):			
10% PVP	2.00		
DMSO	1.00		
5 ug/ul BSA	2.00		
Template DNA volume	1.00		
Total volume without DNA	19.0		

Always add a negative control[8]. Use positive control only if strictly necessary.

- Flick and spin the mix.
- Pipette 19 ul of the mix into each labelled PCR tube. Close the tubes immediately.
- Put back in the freezer all PCR reagents.

On the pre-PCR bench top:

- Flick and spin down DNA templates.
- Add 1 ul of appropriate diluted template to each PCR tube. Spin down any bubbles.

Place tubes into thermo-cycler and start appropriate program (**Table 4**).

- Return DNA templates to the freezer.

Run diagnostic gel to check quality and quantity of the DNA products.

PCR reactions can be stored at –20°C.

Polymerase Chain Reaction recipe **Appendix 2.**

Tab. 4: PCR cycling protocol used for each marker.

Marker	Stage	Step	Repetitions	Hold
COI-5P	1	1. 95° 2’	1	
	2	1. 95° 30” 2. 45° 30” 3. 72° 1’	5	
	3	1. 95° 30” 2. 46.5° 30” 3. 72° 1’	35	
	4	1. 72° 7’	1	4°C
tuf-A	1	1. 94° 4’	1	
	2	1. 94° 1’ 2. 45° 30” 3. 72° 1’	38	
	4	1. 72° 7’	1	4°C

3.2.2.2. Agarose gel electrophoresis

Always wear nitrile gloves (not latex!) while working with EtBr.

✓ **0.8% agarose gel in TAE, small tray:**

0.20 g of agarose

25 ml of **TAE 1X (EtBr free)**

1 µl of **EtBr** (stock solution: 10 mg ml^{-1})

[8] *If assembling more than one PCR mix, consider a negative control for each.*

✓ **0.8% agarose gel in TAE, large tray:**

0.60 g of agarose

75 ml of **TAE 1X (EtBr free)**

3 µl of **EtBr** (stock solution: 10 mg ml^{-1})

With clean gloves:

- Weight agarose e measure TAE 1X (EtBr free) for gel preparation.
- In a flask combine agarose and buffer (do not touch the flask, it is EtBr contamined).
- Prepare two stripes of paper tape for the gel tray

With nitrile gloves:

- Close the gel tray with prepared tapes.
- Put the proper comb (check the teeth number in relation to the number of samples)
- Melt in a microwave the gel mix in the flask and allow to coolbut not to solidify.
- Just before pouring, add 1 µl of EtBr (stock solution: 10 mg ml^{-1}).
- Swirl to completely mix, and pour gel in the tray, sealed with tape and let the gel harden.
- Put the gel in the electrophoretic chamber, after removing the tape and the comb.

With clean gloves:

- Fill the chamber with TAE 1X[9].
- Load DNA sample into gel wells. Always add a line with the proper DNA marker.

With nitrile gloves:

- Connect electrodes and run at 60-80 V[10].
- When the run is complete, check it at UV light. Take a picture if needed.

Discard gel and running buffer (recycle it a few times before discarding) in EtBr-waste.

[9] *Adding EtBr to the chamber buffer is not necessary; when added, the background fluorescence of the gel is more uniform.*

[10] *3.5-5 V cm^{-1}.*

Wipe dry UV screen with clean paper tissue[11] and discard it in EtBr-waste.

Agarose gel electrophoresis recipes **Appendix 2**.

Sequencing reactions were performed by an external company (Macrogen Europe, The Netherlands).

3.2.3. DNA Barcode Analyses

Specimen data, sequences and used primers were deposited in the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org). Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and multiple sequence alignments were constructed in Seaview (v. 4.3.3, Gouy et al., 2010), including sequences of related taxa downloaded from GenBank (Benson et al., 2017). Sequence alignments were subjected to distance analyses with a Neighbor-Joining algorithm under a K2P model of nucleotide substitution (Kimura, 1980) as performed in PAUP* 4b10 for the Macintosh (Swofford, 2002) to visualize clusters of genetic species.

In addition, COI-5' sequences were contrasted with BOLD, with the "identify specimen" interface.

[11] *Do not clean the screen with any detergent.*

4. DNA barcoding shades light on novel records in the Tunisian red algal flora (submitted[12])

[12]Submitted to *Botanica Marina* (IP 1.250)

DE GRUYTER Botanica Marina

DNA barcoding sheds light on novel records in the Tunisian red algal flora

Journal:	*Botanica Marina*
Manuscript ID	BOTMAR.2017.0036.R3
Manuscript Type:	Research article
Date Submitted by the Author:	23-Nov-2017
Complete List of Authors:	Miladi, Ramzi Manghisi, Antonio; Università degli Studi di Messina, Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali Armeli Minicante, Simona Genovese, Guiseppa Le Gall, Line; Museum National dHistoire Naturelle (MNHN), UMR 7138 Systematique, Adaptation et Evolution Abdelkafi, Slim Morabito, Marina
Classifications:	5400 Algal floristics < 5 Algal systematics/floristics/biogeography, 5200 Algal systematics < 5 Algal systematics/floristics/biogeography, 5800 Algal checklists < 5 Algal systematics/floristics/biogeography
Keywords:	Tunisian algal flora, DNA barcoding, alien species, cryptic species, Florideophyceae

DNA barcoding sheds light on novel records in the Tunisian red algal flora

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Running title: DNA barcoding of the Tunisian red algal flora

Abstract

Tunisia holds a key position in the Mediterranean Sea as it constitutes a transitional area with a rich habitat diversity between eastern and western basins. The most recent inventory of marine macrophytes dates back to 1987 with an update in 1995. This is the first DNA barcoding study of macroalgae in Tunisia. Our target was to carry out a survey of macroalgae, to reveal cryptic species and allochthonous introductions, and to identify taxa with convoluted taxonomy.

COI-5P sequence analysis resolved 26 genetic species, including four new reports and three alien species. Of the remaining taxa, several displayed cryptic diversity among the species reported from the Mediterranean or from other geographical regions, six required a taxonomic update and two a nomenclature update. This paper adds records to the Barcode of Life Data (BOLD) System catalogue, increasing the biodiversity knowledge linked to geographical information, and making them freely available for the scientific community.

Keywords: Tunisian algal flora, DNA barcoding, alien species, cryptic species, Florideophyceae.

Introduction

Tunisia holds a key position in the Mediterranean between the eastern and western basins of the Mediterranean Sea and constitutes a transitional area with a rich habitat diversity. The Strait of Sicily, the waters between Tunisia and Sicily, is the crossroad from south to north and from east to west, and is crucial in the analysis of the distribution of marine organisms in the Mediterranean Sea (Coll et al. 2010).

The presence of rocky or sandy coasts, deep bays, some protruding headlands, numerous islands and islets are characteristic features of the Tunisian littoral topography (Le Danois 1925, Azouz 1966, 1973, Ben Mustapha 1966, Poizat 1970, Ben Othman 1973, Blanpied et al. 1979a, 1979b, Pergent and Kempf 1993). Such a diverse topography supports a very rich marine macroalgal diversity, with the northern coast and the Gulf of Tunis being richer than the rest of the coast (Ben Mustapha and Afli 2007).

The first records of Tunisian marine algae were published by Piccone (1879, 1884), who reported a limited number of taxa from the northern coast, while the first inventory was compiled by Debray (1897). After this, few studies were carried out until the valuable works of Meñez and Mathieson (1981) and of Ben Maiz and coworkers (Ben Maiz et al. 1987, Ben Maiz 1995), which represent the latest inventory available of marine macrophytes from this country. These floristic and taxonomic studies of the Tunisian marine flora have been carried out only by means of classic morphological-anatomical identifications.

Subsequently, papers focused on either selected taxa (Djellouli 2000, Ben Said and Ksouri 2002, Ksouri et al. 2002, Langar et al. 2002, Chebil-Ajjabi et al. 2005, Bouafif et al. 2014, 2016), limited areas (Ben Maiz and Boudouresque 1986, Ben Maiz and Shili 2007, Ben Mustapha and Afli 2007, Shili et al. 2007), or specific ecological aims (Ben

Mustapha and Afli 2007, El Ati-Hellal et al. 2007), non-indigenous (alien) species (Sghaier et al. 2016), or dealt with the applications for which macroalgae are exploited (Ksouri et al. 2007, Azaza et al. 2008, Yaich et al. 2011, Kolsi et al. 2015).

Over the last twenty years, DNA sequences have become powerful tools in biodiversity studies and, although different molecular markers and analytical strategies are available for systematic and phylogenetic research (Maggs et al. 2007, De Clerck et al. 2013, Kraft and Saunders 2014), a quick and accurate tool - the DNA barcoding approach (Hebert et al. 2003, Saunders 2005) - has been developed only recently for molecular-assisted alpha taxonomy (MAAT) surveys with the aim of compiling a DNA library of life (Le Gall et al. 2017).

Red algae exhibit simple morphologies, with high phenotypic plasticity and convergence, and an alternation of heteromorphic generations in life histories, which are all features making species identification a very challenging task. Red algal taxonomists have overcome such difficulties using "*the more objective technique of MAAT, which uses differences in gene sequence data to delineate genetic species groups that are subsequently analysed morphologically and assigned to existing species or established as novel taxa*" (Filloramo and Saunders 2016). Different barcodes have been selected as markers for the different branches of the tree of life, including red macroalgal lineages (Saunders and McDevit 2012). Since its beginning, MAAT using the COI-5P region has proven effective and reliable for defining red algal species limits (e.g. Saunders 2005, Manghisi et al. 2010, Le Gall et al. 2015).

To achieve a comprehensive knowledge of the macroalgal flora of a specific area, as well as of a specific taxonomic group, an integrated approach is essential. This includes morphological-anatomical observations, ecological and biogeographic data, together with phylogenetic analyses. However, this approach is time-consuming and requires trained specialists. DNA barcoding has proved useful for facilitating rapid and effective

surveys of macroalgal diversity in a specific area, uncovering cryptic species, assessing the relationships with neighbouring floras, monitoring allochthonous introductions, and for the identification of problematic taxa (Manghisi et al. 2010, Kress et al. 2015, Machín-Sánchez et al. 2016, Kogame et al. 2017).

The aim of the present paper is to provide a MAAT survey of Florideophycean algae collected along the Tunisian coast as a part of a programme of compiling a DNA barcode inventory of macroalgal species. To the best of our knowledge, this is the first study of the Tunisian macroalgal flora using DNA barcoding methods.

Materials and methods

Red macroalgae were sampled at 13 sites distributed along the Tunisian coastline between February 2014 and August 2015 (Figure 1). All samples were hand-collected from the intertidal and shallow subtidal zones.

Samples were transported in seawater at low temperature to the laboratory, where they were washed thoroughly with seawater to remove debris. For each sample, a voucher specimen was prepared by pressing a single individual on an herbarium sheet with a subsample dried in silica gel for molecular analyses. Vouchers are housed in the Phycological Lab Herbarium (PhL) of the University of Messina, Italy (http://grbio.org/institution/phycological-lab-herbarium-university-messina). Additional samples included in the algal collections of PhL and of the Muséum National d'Histoire Naturelle in Paris, France (PC; Thiers, [continuously updated]) were also considered for comparison with Tunisian collections.

DNA was isolated from silica dried thalli as detailed in Manghisi et al. (2010) and the mitochondrial COI-5P region was PCR amplified as described in Saunders and McDevit (2012). Sequencing reactions were performed by Genoscope (Centre National

de Sequencage, www.genoscope.fr, Evry, France) or by an external company (Macrogen Europe, The Netherlands).

Specimen data, sequences and the primers used were deposited in the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org) and submitted to GenBank. Collection information together with BOLD and GenBank accession numbers are listed in supplementary Table S1.

Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and multiple sequence alignments were constructed in Seaview (v. 4.3.3, Gouy et al. 2010), including sequences of related taxa downloaded from GenBank (Benson et al. 2017). Sequence alignments were subjected to distance analyses with a Neighbor-Joining algorithm under a K2P model of nucleotide substitution (Kimura 1980) as performed in PAUP* 4b10 for the Macintosh (Swofford 2002) to visualize clusters of genetic species, and pairwise distances were computed as p-distances. In addition, COI-5P sequences were searched through the BOLD "identification" interface. The closest COI-5P sequences, either from the comparison with GenBank records or as identified in BOLD, were registered for each recognized genetic species with 95% identity regarded as a minimum cut-off value for recording a match.

Results and discussion

A total of 64 COI-5P sequences were generated resolving 26 genetic species groups (Figures 2-5, Figures S1-S18 available as supplementary material online). All presented trees are unrooted.

Of the 26 genetic species groups identified, four were newly reported for Tunisia: *Gastroclonium* sp., *Halymenia floresii* (Clemente) C. Agardh, *Ramicrusta* sp., and the alien species *Spermothamnion cymosum* (Harvey) De Toni.

Of the remaining 22 genetic species groups, two were alien species, *Hypnea cervicornis* J. Agardh and *Hypnea "cornuta"* (Kützing) J. Agardh and, in several instances, DNA analyses also highlighted cryptic diversity at the species level among the reports from the Mediterranean or other geographical regions.

Six records required a taxonomic update (*Champia* sp., *Corallina caespitosa* R.H. Walker, J. Brodie *et* L.M. Irvine, *Ellisolandia* sp., *Hypnea cervicornis, Palisada tenerrima* (Cremades) D. Serio, M. Cormaci, G. Furnari *et* F. Boisset, *Phyllophora* sp.) and two required a nomenclature update (*Chondracanthus acicularis* (Roth) Fredericq, *Pterocladiella capillacea* (S.G. Gmelin) Santelices *et* Hommersand).

Order CERAMIALES

Family Rhodomelaceae

1. *Digenea simplex* (Wulfen) C. Agardh 1822: 389

Basionym: *Conferva simplex* Wulfen 1803:17. Type locality: Trieste, Italy.

Collected specimen: Ksour Essef, 05/02/2014 (RM0001).

Reported in Tunisia by Meñez and Mathieson (1981).

Top match: *Digenea simplex*, 96.18% similarity (BOLD# GRHOD062-10).

Distance analysis including COI-5P sequences available in GenBank did not permit a molecular identification (Figure S1a), while the comparison with the BOLD database showed that the Tunisian sample grouped with isolates identified as *D. simplex* from Bermuda, Caribbean Netherlands, South Africa (Indian Ocean) and Japan (95.41-96.18% sequence identity; Figure S1b). Three subgroups were evident separating samples from the Atlantic, the Indian and Pacific Oceans, and the Mediterranean. The large sequence divergence and the geographical differences likely reflect cryptic diversity at the species level that requires further investigation with more data. Considering that the type locality of *D. simplex* is Trieste, Italy, Mediterranean samples

could be considered as typical.

2. *Palisada tenerrima* (Cremades) D. Serio, M. Cormaci, G. Furnari *et* F. Boisset 2010: 14

Basionym: *Laurencia tenerrima* J. Cremades in J. Cremades *et* J.L. Pérez-Cirera 1990: 490. Type locality: Almería, Cabo de Gata, Spain.

Collected specimens: Kerkennah, 20/04/2014 (RM0033), 01/06/2014 (RM0070); Port of Chebba, 27/04/2014 (RM0051); Chebba, 22/06/2014 (RM0081); Monastir, 03/08/2014 (RM0140).

Reported in Tunisia as *Laurencia papillosa* (C. Agardh) Greville by Meñez and Mathieson (1981).

Top match: *Laurencia ceylanica* J. Agardh (currently regarded as a synonym of *Chondrophycus ceylanicus* (J. Agardh) M.J. Wynne, Serio, Cormaci *et* G. Furnari), 95.45% similarity (BOLD# GRHOD263-10).

Distance analysis including COI-5P sequences available in GenBank (Figure S2) and the comparison with the BOLD database did not facilitate a molecular identification.

According to Furnari *et al.* (2002), Mediterranean entities previously identified as *Chondrophycus papillosus* (C. Agardh) Garbary *et* J. Harper [syn. *Laurencia papillosa*] should be attributed to *Chondrophycus tenerrimus* (J. Cremades) G. Furnari, F. Boisset, M. Cormaci *et* D. Serio, now *Palisada tenerrima* (J. Cremades) D. Serio, M. Cormaci, G. Furnari *et* F. Boisset.

Family Wrangeliaceae

3. *Spermothamnion cymosum* (Harvey) De Toni 1903: 1266

Basionym: *Callithamnion cymosum* Harvey 1855: 560. Type locality: Middleton Bay, King George Sound, Western Australia.

Collected specimen: Tabarka, 04/04/2015 (RM0209).

New report in Tunisia.

Top match: *Spermothamnion cymosum,* 99.54% similarity (BOLD# ABMMC7329-10).

Distance analysis including COI-5P sequences available in GenBank did not facilitate a molecular identification (Figure S3a), while the comparison with the BOLD database showed that the Tunisian specimen is conspecific with *S. cymosum* from Tasmania (Australia), the type area (99.54% sequence identity) (Figure S3b).

Spermothamnion cymosum is here reported for the first time in Tunisia. This species was only known from Australia (Huisman and Walker 1990, Millar and Kraft 1993, Womersley 1998) until it was reported in Venice Lagoon based on combined morphological and molecular data (Armeli Minicante 2013). We also report the presence of the species in Brittany, France.

The total absence of COI-5P sequences of Mediterranean species of *Spermothamnion*, as well as for all other species of this genus worldwide, makes it difficult to evaluate the actual distribution of the species in the Mediterranean, especially considering their minute size and the difficult diagnostic characters.

Order CORALLINALES

Family Corallinaceae

4. *Amphiroa beauvoisii* J.V. Lamouroux 1816: 299

Type locality: coast of Portugal.

Collected specimen: Monastir, 10/04/2015 (RM0254).

Reported in Tunisia by Meñez and Mathieson (1981).

Top match: *Amphiroa beauvoisii*, 99.54% similarity (BOLD# MANC023-13).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that the specimen is conspecific with

Amphiroa beauvoisii from North Carolina, USA (99.54% sequence identity; Figure S4a, b). In the distance tree built with GenBank sequences, the two isolates formed a group with samples from South Africa (both Atlantic and Indian Ocean), however they exhibited low sequence identity (93.96-94.58%), indicating that they are likely to be different species as hypothesized by Kogame et al. (2017) (Figure S4a). Other sequences are present in the BOLD database assigned to this name, which are not closely related each other, suggesting two distinct groups, both from the Atlantic coast of Panama, one of which includes also samples identified as *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux and one sample not identified at the specific level (Figure S4b). On the basis of data publicly available, it is not possible to state whether the other entities represent misidentifications or cryptic species. Morphological and molecular studies of this species are required, including samples from various regions, notably the type locality (Portugal), to assess which of the identified genetic species is the authentic. *Amphiroa beauvoisii* has been reported from various temperate and subtropical coasts of the world and it is common in the Mediterranean, including Tunisia (Meñez and Mathieson 1981).

5. *Amphiroa cryptarthrodia* Zanardini 1843: 43

Type locality: Dalmatia, Croatia.

Collected specimen: Port of Chebba, 27/04/2014 (RM0050).

Reported in Tunisia by Ben Maiz et al. (1987).

No match.

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database did not permit a molecular identification (Figure S4a).

6. *Amphiroa rigida* J.V. Lamouroux 1816: 297, pl. XI: fig. 1

Type locality: Mediterranean Sea.

Collected specimen: Tabarka, 30/07/2014 (RM0119).

Reported in Tunisia by Meñez and Mathieson (1981).

No match.

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database did not facilitate a molecular identification (Figure S4a, c). Three additional sequences are reported in the BOLD database with this name delineating two additional genetic groups, both containing specimens collected on the Atlantic coast of Panama (Figure S4c). The type locality of *A. rigida* is the Mediterranean Sea and, therefore, we maintain the specific attribution of the Tunisian sample. Data publicly available do not permit an assessment of whether the other entities represent misidentifications or cryptic species.

7. *Corallina caespitosa* R.H. Walker, J. Brodie *et* L.M. Irvine in Walker et al. 2009: 290, figs 3a-d, 4a, b, e, f, g

Type locality: Chit Rocks, Sidmouth, Devon, UK.

Collected specimens: Sousse, 01/08/2014 (RM0100), 12/04/2015 (RM0261); Monastir, 10/04/2015 (RM0253).

Presumably reported in Tunisia as *Corallina officinalis* Linnaeus by Meñez and Mathieson (1981)

Top match: *Corallina caespitosa*, 100% similarity (BOLD# COR080-12).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that the Tunisian specimen is conspecific with *Corallina caespitosa* (97.37-100% sequence identity). This grouping contains isolates from the North Atlantic, both from the European (UK, including the holotype

specimen, Portugal, Spain, France) and the USA coasts, from Atlantic Africa, the Mediterranean (Italy, Greece, France), and the Pacific (South Korea, Japan, Australia, USA; Figure 2).

Corallina caespitosa was described as a cryptic member of *C. officinalis* in the Atlantic European coasts with the support of molecular information (Walker et al. 2009). Subsequent DNA studies (Brodie et al. 2013, Hind and Saunders 2013, Pardo et al. 2015, Williamson et al. 2015) indicated that *C. caespitosa* is widespread in most oceans with samples recorded from Africa, Asia, Australia, Europe and North America. More investigations, perhaps with a finer molecular marker, are needed to assess the complex biogeography of this species and to understand if it is actually a cosmopolitan species or if it has been widely introduced around the globe.

In Tunisia and the wider Mediterranean Sea, two species of *Corallina* were reported (Meñez and Mathieson 1981, Bressan and Babbini 2003), *C. officinalis* and *Corallina elongata* J. Ellis *et* Solander, now *Ellisolandia elongata* (J. Ellis *et* Solander) K.R. Hind *et* G.W. Saunders. Reports of *C. officinalis* in Tunisia should be attributed to *C. caespitosa*, the former probably restricted to cool-temperate regions, predominantly in the North Atlantic and absent from the Mediterranean Sea (Brodie et al. 2013, Hind and Saunders 2013, Williamson et al. 2015).

8. *Ellisolandia* sp.

Collected specimen: Korbous, 08/04/2015 (RM0231).

Presumably reported in Tunisia as *Corallina elongata* by Meñez and Mathieson (1981).

Top match: *Corallina* sp., 99.30% similarity (BOLD# COR101-13).

Distance analysis including COI-5P sequences available in GenBank and a search of the BOLD database (Figure 2) indicated that this specimen is conspecific with an undetermined species of *Corallina* (99.12-99.3%) reported both along the

Mediterranean coast of Spain and in Portugal (Pardo et al. 2015). According to Pardo et al. (2015), this species does not match any of the species previously recorded from the Iberian Peninsula, and the Atlantic and Mediterranean isolates showed morphological differences. Reported variations were in the size of thalli, the branching pattern and the shape of intergenicula, with the Atlantic isolate resembling *E. elongata* and the Mediterranean one morphologically similar to *C. officinalis* (Pardo et al. 2015). Further collections would be required to fully ascertain this species' identity, but the considerable divergence of its DNA barcode indicates that this taxon is a cryptic member of *Corallina sensu lato* in Europe. Furthermore, it is possible that this entity may actually belong to the recently proposed genus *Ellisolandia* K.R. Hind *et* G.W. Saunders (2013).

At present, we cannot conclude whether *Ellisolandia* sp. recorded here is new for Tunisia or corresponds to previous reports misidentified as either *E. elongata* or *C. officinalis*.

9. *Jania adhaerens* J.V. Lamouroux 1816: 270

Type locality: Mediterranean Sea.

Collected specimens: Chebba, 22/06/2014 (RM0074); Zarzis, 30/08/2014 (RM0169).

Reported in Tunisia by Ben Maiz *et al.* (1987).

Top match: *Jania adhaerens,* 100% similarity (BOLD# BERMR281-10, GBMIN45316-15, GBMIN45315-15).

Distance analysis including COI-5P sequences available in GenBank showed that Tunisian samples are conspecific with an undetermined species of *Jania* from Sicily (Italy), Malta and the Indian Ocean coast of South Africa (99.81-100% sequence similarity; Figure 3). The comparison with the BOLD database showed that a representative Tunisian sample (RM0169), as well as the GenBank ones from Sicily and

Malta, are conspecific with *Jania adhaerens* from Western Australia and Bermuda (99.49-100%; Figure 4a). Other sequences are present in the BOLD database assigned to this name, which are not closely related each other, delineating two additional genetic groups, one from Japan and South Africa, and another from Western Australia (Figure 4a). The specific attribution of the Tunisian sample is strengthened by its alliance with sequences from the type locality, which is the Mediterranean Sea. With the publicly available data it is not possible to state whether the other entities represent misidentifications or cryptic species.

10. *Jania virgata* (Zanardini) Montagne 1846: 133

Basionym: *Corallina virgata* Zanardini 1839: 136. Type locality: Adriatic Sea.

Collected specimens: Tabarka, 04/04/2015 (RM0202); Korbous, 08/04/2015 (RM0232).

Reported in Tunisia by Meñez and Mathieson (1981).

Top match: Uncultured Corallinales, 100% similarity (GenBank# GQ917514).

Distance analysis including COI-5P sequences available in GenBank did not permit a genetic identification (Figure 3). The Tunisian samples grouped in a large assemblage with *Jania virgata* from Ragusa, Italy, an undetermined species from the French Riviera (GenBank# GQ917514), *Jania subulata* (Ellis *et* Solander) Sonder from Hawaii (as *Haliptilon subulatum*) and *Jania rosea* (Lamarck) Decaisne from South Africa (Indian Ocean).

Similarly, the comparison with the BOLD database (Figure 4b) showed that a representative Tunisian sample (RM0202) is allied with a large group including *J. subulata* from the Atlantic coast of Panama, from Caribbean Netherlands (type locality), South Africa (Indian Ocean), and Western Australia (the last three as *H. subulatum*). The group also includes an undetermined species of *Haliptilon* from South Africa

(Indian Ocean) and *Jania cubensis* Montagne ex Kützing (as *H. cubense*) from the Dutch Caribbean (94.38-97.51%).

Tunisian samples showed low sequence identity (94.38 %) with the topo-type isolate of *J. subulata* highlighting that the group could represent a species complex. N'Yeurt and Payri (2010) noted that the affinities between *J. subulata* and *J. rosea* remain unclear. The latter species was reported from Turkey (Taskin et al. 2008), but the authors stated that its presence in the Mediterranean Sea needs to be confirmed.

Jania subulata is considered as a cosmopolitan tropical and subtropical species, and was reported only for the Adriatic Sea in the Mediterranean in the middle of the 19th century (Kützing 1845, Zanardini 1847) as *H. subulatum* (J. Ellis *et* Solander) H.W. Johansen and later works did not report it (Furnari et al. 2003, Coll et al. 2010, Furnari et al. 2010). Moreover, the very common Mediterranean species *J. virgata* has not been sequenced. The Tunisian alga could be *J. virgata*, which is already reported for Tunisia (Meñez and Mathieson 1981). To define this species complex better, more data from other markers are needed.

Order GELIDIALES

Family Pterocladiaceae

11. *Pterocladiella capillacea* (S.G. Gmelin) Santelices *et* Hommersand 1997: 118, figs 1a, 2b, 3b, 4, 5

Basionym: *Fucus capillaceus* S.G. Gmelin 1768: 146. Type locality: Mediterranean Sea.

Collected specimens: Le Kram, 01/03/2014 (RM0005); Sidi bou Said, 25/04/2014 (RM0043); Sousse, 01/08/2014 (RM0099); Tabarka, 30/07/2014 (RM0125), 04/04/2015 (RM0198); Monastir, 03/08/2014 (RM0139).

Reported in Tunisia as *Pterocladia capillacea* (S.G. Gmelin) Bornet by Meñez and

Mathieson (1981).

Top match: *Pterocladia mexicana* W.R. Taylor (currently regarded as a synonym of *Pterocladiella capillacea*), 99.53% similarity (GenBank# KX427235).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database (Figure S5) showed that the specimens are conspecific with *Pterocladiella capillacea* (98.32-100% sequence identity) from different localities from the Indo-Pacific region (South Korea and Australia), Mexico (as *Pterocladia mexicana*, isotype specimen), Ecuador (as *Pterocladia robusta* W.R. Taylor, holotype specimen) and Brazil.

Tunisian samples allied also with unpublished sequences of Mediterranean samples from France (99.65-100%, data not shown). *Pterocladiella capillacea* is present along all Mediterranean coasts (type locality) and unpublished molecular analyses suggest that COI-5P sequences are useful to separate populations with a distinct geographical origin since Mediterranean samples (both Tunisian and unpublished isolates) are different from Asian and other samples (Ga Hun Boo, personal communication). At present, it is not possible to decide whether these differences will affect the taxonomy at the specific or intraspecific levels. Supplementary sequences and other molecular markers would be useful.

Order GIGARTINALES

Family Cystocloniaceae

12. *Hypnea cervicornis* J. Agardh 1851: 451-452

Type locality: Bahia State, Brazil.

Collected specimens: Tabarka, 04/04/2015 (RM0218).

Reported in Tunisia as *Hypnea* cf. *spinella* (C. Agardh) Kützing by Ben Maiz and Boudouresque (1986).

Top matches: *Hypnea flexicaulis* Y. Yamagishi *et* M. Masuda (currently regarded as a synonym of *H. cervicornis*), 100% similarity (GenBank# KP708661, KP708660, KP708659, KP708658); *Hypnea pannosa* J. Agardh, 100% similarity (BOLD# OZSEA1018-10, OZSEA1029-10).

From the comparison of the COI-5P sequence with published data available in GenBank (Figure S6a), the Tunisian isolate showed 100% identity with sequences of specimens of *Hypnea cervicornis* (as *H. flexicaulis*) from Western Australia and was allied with a larger group including isolates identified as *H. flexicaulis* from several localities in the Indo-Pacific region (Pacific Mexico, Hawaii, South Korea, Hong Kong, Taiwan, Indonesia, Philippines, Maldives, India) and from the Mediterranean lagoons of Venice (Italy) and Thau (France), isolates identified as *Hypnea boergesenii* T. Tanaka from Brazil and South Korea, and isolates of *H. cervicornis* from Brazil (including type locality) and Atlantic Panama (95.70-100% sequence identity).

The comparison with the BOLD database (Figure S6b) showed that the Tunisian sample allied with unpublished specimens of *H. pannosa* from Mexico (type area), New South Wales, Tasmania, Western Australia and South Korea, and an unnamed sample from Brazil (96.77-100%). The large sequence variation makes it doubtful that such a wide group may actually represent a single species. It is likely that the assemblage represents a cluster of cryptic species. BOLD sequences of *H. pannosa*, and related sample data, have not been released and, therefore, it is not yet possible to draw any taxonomic conclusions.

Hypnea cervicornis and *H. pannosa* are reported as sympatric in many regions and their segregation is based only on their habit (Xia and Wang, 1997). Whether the two species belong to the same taxon or not needs further study.

In the Mediterranean Sea, *H. cervicornis* was reported, as *H. flexicaulis,* in Venice Lagoon (Wolf et al. 2011), and considered as a recent introduction from Indo-Pacific

populations via ship traffic or shellfish transfers. However, the species had been listed before in the Mediterranean either as *H. cervicornis* (Djellouli et al. 2000, Zerzeri et al. 2010) or as *H. spinella* (Ben Maiz and Boudouresque 1986, Athanasiadis 1987, Zeybek et al. 1993, Furnari et al. 2003, Bottalico et al. 2016, Gallardo et al. 2016, Sghaier et al. 2016) on the basis of the synonymy proposed for them by Haroun and Prud'Homme van Reine (1993). However, the latter synonymy was recently rejected by de Jesus et al. (2016 and references therein).

Hypnea cervicornis occurs in temperate and tropical waters around the world and several DNA sequences are presently available, making it possible to identify populations with distinct geographycal origin and to speculate on their contemporary distributions (Geraldino et al. 2015, Nauer et al. 2015, de Jesus et al. 2016). The isolates in Venice and Thau lagoons (in Wolf et al. 2016 and the present paper) are likely introduced from a Korean lineage, in agreement with Geraldino et al. (2015). Conversely, the Tunisian isolate is allied with an Australian lineage, suggesting that multiple introductions occurred in the Mediterranean Sea by one or diverse vectors. This would suggest that all Mediterranean reports of *H. cervicornis* should be verified with DNA data in order to confirm their taxonomic identity (*H. cervicornis, H. spinella* or *H. pannosa*) and to understand their geographical origin (Indopacific or Atlantic lineages) in the context of biodiversity management.

13. *Hypnea "cornuta"* (Kützing) J. Agardh 1851: 449-450

Basionym: *Chondroclonium cornutum* Kützing 1849: 741. Type locality: Coast of Guinea.

Collected specimens: Gabes, 10/08/2014 (RM0155), 10/08/2015 (RM0288, RM0289, RM0292); Zarat, 15/08/2014 (RM0167).

Reported in Tunisia by Sghaier et al. (2016).

Top matches: *Hypnea cornuta*, 100% similarity (BOLD# ITRED091-13, ITRED090-13, ITRED089-13, ITRED088-13, ITRED087-13, ITRED086-13, ITRED085-13, ITRED084-13, ITRED083-13, ITRED081-13, ITRED080-13).

From the comparison of the COI-5P sequences with published data available in GenBank (Figure S7a) and comparison with the BOLD database (Figure S7b), the Tunisian isolates are conspecific with *Hypnea "cornuta"* from Sicily (Lake Ganzirri and Torre Faro), Italy (100% sequence identity). It is noteworthy that two additional samples named as *H. "cornuta"* are present in the distance analyses, which are not closely related to the entity from Tunisia and Sicily, one from Korea (83.84% sequence identity; Figure S7a), and another from Western Australia (92.39% sequence identity; (Figure S7b). The type locality of *H. cornuta* is Guinea (eastern Atlantic Ocean) and a comparison with the type specimen or with samples from the type locality is needed to draw conclusions on their taxonomic affinities.

In both distance analyses, the sequences of the Tunisian and Sicilian samples were allied with two species both listed as *Hypnea stellulifera* (J. Agardh) Yamagishi *et* Masuda, one from Brazil and the other from the type area in the Philippines (85.34-96.39%). The three allied taxa showed clear barcoding gaps between each other proving that they are three well distinguished species.

Hypnea cornuta was recently reported in Tunisia as a potentially invasive species (Sghaier et al. 2016). It was also reported from various localities in the world, from temperate to tropical areas in both the Atlantic and Indo-pacific basins (Guiry and Guiry 2017), and in the Mediterranean Sea (Manghisi et al., 2011, and reference therein).

The origin of the Tunisian and Sicilian entity is uncertain. It might be introduced as a Lessepsian migrant, as also speculated by Verlaque et al. (2015), or by a secondary introduction from the eastern Mediterranean basin (Manghisi et al., 2011). Zenetos et al. (2010) listed it as established in both Central and Eastern Mediterranean basins, but

more recently, Verlaque et al. (2015) noted that caution should be used while discussing both the distribution and the means of introduction of *Hypnea* species because of the difficulties in identification.

14. *Hypnea musciformis* (Wulfen) J.V. Lamouroux 1813: 43

Basionym: *Fucus musciformis* Wulfen 1791: 54. Type locality: Trieste, Italy.

Collected specimens: Le Kram, 01/03/2014 (RM0007); Sousse, 01/08/2014 (RM0097, RM0098), 12/04/2015 (RM0271), 13/08/2015 (RM0295).

Reported in Tunisia by Meñez and Mathieson (1981).

Top matches: *Hypnea musciformis*, 100% similarity (BOLD# ITRED082-13, GenBank# GQ141881, KT428779).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that Tunisian specimens are included in a large group of isolates identified as *Hypnea musciformis* (96.31-100%) (Figure S8a, b). From the comparison of the two distance trees, the wide assemblage comprised four lineages: one with samples from the Mediterranean (Spain, France and Italy including Venice lagoon, which is in the type area) and the Atlantic Ocean (Namibia, North Carolina and Florida, USA) (Figure S8a, b); the second with an isolate from the Dominican Republic (Figure S8a); the third with samples from tropical central America, both Atlantic (Mexico, Colombia, Barbados and Caribbean Netherlands) and Pacific (Hawaii) (Figure S8a, b); the fourth with an isolate from Texas (as *Hypnea* sp. *"1musciformis"*, Figure S8b). It is not possible to infer whether the second and fourth lineages actually represent distinct species or not because COI-5P sequence of the Texan sample is not public. Based on the observed large sequence divergence, the group may represent a complex of closely related species rather than a single cosmopolitan taxon.

Hypnea musciformis is a common species native for the Mediterranean Sea and is widely reported in warm and temperate seas (Guiry and Guiry 2017).

Family Gigartinaceae

15. *Chondracanthus acicularis* (Roth) Fredericq in Hommersand, Guiry, Fredericq *et* Leister 1993: 117

Basionym: *Ceramium aciculare* Roth 1806: 114. Type locality: Adriatic Sea.

Collected specimen: Korbous, 08/04/2015 (RM0233).

Reported in Tunisia as *Gigartina acicularis* (Roth) J.V. Lamouroux by Meñez and Mathieson (1981).

Top matches: *Chondracanthus acicularis*, 100% similarity (BOLD# GIGSP005-10, REDEU161-11).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that the specimen is conspecific with *Chondracanthus acicularis* from various localities along the Mediterranean coast of France and Spain, the Atlantic (France, North Carolina, Brazil) and Indian Oceans (Western and South Australia) (99.21-100% sequence identity) (Figure S9a, b). Other sequences are present in the BOLD database assigned to this name (as *C. acicularis* and *Chondracanthus* sp. *"2acicularis"*, Figure S9b), but they closely ally with *Chondracanthus saundersii* C.W. Schneider *et* C.E. Lane, which was described on samples previously known in Bermuda and much of the western Atlantic as *C. acicularis* or *Chondracanthus teedei* (Mertens ex Roth) Kützing (Schneider and Lane 2005).

Family Phyllophoraceae

16. *Phyllophora* sp.

Collected specimen: Tabarka, 04/04/2015 (RM0184).

Reported in Tunisia as *Phyllophora crispa* (Hudson) P.S. Dixon by Meñez and Mathieson (1981).

Top match: *Phyllophora* sp., 99.39% identity (BOLD# ABMMC1750-07).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that the specimen is conspecific with an undetermined species of *Phyllophora* from Catalonia, Spain (99.39%) (Figure S10a, b). The species is closely related to Atlantic samples isolated from France, Ireland, Norway, Spain of *Phyllophora crispa* (96.88-97.95% sequence identity), whose type locality is England.

The Spanish entity had already been identified as a cryptic species by a phylogenetic study of the family Phyllophoraceae (Le Gall and Saunders 2010). Considering that *P. crispa* is widely reported in the Mediterranean Sea (Guiry and Guiry 2017), further sampling, a careful morphological revision and DNA sequencing of additional markers are necessary to assess the actual distribution of the two cryptic taxa, together with a revision of all synonyms of *P. crispa*.

17. *Schottera nicaeensis* (J.V. Lamouroux ex Duby) Guiry *et* Hollenberg 1975: 153, figs 4-9, 11-15

Basionym: *Halymenia nicaeensis* J.V. Lamouroux ex Duby 1830: 942. Type locality: Marseilles, France.

Collected specimens: Korbous, 08/04/2015 (RM0230, RM0236).

Reported in Tunisia by Ben Maiz et al. (1987).

Top matches: *Schottera nicaeensis*, 99.85% similarity (BOLD# REDEU463-11, REDEU555-11, REDEU579-11, REDEU613-11).

Distance analysis including COI-5P sequences available in GenBank (Figure S11a) and the comparison with the BOLD database (Figure S11b) showed that the specimens are conspecific with Mediterranean samples of *Schottera nicaeensis* from the Riviera, France, close to the type locality, and Catalonia, Spain (99.54-99.85% sequence identity). They are closely allied with an Atlantic French population from Brittany, also identified as *S. nicaeensis*, or with the unpublished name *Schottera palmettoides*, presumably based on *Phyllophora palmettoides* J. Agardh, in some BOLD records (98.11-98.22%) (Figure S11a, b). In the distance analysis, a third genetic species named as *S. nicaeensis* from Victoria, Australia, placed in a distant branch (Figure S11a, b). Le Gall and Saunders (2010) in a DNA barcoding survey of Canadian Phyllophoraceae, highlighted that the genus *Schottera* is not monophyletic and included divergent specimens identified as *S. nicaeensis*, advocating the need to further investigate this widespread genus.

18. *Sphaerococcus coronopifolius* Stackhouse 1797: xxiv

Type locality: Cornwall, England.

Collected specimens: Tabarka, 30/07/2014 (RM0113), 04/04/2015 (RM0181, RM0182, RM0196, RM0212).

Reported in Tunisia by Meñez and Mathieson (1981).

Top matches: *Sphaerococcus coronopifolius*, 98.66% similarity (BOLD# REDEU478-11, REDEU499-11).

Distance analysis including COI-5P sequences available in GenBank (Figure S12a) did not facilitate a molecular identification, while the comparison with the BOLD database (Figure S12b) showed that a representative Tunisian sample (RM0212) is allied with *Sphaerococcus coronopifolius* (97.40-98.66%). The sample is placed in a large group

with others from the Atlantic French coast (type area), and from the Mediterranean, namely from France, Spain and Croatia (Figure S12b).

Based on the observed sequence variation the assemblage might represent a cluster of cryptic species, with the Tunisian samples distinct from all others. It is unfortunate that BOLD sequences, and related sample data, are not public and, therefore, it is not yet possible to draw any taxonomic conclusions.

Order HALYMENIALES

Family Halymeniaceae

19. *Grateloupia filicina* (J.V. Lamouroux) C.Agardh 1822: 223

Basionym: *Delesseria filicina* J.V. Lamouroux 1813: 125. Type locality: Trieste, Italy.

Collected specimen: Port of Chebba, 27/04/2014 (RM0054).

Reported in Tunisia by Djellouli et al. (2000).

Top match: *Grateloupia* sp., 99.67% similarity (BOLD# REDEU254-11).

Distance analysis showed that the Tunisian sample is conspecific with *Grateloupia filicina* from Asturias, Spain (GenBank# KP271165, 99.62% sequence identity) (Figure S13a), while the comparison with the BOLD database showed it allying with an unidentified species from Brittany, France (98.85-99.67%) (Figure S13b). Other sequences are present in the BOLD database assigned to this name, which are not closely related each other, delineating two additional genetic species, one from China and another from South Africa (Figure S13b). The specific attribution of the Tunisian sample is strengthened by *rbc*L sequences analysis (Morabito unpublished) including other samples from the Mediterranean Sea. In light of data publicly available, it is not possible to state whether the other entities represent misidentifications or cryptic species.

G. filicina is widely distributed in the Mediterranean (Guiry and Guiry 2017) and it was recently reported in Tunisia (Djellouli et al. 2000).

20. *Halymenia floresii* (Clemente) C. Agardh 1817: xix

Basionym: *Fucus floresii* Clemente 1807: 312 (as *'floresius'*). Type locality: Sanlúcar de Barrameda, Cádiz, Spain.

Collected specimens: Kerkennah, 01/06/2014 (RM0062); Gabes, 10/08/2015 (RM0281).

Presumably new report.

Top matches: *Halymenia floresii*, 100% similarity (BOLD# REDEU726-11, REDEU709-11).

In distance analysis including COI-5P sequences available in GenBank Tunisian samples allied with *Halymenia floresii* from Portugal, close to type locality, but with low sequence identity (97.57-97.72%) (Figure S13a). In the comparison with the BOLD database two lineages were present, one with specimens from the Mediterranean coast of France and Portugal, the other with specimens from the Mediterranean coasts of France and Tunisia (97.54-100%) (Figure S13c). The taxonomic status of the two lineages should be investigated with other genetic markers.

Halymenia floresii is widely distributed in the Mediterranean Sea (Guiry and Guiry 2017). In Tunisia, Ben Maiz et al. (1987) reported an unidentified *Halymenia* species without any description, making it impossible to assess whether it is conspecific to *H. floresii* reported here. Furthermore, an unidentified *Halymenia* species was also recorded by Ben Mustapha and Afli (2007) described as having a *"thalle cartilagineux à surface «glissante»"* (cartilagineous thallus with a slippery surface) and, therefore, clearly different from *H. floresii.*

Order PEYSSONNELIALES

Family Peyssonneliaceae

21. *Peyssonnelia rubra* (Greville) J. Agardh 1851: 502

Basionym: *Zonaria rubra* Greville 1827: 340-341. Type locality: Ionian Islands, Greece.

Collected specimens: Chebba, 27/04/2014 (RM0052); Tabarka 30/07/2014 (RM0116).

Reported in Tunisia by Meñez and Mathieson (1981).

Top matches: *Peyssonnelia rubra*, 100% similarity (BOLD# REDEU538-11, REDEU149-11, ABMMC11219-10).

Distance analysis including COI-5P sequences available in GenBank did not permit a molecular identification (Figure S14a). The comparison with the BOLD database showed that a representative Tunisian sample (RM0052) is conspecific with *Peyssonnelia rubra* (99.85-100%), in a group of Mediterranean samples from Greece (type area), France, Italy and Croatia (Figure S14b).

22. *Peyssonnelia squamaria* (S.G. Gmelin) Decaisne *ex* J. Agardh 1842: 93

Basionym: *Fucus squamarius* S.G. Gmelin 1768: 171. Type locality: Mediterranean Sea.

Collected specimens: Tabarka, 30/07/2014 (RM0123), 04/04/2015 (RM0180); Monastir, 03/08/2014 (RM0137).

Reported in Tunisia by Meñez and Mathieson (1981).

Top matches: *Peyssonnelia squamaria*, 99.85% similarity (BOLD# REDEU189-11, REDEU473-11, REDEU588-11).

Distance analysis including COI-5P sequences available in GenBank and the comparison with the BOLD database showed that the specimen is conspecific with

Peyssonnelia squamaria (98.77-99.85%) in a group including Mediterranean (type locality) samples from Croatia, Italy and France (Figures S14a, c).

23. *Ramicrusta* sp.

Collected specimen: Tabarka, 30/07/2014 (RM0115).

New report in Tunisia.

No match.

Distance analysis including COI-5P sequences available in GenBank as well as the comparison with the BOLD database did not facilitate a molecular identification at the species level, but clearly showed an alliance with the holotype specimen of *Ramicrusta australica* K.R. Dixon (91.13% sequence identity) and other species of *Ramicrusta* D.R. Zhang and J.H. Zhou from the Pacific Ocean (Vanuatu, China, Philippines, Australia) and the Caribbean Sea (Bonaire, Jamaica) (Figure 5).

This is the first report of a species of *Ramicrusta* in the Mediterranean and the second in a temperate sea. It is also present along the French Riviera (Le Gall unpublished data). *Ramicrusta* is likely an overlooked genus, described at first as monospecific from South China Sea (Zhang and Zhou 1981) and later expanded with the description of other species with a potentially widespread tropical distribution, both in the Pacific Ocean and the Caribbean Sea (Pueschel and Saunders 2009, Dixon and Saunders 2013, Ballantine et al. 2016). The only known temperate species is *R. australica* (Dixon and Saunders 2013) the closest ally of the Tunisian entity here reported.

Whether the Tunisian taxon is a new species or a *Peyssonnelia* species that should be combined to the genus *Ramicrusta,* will be better investigated after a larger sampling of species of Peyssonneliaceae in the Mediterranean and the use of an integrated taxonomic approach.

Order RHODYMENIALES

Family Champiaceae

24. *Champia* sp.

Collected specimen: Gabes, 10/08/2015 (RM0282).

Reported in Tunisia as *Champia parvula* (C. Agardh) Harvey by Meñez and Mathieson (1981).

No match.

Distance analysis including COI-5P sequences available in GenBank as well as the comparison with the BOLD database did not permit a molecular identification (Figures S15-16).

In the Mediterranean Sea, the genus *Champia* is represented by *Champia parvula* (Coll et al. 2010) which was also reported for Tunisia (Meñez and Mathieson 1981) and by the recently added *Champia compressa* Harvey (Moussa et al. 2015).

A COI-5P sequence of a specimen of *C. parvula* from the type locality (Cadiz, Spain) is represented in the distance analysis with sequences from GenBank and it is distantly related to the Tunisian sample (89.50% sequence identity) (Figure S15).

Specimens identified as *C. parvula* both in GenBank (Hawaii, Figure S15) and in BOLD (France, Figure S16) highlighted the presence of cryptic diversity at the species level under this taxon name that should be further investigated.

Champia parvula is presently considered a cosmopolitan species although its wide distribution has been questioned by the segregation of new species and variety using morphological characteristics and genetic sequencing (Reedman and Womersley 1976, Lozada-Troche and D.L. Ballantine 2010, Suzuki et al. 2013, Koh et al. 2013, Griffith et al. 2017, Koh et al. 2013). Consequently, it is likely that the "cosmopolitan" *C. parvula* represents a species complex, including further cryptic species yet to be discovered.

25. *Gastroclonium* sp.

Collected specimen: Korbous, 08/04/2015 (RM0223).

New report in Tunisia.

Top match: *Gastroclonium reflexum* (Chauvin) Kützing, 98.92% similarity (BOLD# REDEU618-11).

Distance analysis including COI-5P sequences available in GenBank did not facilitate a molecular identification (Figure S15), however the comparison with the BOLD database (Figure S17) showed that the Tunisian sample is conspecific with *Gastroclonium* sp. (as *G. reflexum*) from the Mediterranean French coast (98.92% sequence identity).

In both distance trees (with GenBank and BOLD sequences) another entity is represented under the name *G. reflexum*, including samples from the French Atlantic coast (type area) and from Atlantic Spain (Figures S15, S17). This is likely the authentic species and is only distantly related to the Tunisian taxon (86.56-86.59%).

G. reflexum was reported from various localities in the world including the Mediterranean Sea (Guiry and Guiry 2017) although not reported along Tunisian coast. Further sampling and morphological and molecular observations are necessary to assess the actual specific identity of the Tunisian sample.

Family Rhodymeniaceae

26. *Rhodymenia ardissonei* (Kuntze) Feldmann 1937: 274.

Basionym: *Palmaria ardissonei* Kuntze 1891: 909. Type locality: Porto Maurizio, Ligurian Sea, Italy.

Collected specimen: Zarat, 15/08/2014 (RM0165).

Reported in Tunisia by Ben Maiz et al. (1987).

Top matches: *Rhodymenia ardissonei*, 99.84% similarity (BOLD# REDEU734-11, REDEU447-11, REDEU571-11, REDEU531-11).

Distance analysis including COI-5P sequences available in GenBank did not permit a molecular identification (Figure S18a) while the comparison with the BOLD database (Figure S18b) showed that the Tunisian sample is conspecific with *Rhodymenia ardissonei* from the French Riviera (type area) (99.52-99.84% sequence identity).

Conclusions

In the present paper, we conducted a molecular-assisted alpha taxonomy (MAAT) survey of the macroalgal diversity of Tunisia. This allowed us to reveal cryptic species, allochthonous introductions and to identify problematic taxa. This is the first report of the presence of the alien species, *Spermothamnion cymosum* (Harvey) De Toni, collected in Tabarka, close to a tourist harbour. We confirm the presence of two additional alien species, *Hypnea cervicornis* J. Agardh and *Hypnea "cornuta"* (Kützing) J. Agardh, collected at Gabes and Zarat, close to commercial and fishing harbours. We also highlight the cryptic diversity among the Mediterranean reports of this taxon.

At the best of our knowledge, this is the first study dealing with macroalgal taxonomy in Tunisia using a DNA approach.

As opposed to morphological identifications, MAAT methods are more useful tools for a first approach towards screening and uniting biological specimens in genetic groups as a first step to assigning them to species and genera (Saunders 2005). This methodology is functional in organisms with simple morphologies, a high degree of phenotypic plasticity or convergence and heteromorphic life histories, like marine macroalgae (e.g., Le Gall and Saunders 2010, Manghisi et al. 2015, Filloramo and Saunders 2016).

The introduction of new invasive species is a crucial factor that will continue to change the biodiversity of the Mediterranean Sea (Cecere et al. 2016). In future, the situation

could become significantly changed by to the recent enlargement of the Suez Canal. This is currently is considered one of the main vectors of introduction of alien species (Galil et al. 2015). Aquaculture is also a key vector of introduction from cold to temperate regions of the North Pacific Ocean (Verlaque et al. 2007, Kim et al. 2014) along with maritime and coastal development and other anthropogenic activities (Ben Mustapha and Afli 2007).

Records of alien marine species from Tunisia are sparse and scattered because of coastline topography and lack of research programs focusing on the subject (Sghaier et al. 2016). The Tunisian coast, particularly the Gulf of Gabes region, suffers the consequences of the above-mentioned disturbances. Hotspots for marine species introduction include harbours where human activities affect the health of ecosystems and facilitate the introduction and the secondary dispersal of alien species (Occhipinti-Ambrogi et al., 2011, Kim et al. 2014).

MAAT surveys contribute to aggregate new records in a DNA library of life such as the BOLD system catalogue, amplifying the biodiversity knowledge linked to geographical information and making them freely available to the scientific community. Their added value in comparison to a classic floristic list is that DNA barcodes are permanent labels assigned to specimens regardless of any subsequent taxonomic or nomenclature variation.

An effective monitoring of the biodiversity changes by means of a quick and accurate tool, such as DNA barcoding, is essential to provide the basis for an environmental management.

Acknowledgements

The Authors are indebted to Drs. Sung Min Boo and Ga Hun Boo for discussions about *Pterocladiella* and for sharing unpublished data, and to Dr. Gary W. Saunders for sharing valuable information about *Ramicrusta*.

Dr. Donatella Serio is acknowledged for discussing the *Laurencia* complex.

The authors are grateful to Dr. Robert J. Wilkes for the linguistic check of the manuscript.

Two anonymous referees and prof. Matthew J. Dring are kindly acknowledged for their valuable suggestions to improve the manuscript.

This study was supported by grants to R.M. (Emmag Erasmus Mundus fellowship) and to G.G. and M.M. (INNOVAQUA PON02_00451_3362185).

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Legend of figures

Figure 1: Collection sites along the Tunisian coastline.

Figure 2: Molecular-assisted identification of *Corallina caespitosa* R.H. Walker, J. Brodie et L.M. Irvine and *Ellisolandia* sp. Neighbor-Joining subtree of COI-5P sequences of Corallinales downloaded from GenBank. Sequences generated in the present work are in coloured bold type, where different colours indicate distinct genetic species. Each clade including Tunisian samples and interpreted here as a genetic species has branches of the same colour of included samples. Scale bar: substitutions per site.

Figure 3: Molecular-assisted identification of *Jania adhaerens* J.V. Lamouroux and *Jania virgata* (Zanardini) Montagne. Neighbor-Joining subtree of COI-5P sequences of Corallinales downloaded from GenBank. Other details as in Fig. 2.

Figure 4: Molecular-assisted identification of *Jania adhaerens* J.V. Lamouroux and *Jania virgata* (Zanardini) Montagne. a) Subtree obtained through the "identification" interface in the Barcode of Life Datasystem (BOLD) for *J. adhaerens*; b) subtree obtained through the "identification" interface in BOLD for *J. virgata*. Other details as in Fig. 2.

Figure 5: Molecular-assisted identification of *Ramicrusta* sp. Neighbor-Joining tree of COI-5P sequences of Peyssonneliales downloaded from GenBank. Other details as in Fig. 2.

Legends of supplementary figures

Figure S1: Molecular-assisted identification of *Digenea simplex* (Wulfen) C. Agardh. a) Neighbor-Joining subtree of COI-5P sequences of Ceramiales downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Sequences generated in the present work are in coloured bold type, where different colours indicate distinct genetic species. Each clade including Tunisian samples and interpreted here as a genetic species has branches of the same colour of included samples. Scale bar: substitutions per site.

Figure S2: Molecular-assisted identification of *Palisada tenerrima* (Cremades) D. Serio, M. Cormaci, G. Furnari et F. Boisset. Neighbor-Joining subtree of COI-5P sequences of Ceramiales downloaded from GenBank. Other details as in Fig. S1.

Figure S3: Molecular-assisted identification of *Spermothamnion cymosum* (Harvey) De Toni. a) Neighbor-Joining subtree of COI-5P sequences of Ceramiales downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S4: Molecular-assisted identification of *Amphiroa beauvoisii* J.V. Lamouroux, *Amphiroa cryptarthrodia* Zanardini and *Amphiroa rigida* J.V. Lamouroux. a) Neighbor-Joining subtree of COI-5P sequences of Corallinales downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem of *A. beauvoisii*; c) subtree obtained through the "identification" interface in the Barcode of Life Datasystem of *A. rigida*. Other details as in Fig. S1.

Figure S5: Molecular-assisted identification of *Pterocladiella capillacea* (S.G. Gmelin) Santelices et Hommersand. Neighbor-Joining subtree of COI-5P sequences of Gelidiales downloaded from GenBank. Other details as in Fig. S1.

Figure S6: Molecular-assisted identification of *Hypnea cervicornis* J. Agardh. a)

Neighbor-Joining subtree of COI-5P sequences of *Hypnea* downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S7: Molecular-assisted identification of *Hypnea "cornuta"* (Kützing) J. Agardh. a) Neighbor-Joining tree of COI-5P sequences of *Hypnea* downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life. Other details as in Fig. S1.

Figure S8: Molecular-assisted identification of *Hypnea musciformis* (Wulfen) J.V. Lamouroux. a) Neighbor-Joining tree of COI-5P sequences of *Hypnea* downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S9: Molecular-assisted identification of *Chondracanthus acicularis* (Roth) Fredericq in Hommersand, Guiry, Fredericq et Leister. a) Neighbor-Joining subtree of COI-5P sequences of Gigartinaceae downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S10: Molecular-assisted identification of *Phyllophora* sp. a) Neighbor-Joining tree of COI-5P sequences of Phyllophoraceae downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S11: Molecular-assisted identification of *Schottera nicaeensis* (J.V. Lamouroux ex Duby) Guiry et Hollenberg. a) Neighbor-Joining subtree of COI-5P sequences of Phyllophoraceae downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S12: Molecular-assisted identification of *Sphaerococcus coronopifolius* Stackhouse. a) Neighbor-Joining tree of COI-5P sequences of Phyllophoraceae

downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S13: Molecular-assisted identification of *Grateloupia filicina* (J.V. Lamouroux) C. Agardh and *Halymenia floresii* (Clemente) C. Agardh. a) Neighbor-Joining tree of COI-5P sequences of Halymeniales downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem (BOLD) for *G. filicina*; c) subtree obtained through the "identification" interface in BOLD for *H. floresii*. Other details as in Fig. S1.

Figure S14: Molecular-assisted identification of *Peyssonnelia rubra* (Greville) J. Agardh, *Peyssonnelia squamaria* (S.G. Gmelin) Decaisne *ex* J. Agardh. a) Neighbor-Joining tree of COI-5P sequences of Peyssonneliales downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem (BOLD) for *P. rubra*; c) subtree obtained through the "identification" interface in BOLD for *P. squamaria*. Other details as in Fig. S1.

Figure S15: Molecular-assisted identification of *Champia* sp. and *Gastroclonium* sp. Neighbor-Joining tree of COI-5P sequences of Champiaceae downloaded from GenBank. Other details as in Fig. S1.

Figure S16: Molecular-assisted identification of *Champia* sp. Tree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

Figure S17: Molecular-assisted identification of *Gastroclonium* sp. Subtree obtained through the "identification" interface in the Barcode of Life Datasystem for *Gastroclonium* sp. Other details as in Fig. S1.

Figure S18: Molecular-assisted identification of *Rhodymenia ardissonei* (Kuntze) Feldmann. a) Neighbor-Joining subtree of COI-5P sequences of Rhodymeniaceae downloaded from GenBank; b) subtree obtained through the "identification" interface in the Barcode of Life Datasystem. Other details as in Fig. S1.

	Collection site	Latitude	Longitude
1	Tabarka	36.9579 N	8.7554 E
2	Sidi Bou Saïd	36.8664 N	10.3501 E
3	Le Kram	36.8305 N	10.3188 E
4	Korbous	36.8279 N	10.5691 E
5	Sousse	35.8335 N	10.6417 E
6	Monastir	35.7737 N	10.8377 E
7	Ksour essef	35.4190 N	11.0406 E
8	Chebba	35.2421 N	11.1392 E
9	Port of Chebba	35.2308 N	11.1629 E
10	Kerkennah	34.6622 N	11.1222 E
11	Gabes	33.8918 N	10.1188 E
12	Zarat	33.6901 N	10.3819 E
13	Zarzis	33.5159 N	11.1178 E

1

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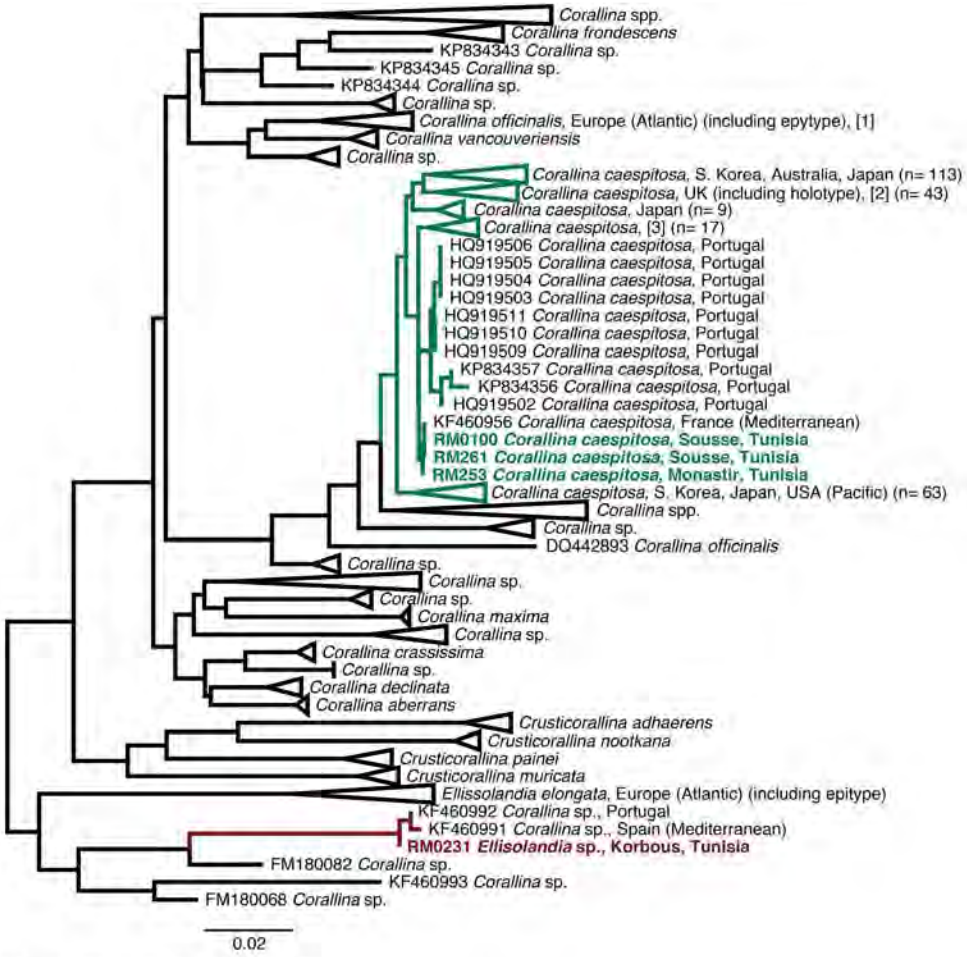

[1]: N. America (Atlantic and Pacific), Asia
[2]: Portugal, Spain (Atlantic), France (Atlantic), Portugal, USA (Atlantic), Japan.
[3]: France (Atlantic), Portugal, Spain (Atlantic), Africa (Atlantic), Italy, Greece

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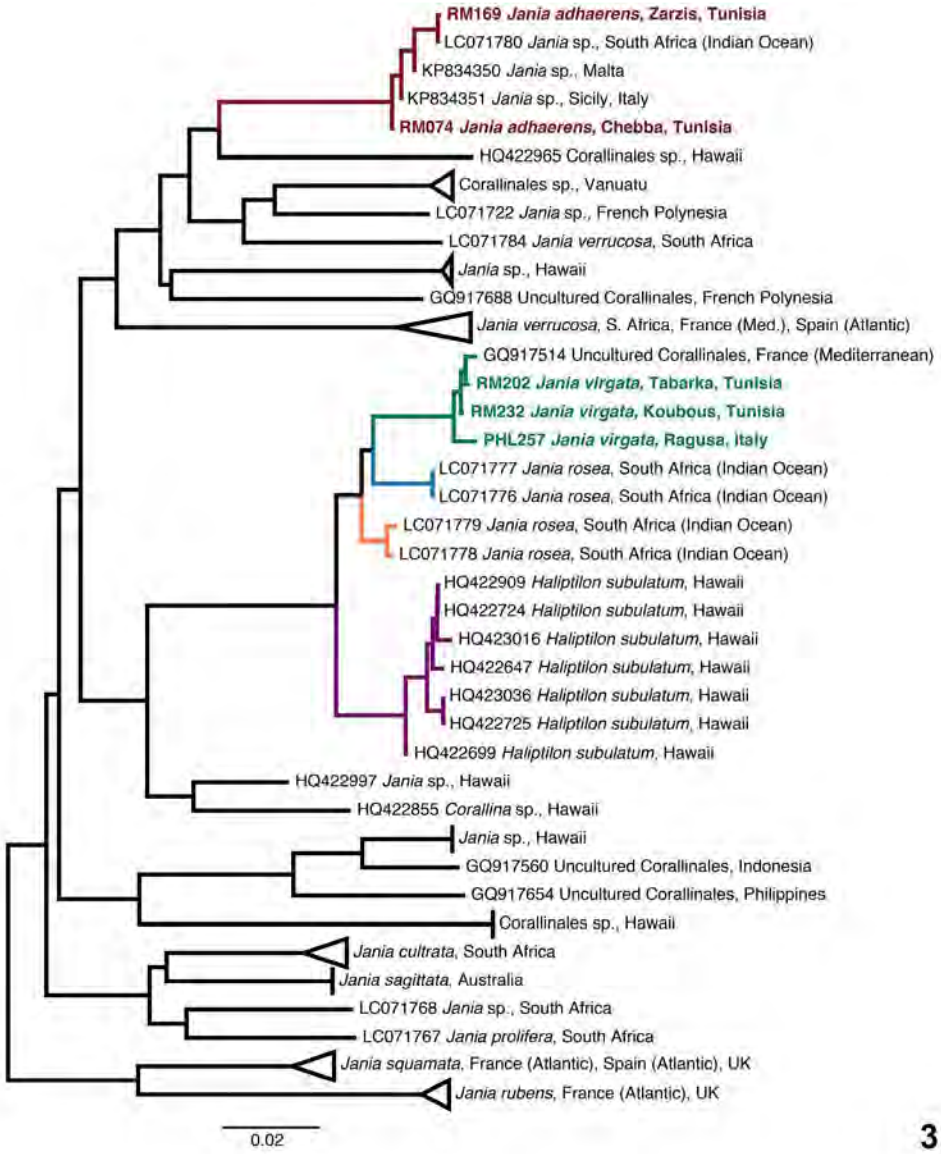

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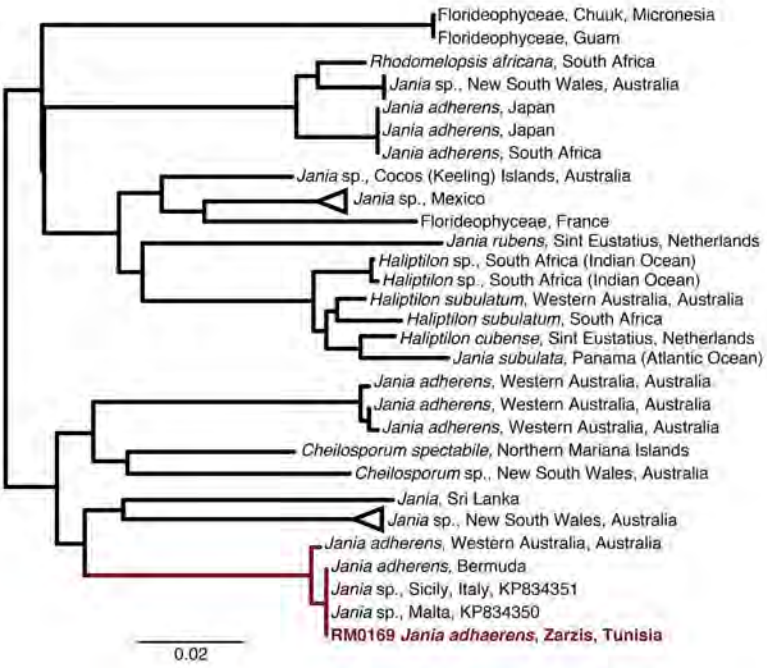

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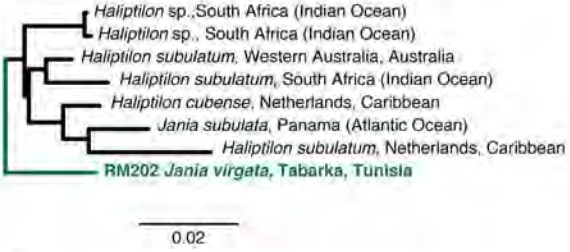

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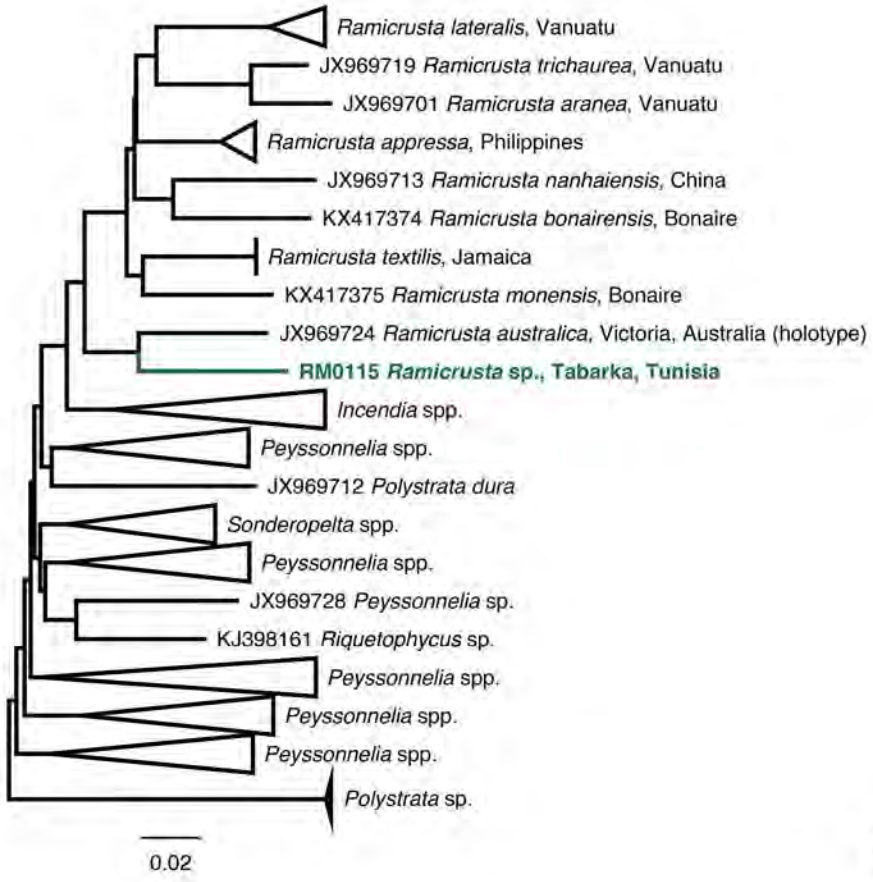

5

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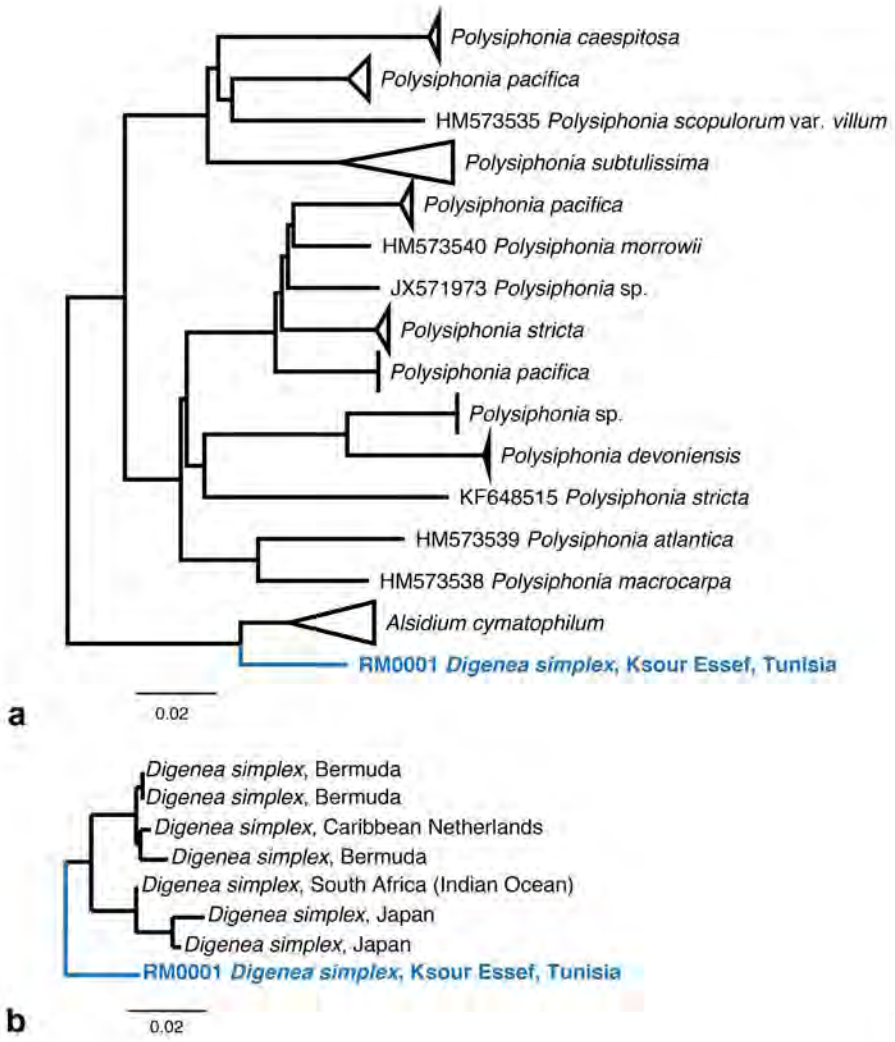
Table S1. Collection information of samples used in this study together with Barcode of Life Datasystem (BOLD) and Genbank (GB) accession numbers.

Order	Family	Species	Sample ID	BOLD ID	GB ID	Collection Date	Collection Site
Ceramiales	Rhodomelaceae	*Digenea simplex*	RM0001	TURED010-17	MG030761	05/02/14	Ksour Essef, Tunisia
		Palisada tenerrima	RM0033	TURED030-17	MG030786	20/04/14	Kerkennah, Tunisia
			RM0070	TURED031-17	MG030784	01/06/14	Kerkennah, Tunisia
			RM0051	TURED032-17	MG030785	27/04/14	Port of Chebba, Tunisia
			RM0081	TURED033-17	MG030782	22/06/14	Chebba, Tunisia
			RM0140	TURED034-17	MG030783	03/08/14	Monastir, Tunisia
			PhL686	REAPP001-17	MF544099	08/10/09	Torre Faro, Messina, Italy
	Wrangeliaceae	*Spermothamnion cymosum*	RM0209	TURED054-17	MG030803	04/04/15	Tabarka, Tunisia
			LLG5051	TURED055-17	MG030804	08/07/14	Finistère, Brittany, France
			SAM390	REDVE004-17	MF597664	04/10/10	Isola della Certosa, Venice Lagoon, Italy
Corallinales	Corallinaceae	*Amphiroa beauvoisii*	RM0254	TURED001-17	MG030753	10/04/15	Monastir, Tunisia
		Amphiroa cryptarthrodia	RM0050	TURED002-17	MG030754	27/04/14	Port of Chebba, Tunisia
		Amphiroa rigida	RM0119	TURED003-17	MG030755	30/07/14	Tabarka, Tunisia
		Corallina caespitosa	RM0100	TURED006-17	MG030759	01/08/14	Sousse, Tunisia
			RM0261	TURED007-17	MG030758	12/04/15	Sousse, Tunisia
			RM0253	TURED008-17	MG030760	10/04/15	Monastir, Tunisia
		Ellisolandia sp.	RM0231	TURED009-17	MG030762	08/04/15	Korbous, Tunisia
		Jania adhaerens	RM0074	TURED028-17	MG030778	22/06/14	Chebba, Tunisia
			RM0169	TURED029-17	MG030779	30/08/14	Zarzis, Tunisia
		Jania virgata	RM0202	TURED013-17	MG030780	04/04/15	Tabarka, Tunisia
			RM0232	TURED014-17	MG030781	08/04/15	Korbous, Tunisia
			PhL257	ITRED043-11	MG191361	21/03/10	Ragusa, Italy
Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	RM0005	TURED041-17	MG030794	01/03/14	Le Kram, Tunisia

Order	Family	Species	Sample ID	BOLD ID	GB ID	Collection Date	Collection Site
			RM0043	TURED042-17	MG030795	25/04/14	Sidi Bou Saïd, Tunisia
			RM0099	TURED043-17	MG030796	01/08/14	Sousse, Tunisia
			RM0125	TURED044-17	MG030797	30/07/14	Tabarka, Tunisia
			RM0198	TURED045-17	MG030793	04/04/15	Tabarka, Tunisia
			RM0139	TURED046-17	MG030798	03/08/14	Monastir, Tunisia
Gigartinales	Cystocloniaceae	*Hypnea cervicornis*	RM0218	TURED022-17	MG030767	04/04/15	Tabarka, Tunisia
			PhL692	REAPP003-17	MF544097	15/06/10	Thau Lagoon, France
			SAM351	REDVE002-17	MF597661	30/06/10	Ottagono S. Pietro in Volta, Venice Lagoon, Italy
			SAM321	REDVE001-17	MF597662	30/06/10	Porto S. Leonardo, Venice Lagoon, Italy
			SAM443	REDVE005-17	MF597660	04/10/10	Porto S. Leonardo, Venice Lagoon, Italy
		Hypnea cornuta	RM0155	TURED017-17	MG030768	10/08/14	Gabes, Tunisia
			RM0288	TURED018-17	MG030769	10/08/15	Gabes, Tunisia
			RM0289	TURED019-17	MG030770	10/08/15	Gabes, Tunisia
			RM0292	TURED020-17	MG030771	10/08/15	Gabes, Tunisia
			RM0167	TURED021-17	MG030772	15/08/14	Zarat, Tunisia
			PhL691	REAPP002-17	MF544098	12/10/09	Torre Faro, Messina, Italy
		Hypnea musciformis	RM0007	TURED023-17	MG030774	01/03/14	Le Kram, Tunisia
			RM0097	TURED024-17	MG030775	01/08/14	Sousse, Tunisia
			RM0098	TURED025-17	MG030776	01/08/14	Sousse, Tunisia
			RM0271	TURED026-17	MG030773	12/04/15	Sousse, Tunisia
			RM0295	TURED027-17	MG030777	13/08/15	Sousse, Tunisia
			SAM352	REDVE003-17	MF597663	30/06/10	Ottagono S. Pietro in Volta, Venice lagoon, Italy
	Gigartinaceae	*Chondracanthus acicularis*	RM0233	TURED005-17	MG030757	08/04/15	Korbous, Tunisia
	Phyllophoraceae	*Phyllophora* sp.	RM0184	TURED040-17	MG030792	04/04/15	Tabarka, Tunisia
		Schottera nicaeensis	RM0230	TURED049-17	MG030801	08/04/15	Korbous, Tunisia

Order	Family	Species	Sample ID	BOLD ID	GB ID	Collection Date	Collection Site
			RM0236	TURED050-17	MG030802	08/04/15	Korbous, Tunisia
		Sphaerococcus coronopifolius	RM0113	TURED051-17	MG030807	30/07/14	Tabarka, Tunisia
			RM0182	TURED052-17	MG030805	04/04/15	Tabarka, Tunisia
			RM0212	TURED053-17	MG030806	04/04/15	Tabarka, Tunisia
Halymeniales	Halymeniaceae	*Grateloupia filicina*	RM0054	TURED012-17	MG030764	27/04/14	Port of Chebba, Tunisia
		Halymenia floresii	RM0062	TURED015-17	MG030765	01/06/14	Kerkennah, Tunisia
			RM0281	TURED016-17	MG030766	10/08/15	Gabes, Tunisia
Peyssonneliales	Peyssonneliaceae	*Peyssonnelia rubra*	RM0052	TURED039-17	MG030787	27/04/14	Chebba, Tunisia
			RM0116	TURED035-17	MG030788	30/07/14	Tabarka, Tunisia
		Peyssonnelia squamaria	RM0123	TURED036-17	MG030789	30/07/14	Tabarka, Tunisia
			RM0180	TURED037-17	MG030790	04/04/15	Tabarka, Tunisia
			RM0137	TURED038-17	MG030791	03/08/14	Monastir, Tunisia
		Ramicrusta sp.	RM0115	TURED047-17	MG030799	30/07/14	Tabarka, Tunisia
Rhodymeniales	Champiaceae	*Champia* sp.	RM0282	TURED004-17	MG030756	10/08/15	Gabes, Tunisia
		Gastroclonium sp.	RM0223	TURED011-17	MG030763	08/04/15	Korbous, Tunisia
	Rhodymeniaceae	*Rhodymenia ardissonei*	RM0165	TURED048-17	MG030800	15/08/14	Zarat, Tunisia



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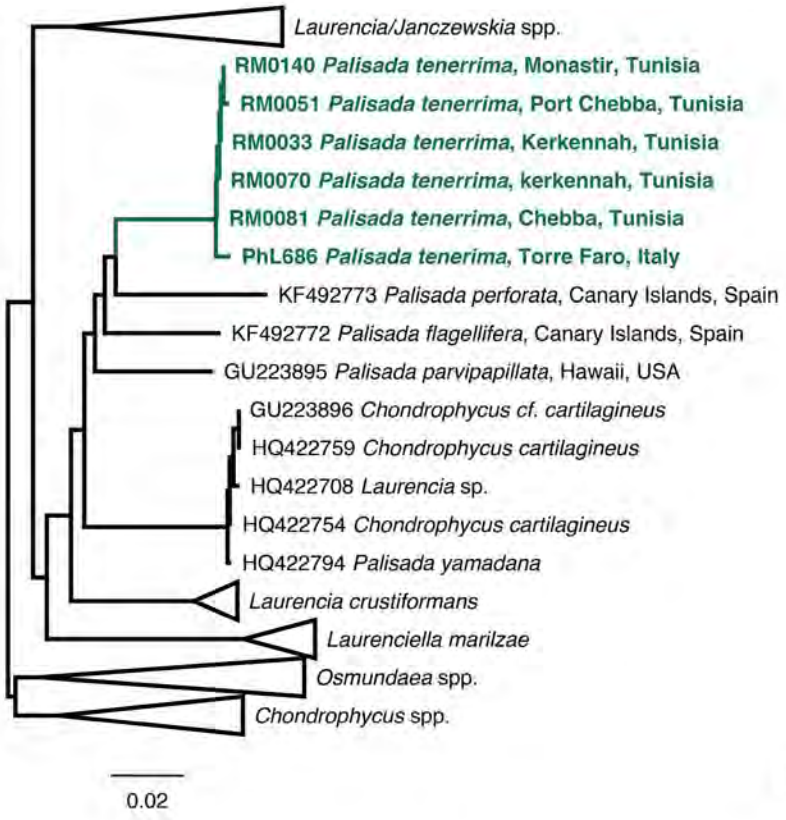

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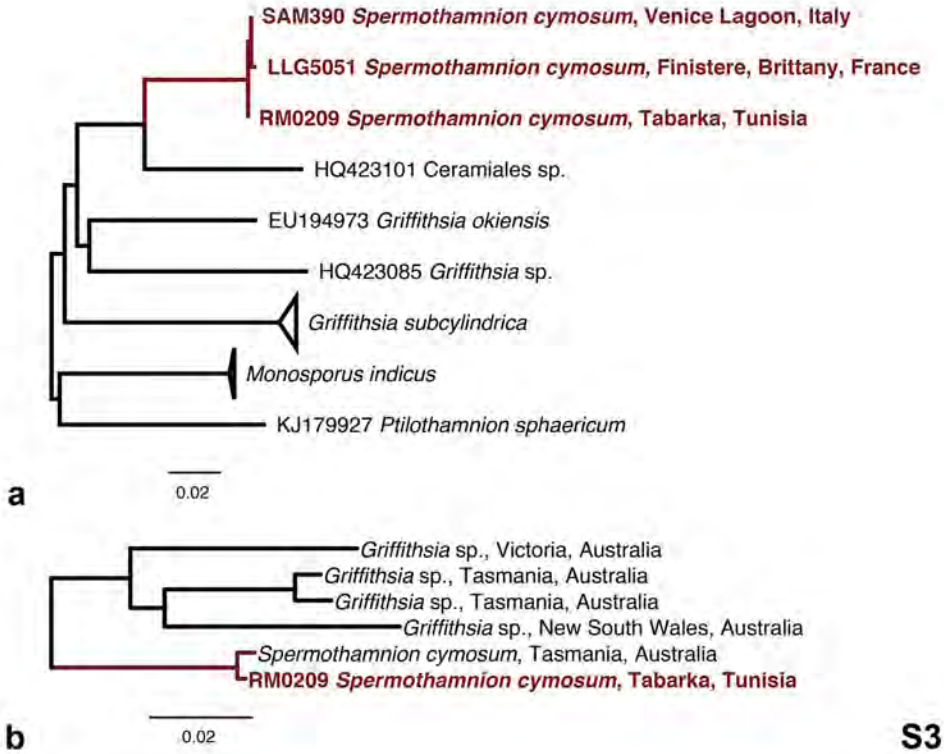

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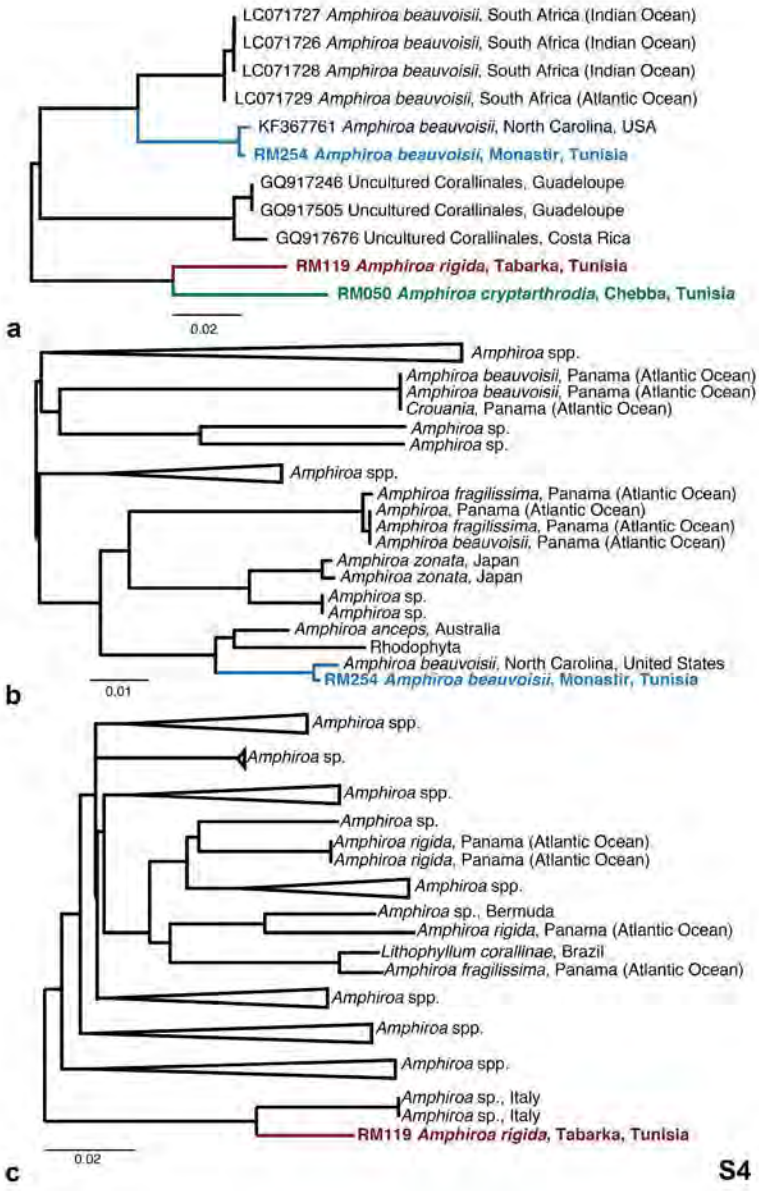

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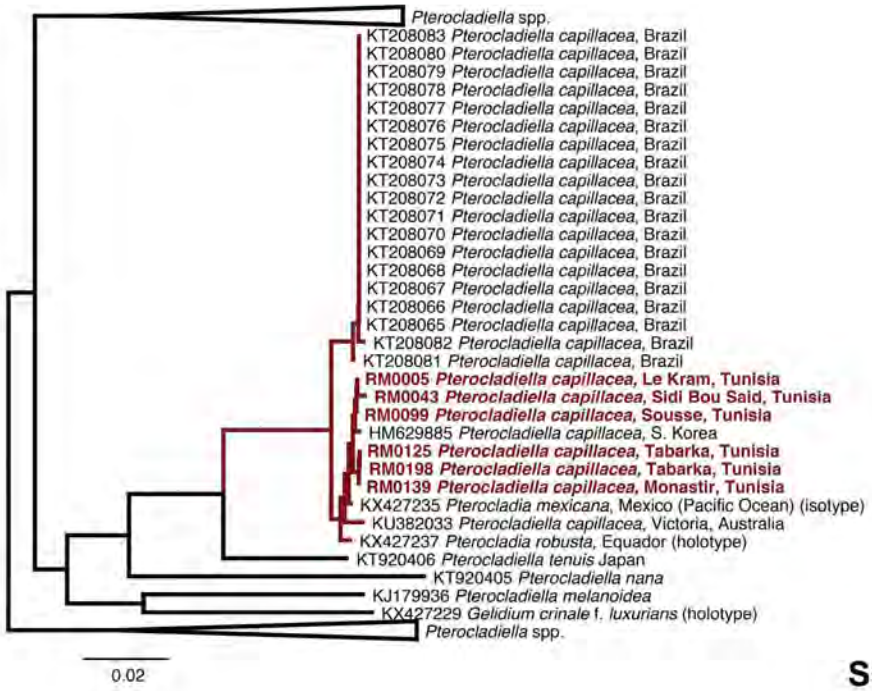

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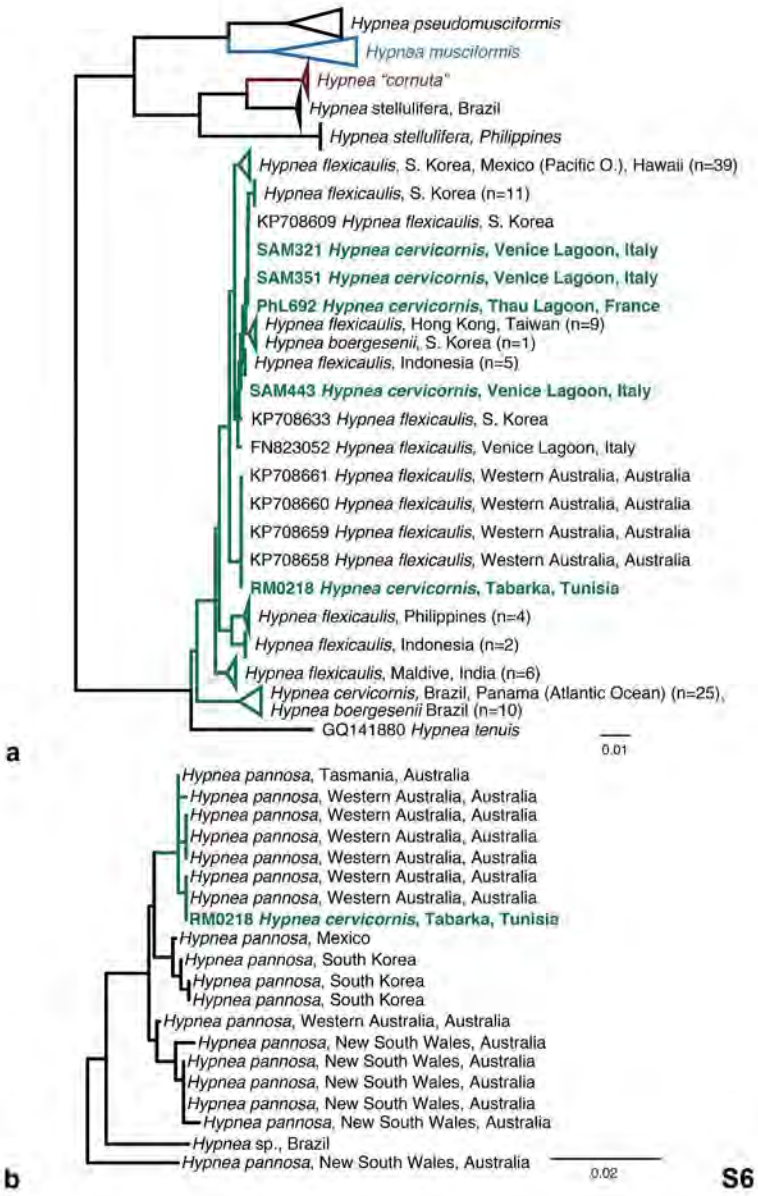

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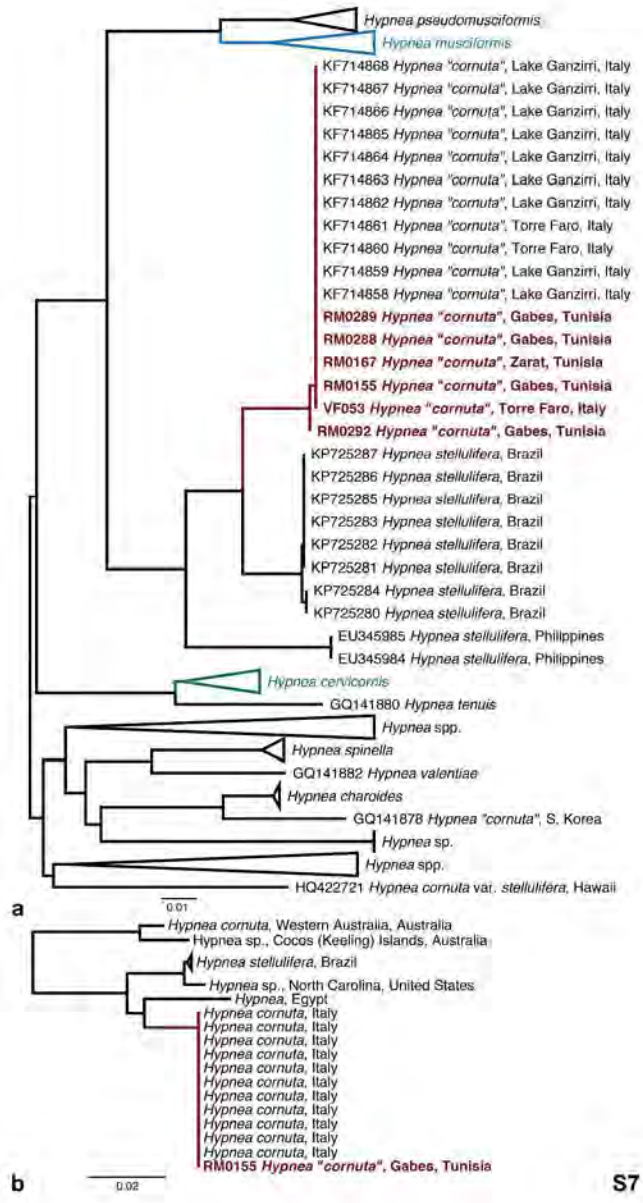

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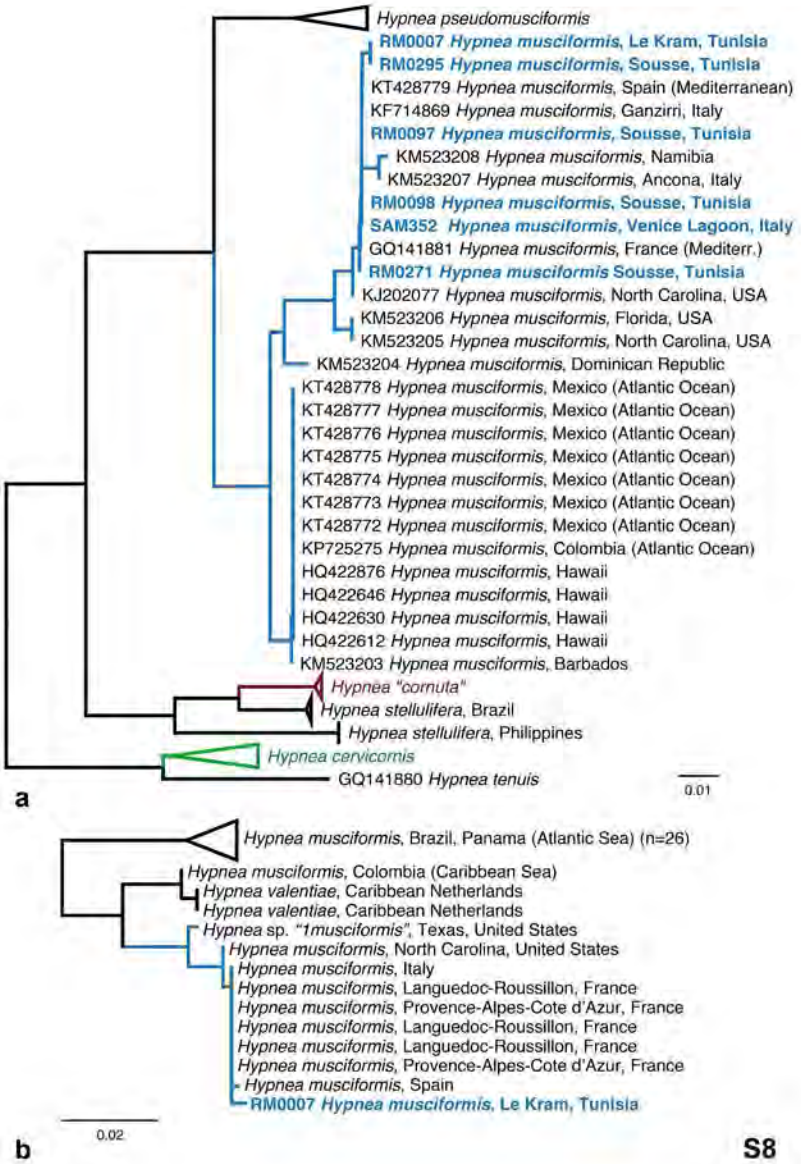

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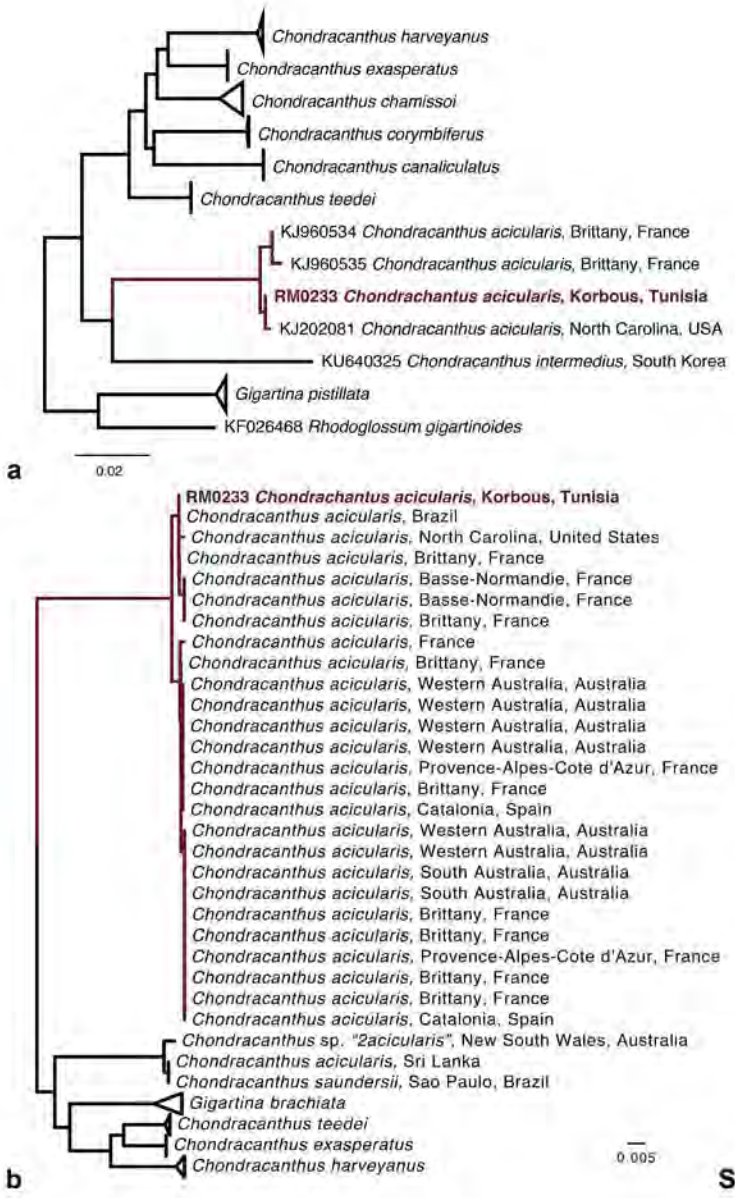

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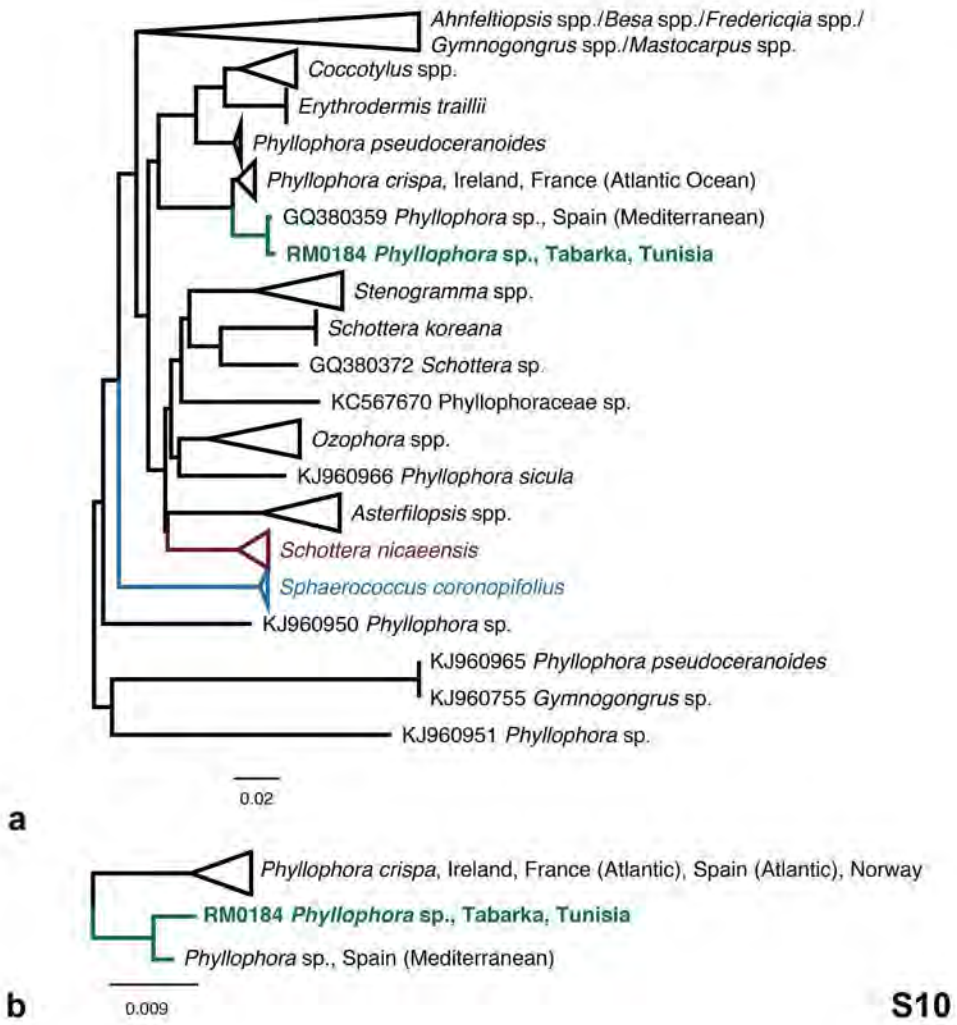

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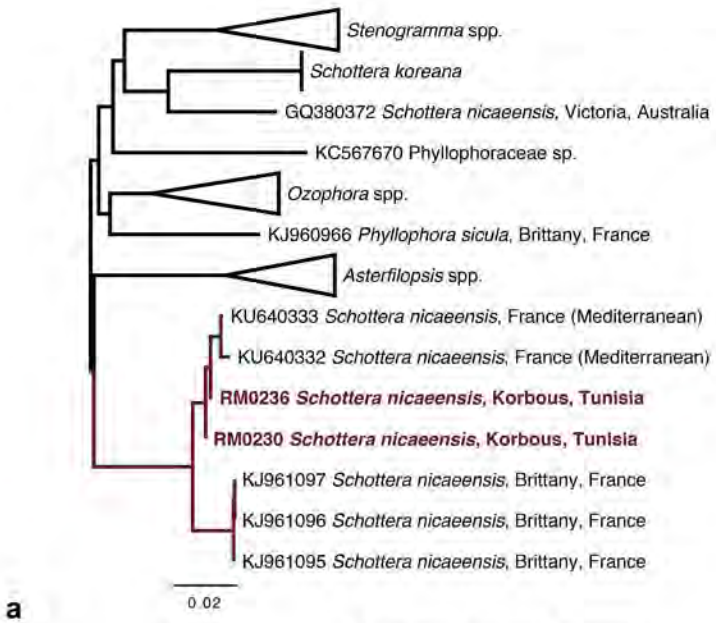

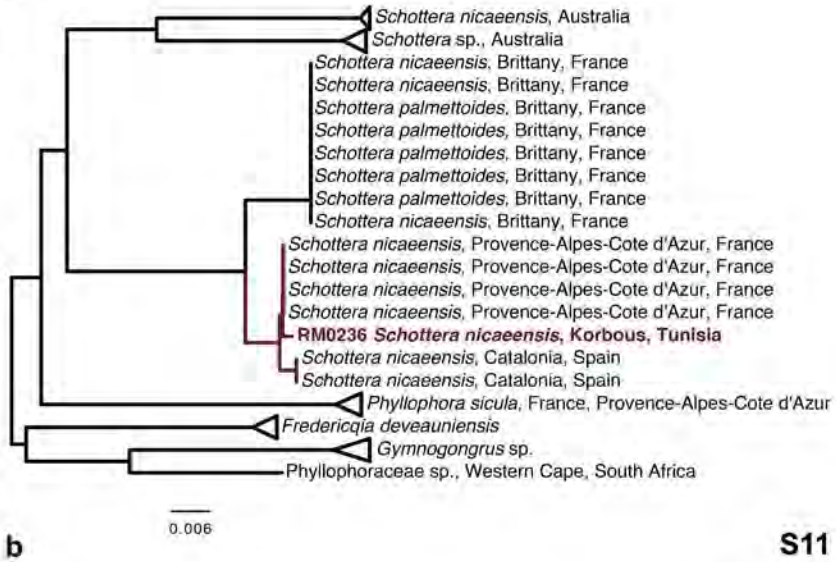

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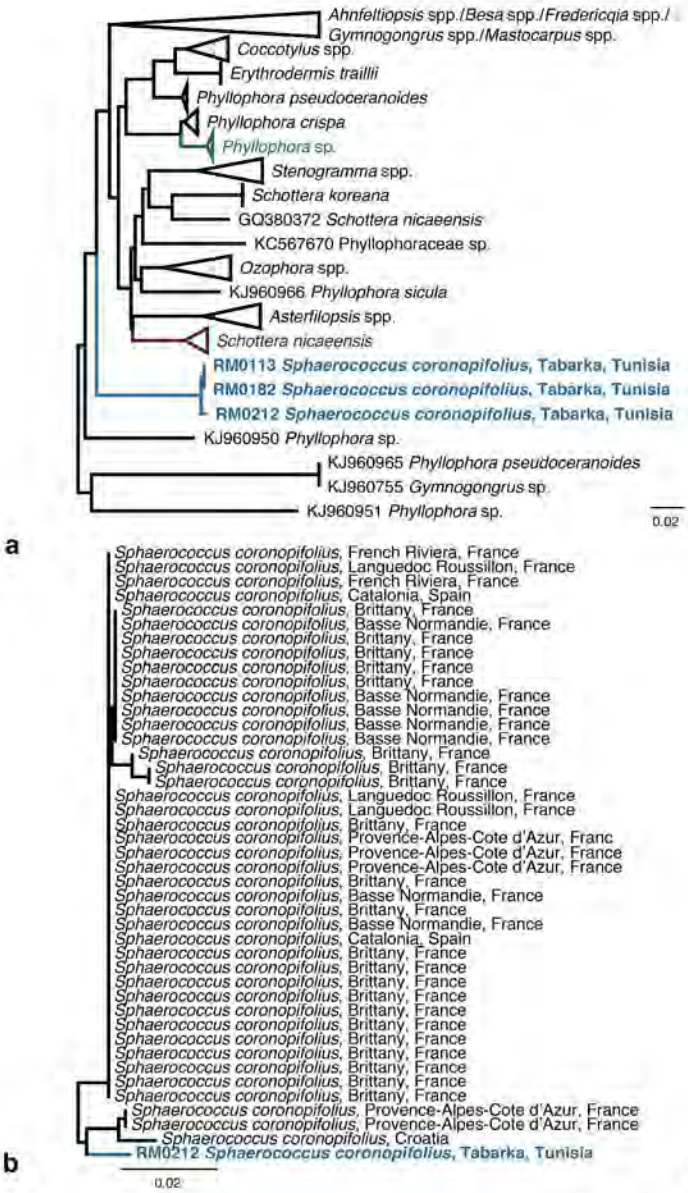

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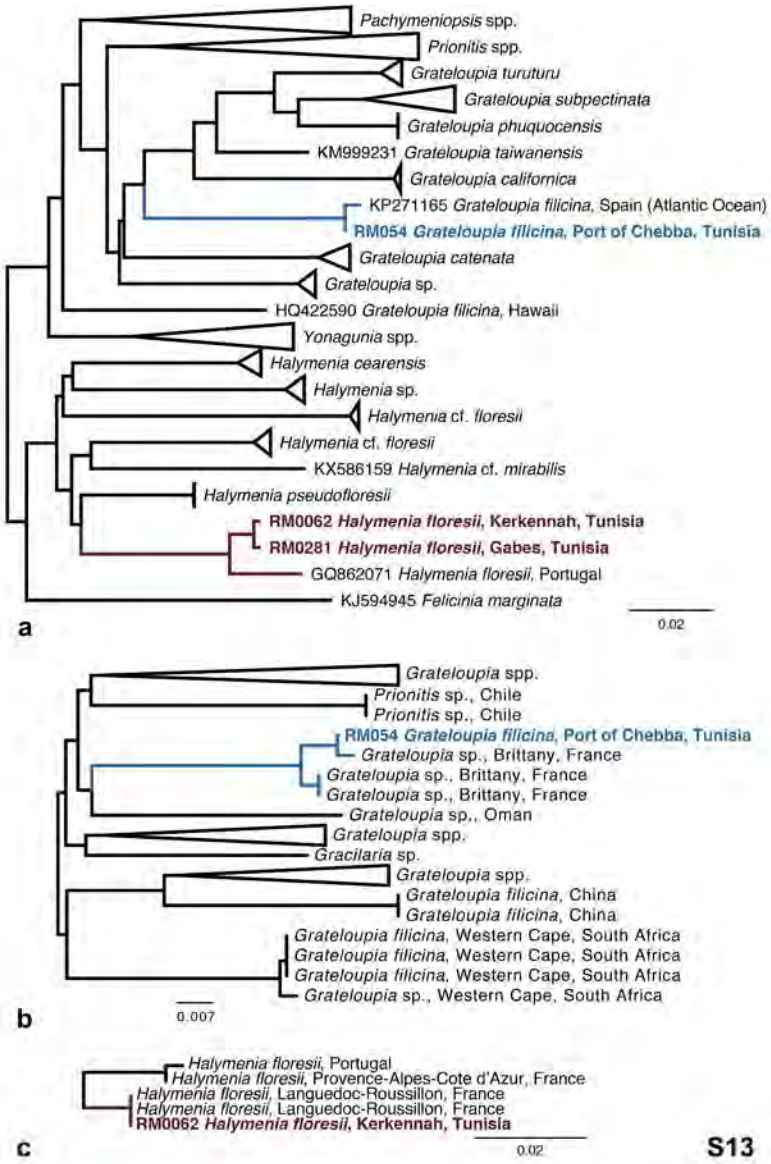

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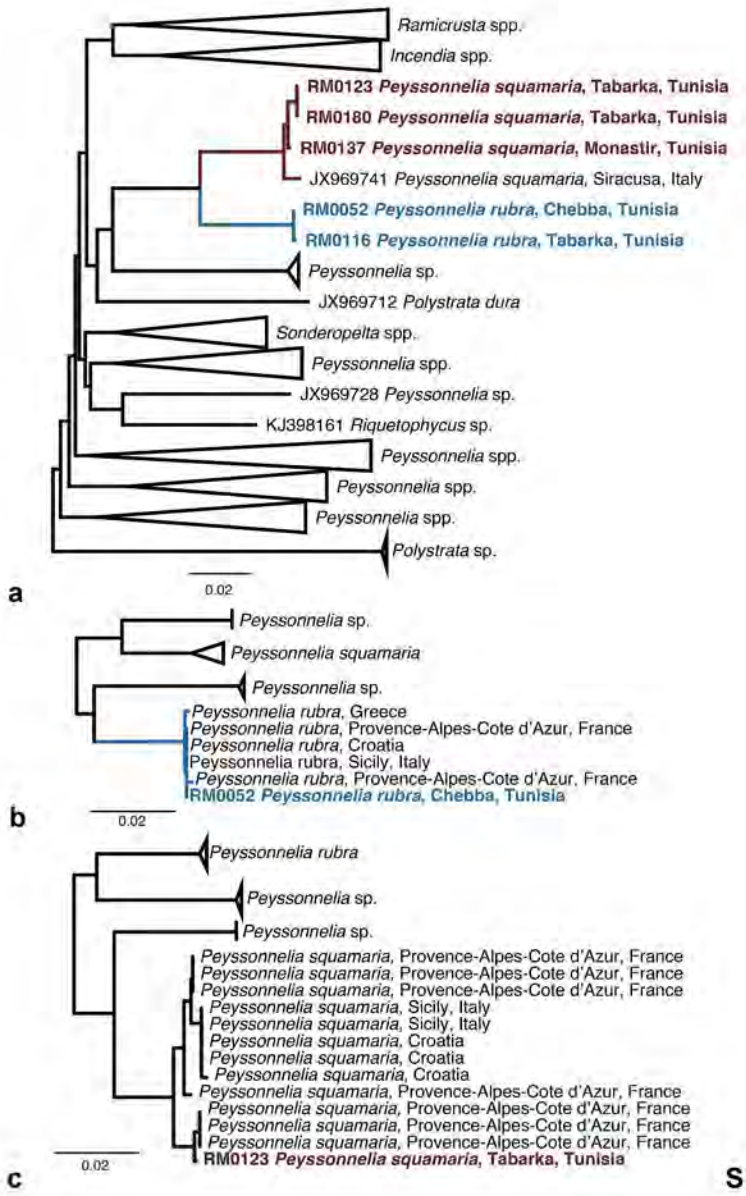

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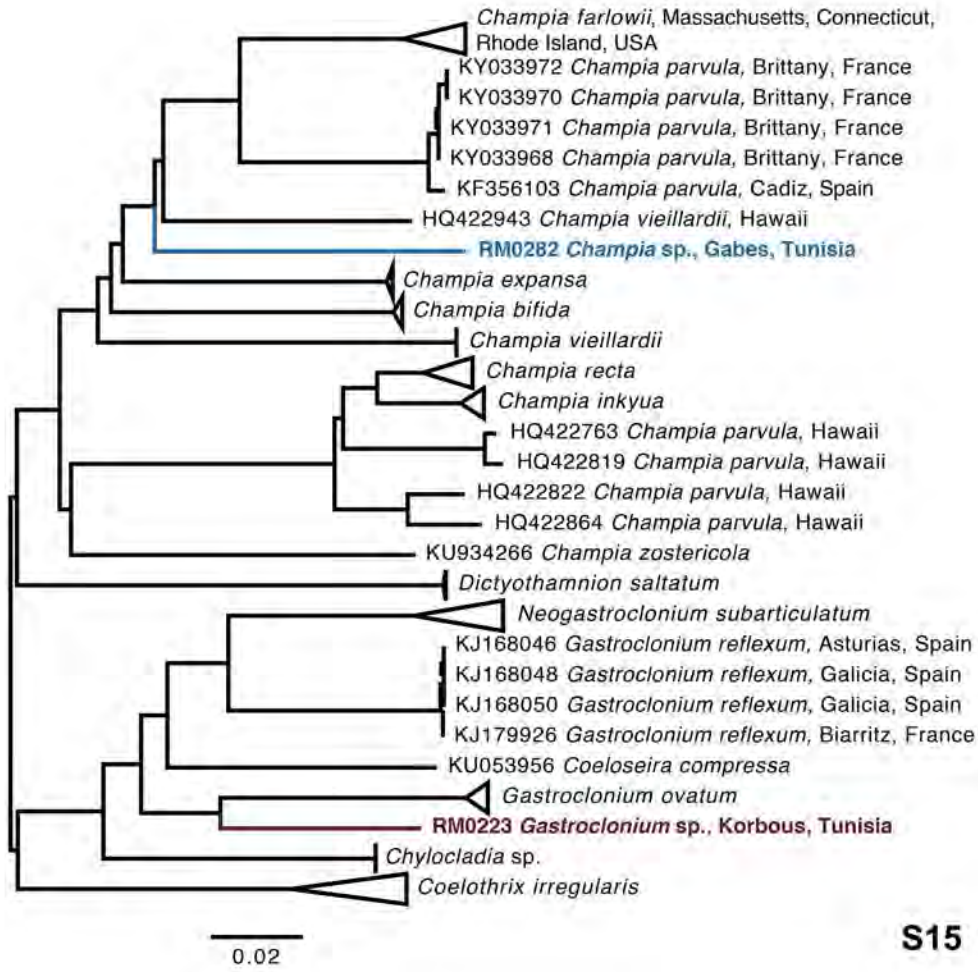

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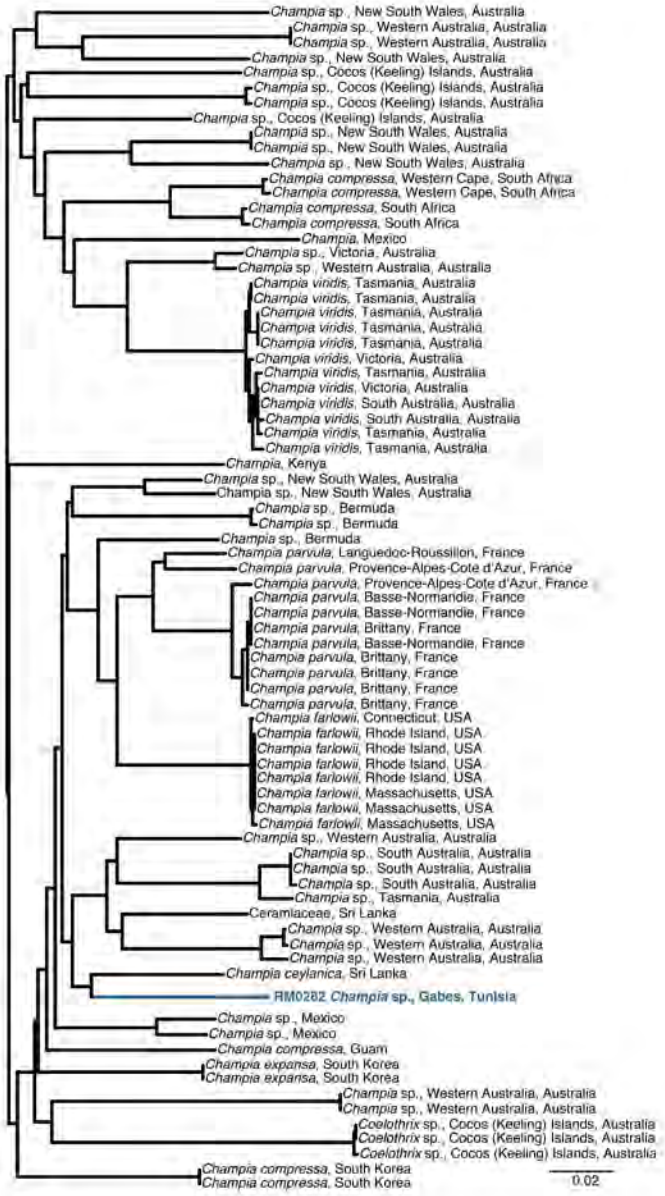

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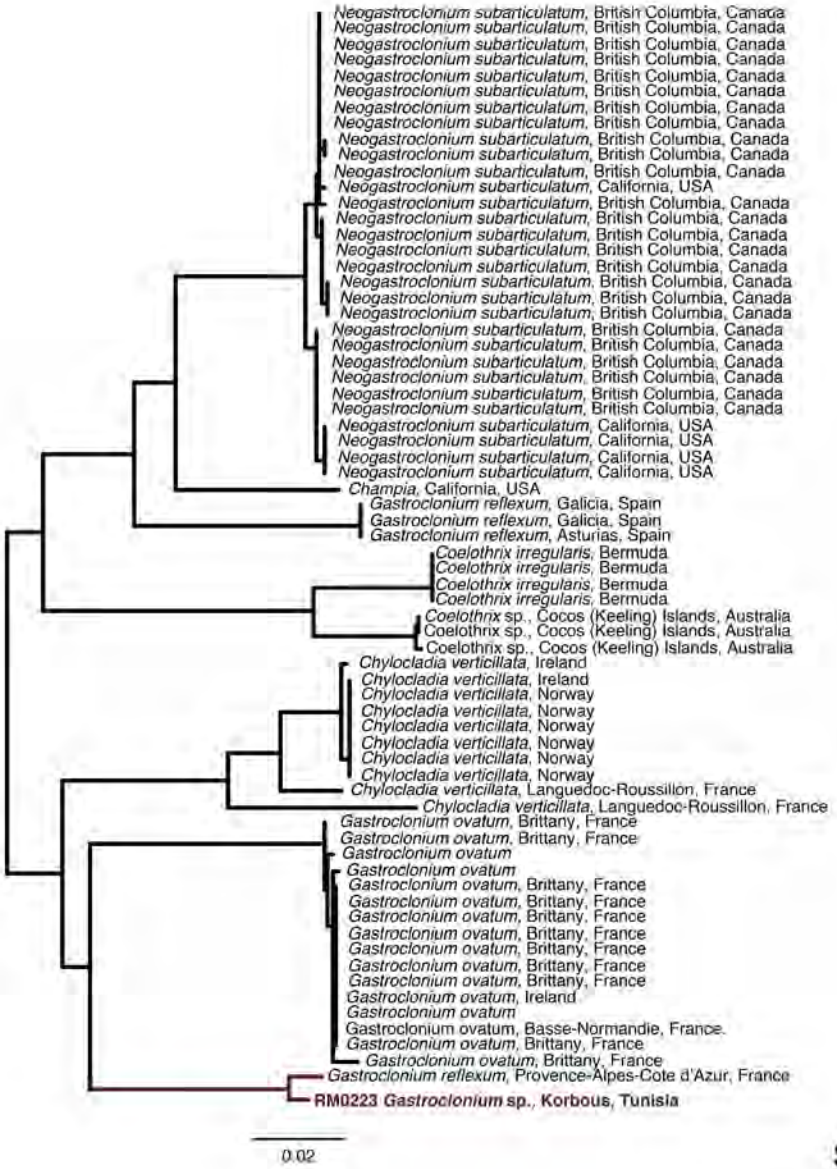

S17

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S18

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5. A DNA barcoding survey of *Ulva* (Chlorophyta) in Tunisia reveals the presence of the overlooked alien *U. ohnoi* (accepted for publication[13]).

[13] Accepted for publication in Cryptogamie, Algologie (IP 1.487)

cryptogamie
algologie | bryologie | mycologie

Line LE GALL
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fax: 33 1 40 79 35 94
legall@mnhn.fr

October 8th 2017

Dear Marina Morabito,

I am please to let you know that the manuscript entitled "**A DNA barcoding survey of *Ulva* (Chlorophyta) in Tunisia and Italy reveals the presence of the overlooked alien *U. ohnoi***" and co-authored by Ramzi MILADI, Antonio MANGHISI, Simona ARMELI MINICANTE, Giuseppa GENOVESE, Slim ABDELKAFI, and Marina Morabito has been accepted for publication in Cryptogamie Algologie an international journal devoted to all aspect of algae. I sincerely thank the authors for considering Cryptogamie Algologie for their fine paper.

If you desire any further information please do not hesitate to contact me.
Sincerely,

Dr. Line Le Gall (Editor in chief of Cryptogamie Algologie)

A DNA barcoding survey of *Ulva* (Chlorophyta) in Tunisia and Italy reveals the presence of the overlooked alien *U. ohnoi*.

Ramzi MILADI[a,b], *Antonio MANGHISI*[a]*, *Simona ARMELI MINICANTE*[c], *Giuseppa GENOVESE*[a], *Slim ABDELKAFI*[b] *and Marina MORABITO*[a]

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[b] *Unité de Biotechnologie des Algues, Département de Génie Biologique. Ecole Nationale d'Ingénieurs de Sfax, Université de Sfax, Route de Soukra Km 4, Sfax, Tunisia*

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Abstract – The cosmopolitan genus *Ulva* Linnaeus includes species of green macroalgae found in marine, brackish and some freshwater environments. Although there is a wide literature for the determination of *Ulva* taxa in Europe, they are among the most problematic algae to accurately identify, because they have few distinctive features, as well as a high intraspecific variation.

At present, the knowledge of both diversity and distribution of the genus *Ulva* in the Mediterranean Sea is almost entirely based on morphological studies and there is only a few published papers dealing with molecular data. Tunisia has a key position in the Mediterranean and constitutes a transition area with a rich habitat diversity between eastern and western basins. The latest inventory of marine macrophytes dates back to 1987, updated in 1995. The aim of the present paper is to provide a molecular-assisted alpha taxonomy survey of *Ulva* spp. along Tunisian coasts, in comparison with a few Italian sites, using the *tuf*A marker. Nine genetic species groups were resolved, including the non indigenous species *Ulva ohnoi*, newly reported for Tunisia. The actual picture of the taxonomy of *Ulva* spp. in the Mediterranean as a whole is far to be clarified and the present data on Tunisian collections aim to be a step towards its clarification. This paper is the first DNA barcoding study on green macroalgae in Tunisia and it contributes to add records to the Barcode Of Life Data Systems which are publically available.

Alien species / DNA barcoding / non indigenous species / *tufA* / Tunisia / *Ulva ohnoi*

INTRODUCTION

Ulva Linnaeus includes species of cosmopolitan green macroalgae found in marine, brackish and some freshwater environments. It includes almost 600 described species of which 125 are currently accepted taxonomically (Guiry & Guiry, 2017), some are a major component in transitional environments and among the most common members of rocky intertidal and subtidal marine habitats worldwide.

Ulva species also include some common bloom-forming taxa (Hiraoka *et al.*, 2003; Leliaert *et al.*, 2009), and species have been recently used for bioremediation applications (El-Sikaily *et al.*, 2007), in chemical ecology (Van Alstyne *et al.*, 2007), as bioindicators of eutrophic or stressed environments (Kozhenkova *et al.*, 2006), and as food (Abbott, 1979; Xia & Abbott,

1987). However, most applicative studies on *Ulva* spp. lack reliable taxonomic identifications (Kirkendale *et al.*, 2013). This is a potential problem because it has been shown that even closely related species exhibit unique ecophysiological and chemical characteristics (Eswaran *et al.*, 2002; Michael, 2009; Paulert *et al.*, 2010; Winberg *et al.*, 2011).

Although there is a wide taxonomic literature on *Ulva* in Europe (Bliding, 1963, 1968; Koeman & van den Hoek, 1982a, b, 1984; Cormaci *et al.*, 2014), they are among the most problematic algae to accurately identify, because they have few distinctive features, as well as a high intraspecific variation. A direct consequence is the number of superfluous and synonymized species recorded (Guiry & Guiry, 2017). More difficulties are added by the knowledge that changes in morphology are induced by low salinity or salinity shock and laboratory culture experiments showed that bacteria and their exudates associated with *Ulva* may induce extensive morphological changes that cross supposedly well-defined species boundaries (Provasoli & Pintner, 1980; Blomster *et al.*, 1998; Marshall *et al.*, 2006). Species of *Ulva* are also known for their rapid proliferous growth and their capacity to tolerate a wide range of environmental conditions, and, as a consequence, are ideal candidates for human-mediated dispersal and have been detected as the most common component of the macroalgal biomass present on hulls (Mineur *et al.*, 2007; Heesch *et al.*, 2009; Couceiro *et al.*, 2011). They are among the most widely introduced species of macroalgae, even by wooden sailing vessels in previous centuries (Santelices *et al.*, 2002; Schaffelke *et al.*, 2006; Kraft *et al.*, 2010). To further complicate the issue, an understanding of native ranges is lacking for many species of *Ulva* and due to their cryptic diversity, many non-indigenous species (NIS) may go unnoticed (Melton *et al.*, 2016a). Previous unrecorded taxa could be possibly overlooked due to the variability and unreliability of accepted morphological characters, and many authors stated that, without the use of DNA sequence data, some species groups would remain with uncertain identification (Loughnane *et* al., 2008; Coll *et* al., 2010; Kang *et* al., 2014). However, accurate biodiversity assessment is essential for monitoring biological introductions, and is critical for environmental management as well as to adequately evaluate temporal changes (Loughnane *et al.*, 2008; Hofmann *et al.*, 2010; Melton *et al.*, 2016a).

For *Ulva* spp. molecular identification, previous studies used the chloroplast-encoded *rbc*L and *tuf*A markers and the nuclear internal transcribed spacer region 1 (ITS1) of the ribosomal cistron (Heesch *et al.*, 2009; Saunders & Kucera, 2010; Couceiro *et al.*, 2011; Kirkendale *et al.*, 2013; Melton *et al.*, 2016a). However, Kirkendale *et al.* (2013) noted that ITS1 commonly had poor amplification success producing double bands indicative of divergent copies (Saunders & Kucera, 2010), whereas *rbc*L showed low levels of genetic diversity reducing species-level resolution (Heesch *et al.*, 2009). Differently, *tuf*A resulted as a viable marker with a higher resolution power at species level in comparison to the other molecular markers (Saunders & Kucera, 2010).

At present, the knowledge of both diversity and distribution of the genus *Ulva* in the Mediterranean Sea is almost entirely based on morphological studies and there are few published papers dealing with molecular data (Wolf *et al.*, 2012; Armeli Minicante *et al.*, 2014). Checklists for Mediterranean algal taxa include a large number of species poorly known, and several taxa *inquirenda* (Coll *et al.*, 2010; Cormaci *et al.*, 2014; Gallardo *et al.*, 2016; Zenetos *et al.*, 2017) and highlight the need of an accurate revision to decipher the taxonomy of several genera, including *Ulva*.

Tunisia has a key position in the Mediterranean and constitutes a transition area with a rich habitat diversity between eastern and western basins. The Sicilian Channel is the crossroad from south to north and from east to west, and is crucial in the analysis of the marine organism distribution into the Mediterranean Sea. The presence of rocky or sandy coasts, deep bays, some protruding caps, numerous islands and islets and a shallow belt are characteristic features of the Tunisian littoral topography (Blanpied *et al.*, 1979a, b; Pergent & Kempf, 1993).

All floristic and taxonomic studies for Tunisian marine flora, have been carried out by means of a morpho-anatomic identification approach, and the latest inventory dates back to 1987, (Ben Maiz *et al.*, 1987), with an update in 1995 (Ben Maiz, 1995). Subsequently, only papers on a

limited area (Ben Maiz & Shili, 2007; Shili *et al.*, 2007), dealing with NIS (Sghaier *et al.*, 2016) or with applicative purposes (Azaza *et al.*, 2008; Yaich *et al.*, 2011; Kolsi *et al.*, 2015), have been published on *Ulva* spp.; none of them used a molecular systematics approach.

The aim of the present paper is to provide a molecular-assisted alpha taxonomy (MAAT) survey of *Ulva* spp. along Tunisian coasts, as well as a few Italian sites to serve as comparison, using the *tuf*A marker for the compilation of a DNA barcode inventory.

MATERIAL AND METHODS

Samples of *Ulva* were collected in 9 sites distributed along the Tunisian coastline from March 2014 to March 2016 (Fig. 1). All samples were hand collected from the intertidal zone. Samples were transported in seawater at low temperature to the laboratory, where they were washed thoroughly with seawater to eliminate debris. For each sample, a voucher specimen was prepared by pressing a single individual on an herbarium sheet with a subsample of thallus dried in silica gel for molecular analyses. Vouchers are housed in the Phycological Lab Herbarium (PhL) of the University of Messina, Italy (http://grbio.org/institution/phycological-lab-herbarium-university-messina). Additional samples collected along Italian coasts included in PhL were also considered for comparison with Tunisian collections.

DNA was extracted from silica dried specimens using a standard CTAB-extraction method (Doyle & Doyle, 1987), with few modifications: 2-mercaptoethanol was excluded from the extraction buffer, while 1% PVP and 0.02% of proteinase K were added; lysis was performed at room temperature for 2 hours on a rotary shaker.

The plastidial *tuf*A gene was PCR amplified as described in Saunders and Kucera (2010). Sequencing reactions were performed by an external company (Macrogen Europe, The Netherlands). Some of the Italian specimens were processed and sequenced at the University of New Brunswick, Canada (Saunders & McDevit, 2012).

Specimen data, sequences and used primers were deposited in the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org). Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and multiple sequence alignments were constructed in Seaview (v. 4.3.3, Gouy *et al.*, 2010), including sequences of the family Ulvaceae downloaded from GenBank (Benson *et al.*, 2017). Sequence alignments were subjected to distance analyses with a Neighbor-Joining algorithm under a K2P model of nucleotide substitution (Kimura, 1980) as performed in PAUP* 4b10 (Swofford, 2002) to visualize genetic clusters that may correspond to species.

RESULTS AND DISCUSSION

It is widely acknowledged that taxonomic identification within the large genus *Ulva* Linnaeus, with 125 species and infraspecific taxa currently accepted (Guiry & Guiry, 2017), by means of dichotomous keys based on morphological characters, is a challenging task because of the paucity of available characters and their significant plasticity in response to environmental factors (Heesch *et al.*, 2009; Hofmann *et al.*, 2010; Couceiro *et al.*, 2011). As a consequence, DNA data have been progressively used as an easier alternative for species recognition.

Two categories of DNA-based species delimitation exist, namely single-locus and multi-locus methods (see Leliaert et al., 2014 for a review). While it is now generally recognized that multi-locus data are essential for accurate species delimitation, single-locus approaches have proven effective for species identification (e.g. Manghisi *et al.*, 2011; Geoffroy et al., 2012; Kogame *et al.*, 2016). Single-locus methods basically rely on three assumptions: a) the gene genealogy is representative of the species phylogeny; b) species are monophyletic; c) the transition between

intra- and interspecific sequence variation is visible as a distinct gap in the frequency distribution, the so-called DNA barcoding gap (Hebert et al., 2004). Although in several studies dealing on algal species delimitation clear discontinuities between intra- and interspecific genetic distances have been observed (e.g. Saunders, 2005; Zimmermann et al., 2011), the use of distance methods may be problematic in some cases, such as among closely related species, in which intra- and interspecific genetic distances may overlap (Hamsher et al., 2011; Hoef-Emden, 2012), or with increased geographic sampling resulting in a blur of barcoding gaps (Meyer & Paulay, 2005; Bittner et al., 2010; Bergsten et al., 2012).

Several taxonomists have claimed that adequate delimitation of species based solely on DNA sequence data is not acceptable, rather should be based on additional lines of evidence, including morphological, ultrastructural, biochemical, geographic, ecological and breeding data (e.g. Pröschold et al., 2001; Vanormelingen et al., 2007; Walker et al., 2009; Bendif et al., 2011; McManus & Lewis, 2011; Neustupa et al., 2011; Ni-Ni-Win et al., 2011; Lam et al., 2012; Milstein & Saunders, 2012; Škaloud & Rindi, 2013). Nevertheless, molecular methods are effective tools for a first screening in order to merely identify genetic species groups in a strategy named molecular-assisted alpha taxonomy (MAAT) (Saunders, 2005). This methodology is especially useful in organisms with simple morphologies, with a high degree of phenotypic plasticity or convergence depending on environmental conditions, like the genus *Ulva*. The chloroplast-encoded *tuf*A gene resulted as a feasible marker for identification of green algae, with a higher resolution power at the species level in comparison to other molecular markers commonly used in studies dealing with *Ulva* spp. molecular identification, such as the nuclear internal transcribed spacer region 1 (ITS1) and the chloroplast-encoded *rbc*L gene (Saunders & Kucera, 2010; Kirkendale *et al.*, 2013).

The main limit of MAAT, however, is that type sequences, generated from type specimens, or at least topotype material, should be used as a reference to define species or clarify species concepts (Heesch *et al.*, 2009; Hofmann *et al.*, 2010). Nevertheless, these data are rarely available, particularly when type specimens are very old, or lost, and/or type localities are unknown or vague, as is, for instance, the case with most of the *Ulva* type specimens described by Linnaeus (1753) (Womersley, 1984), and the names used in sequence data retrieved from GenBank need to be used with caution (Heesch *et al.*, 2009) as the DNA library of life is still under construction (Le Gall *et al.*, 2017). The only historical type specimen, which DNA was sequenced, is the lectotype of *Ulva lactuca* Linnaeus (selected by Papenfuss, 1960) and, according to unpublished data by C. Maggs and F. Mineur (Butler, 2007; Holden, 2007; O'Kelly *et al.*, 2010; Delnatte & Wynne, 2016), the specimen in the Linnean Herbarium matched with *Ulva fasciata* Delile. What is currently known as *U. lactuca* is in fact another species, namely *Ulva stipitata* Areschoug (presently considered as a synonym of *U. lactuca*), even if the authors stated that further work was needed to clarify the matter. However, DNA results on the lectotype of *U. lactuca* has not yet been published.

At present, published papers using MAAT approaches for Mediterranean Ulvaceae are very limited. In 2012, Wolf *et al.* presented a survey on *Ulva* spp. biodiversity in Venice lagoon, pointing the presence of the non-indigenous species (NIS) *Ulva australis* Areschoug (as *Ulva pertusa* Kjellman, already reported by Manghisi *et al.*, 2011) and *Ulva californica* Wille (the first Mediterranean record). Later, Armeli et al. (2014) reported the NIS *Ulva ohnoi* Hiraoka *et* Shimada in Cape Peloro lagoon, north-eastern Sicily, for the first time in Europe, and Bertuccio *et al.* (2013/2014) updated the floristic list of the same site.

In the present work, a total of 43 *tuf*A sequences of *Ulva* from Tunisia and Italy were generated. These sequences were resolved in nine genetic clusters, which are here regarded as species (Table 1, Fig. 2). Five species included specimens collected along Tunisian coasts, namely *Ulva compressa* Linnaeus, *Ulva mediterranea* Alongi, Cormaci et G. Furnari, *Ulva torta* (Mertens) Trevisan, *Ulva laetevirens* Areschoug and *U. ohnoi*, the latter two newly reported for Tunisia. *U. compressa, U. laetevirens* and *U. ohnoi* were also found along Italian coasts, together with *U. australis, Ulva linza* Linnaeus and *Umbraulva dangeardii* Wynne *et* Furnari, and the doubtful record of *U. fasciata*.

***Ulva compressa* Linnaeus.** Distance analysis of *tuf*A sequences showed that Tunisian specimens grouped with others from various Mediterranean sites, including Venice lagoon and Torre Faro, Italy, Banyuls, France, other localities from Canada, Australia and China, as well as with an unidentified species from UK, within the type area (probably Bognor, Sussex, England, fide Hayden *et al.*, 2003: 289) (Fig. 2). Samples included in the present work were collected in various habitats, both undisturbed and with anthropogenic impact, and displayed a highly variable morphology (Figs 3-8), as already observed in other regions of the world (Tan *et al.*, 1999; Hofmann *et al.*, 2010; Kraft *et al.*, 2010).

Ulva compressa is regarded as a common native species in the Mediterranean Sea, thriving in non-exposed sites (Cormaci *et al.*, 2014), already reported for Tunisia (Ben Maiz *et al.*, 1987), and widely distributed in all oceans (Guiry & Guiry, 2017). It is noteworthy that Kirkendale *et al.* (2013) hypothesized that *Ulva compressa* could be introduced from Australia to the rest of the world as it display higher genetic diversity within than outside Australia. The analysis of all publicly available *tuf*A sequences, including Mediterranean samples generated in the present works, showed distance values ranging from 0.00 to 1.35% (0-9 bp) within species. These values are driven by the diversity of Australian samples whereas Mediterranean isolates showed distance values ranging from 0.00 to 0.54% (0-4 bp). The large sequence divergence is comparable to that observed among species currently regarded as distinct (e.g. *U. fasciata* vs *U. ohnoi*). Therefore, it could be an evidence of cryptic diversity at the specific level, which should be investigated with supplementary sequences from other geographic sites and additional molecular markers. Meanwhile, there is no apparent reason to consider it as a NIS in the Mediterranean basin.

***Ulva mediterranea* Alongi, Cormaci et G. Furnari.** The Tunisian sample collected in the present work, found in an habitat with anthopogenic impact, grouped in *tuf*A analysis with isolates from Venice lagoon, Italy, and other localities from Canada, Australia, China and Korea (0.00-0.47%, 0-3 bp sequence divergence) identified as *Ulva flexuosa* Wulfen (Figs 2, 9). Hiraoka et al. (2017), basing on DNA data coupled with culturing and hybridisation experiments, proved that the previously listed samples do not belong to the genuine *U. flexuosa*, which had been characterized by Mareš *et al.* (2011) using molecular systematics in combination with morphological observations (including re-examination of the holotype). Hiraoka et al. (2017) merged the former samples with *U. mediterranea,* also including *Ulva flexuosa* Wulfen var. *linziformis* Alongi, Cormaci *et* G. Furnari and *Ulva linzoides* Alongi, Cormaci *et* G. Furnari among synonyms.

Ulva mediterranea has been reported as a common native species in the Mediterranean Sea and it is widely distributed in all oceans (Guiry & Guiry, 2017). It is not possible to assess whether *U. mediterranea* is a new report for Tunisia or rather it was misidentified as *U. flexuosa* by Ben Maiz et al. (1987).

***Ulva torta* (Mertens) Trevisan.** Distance analysis showed that the Tunisian specimen grouped with *U. torta* from Pacific North America and Australia (0.00-0.40%, 0-3 sequence divergence) (Fig. 2). However, a comparison with sequences from type locality (Germany) is missing.

It has been reported as a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), already reported in Tunisia (Ben Maiz *et al.*, 1987), widely distributed in all oceans (Guiry & Guiry, 2017). Samples collected in the present work were found in an undisturbed habitat (Fig. 10).

***Ulva linza* Linnaeus.** Distance analysis showed that the specimen from Venice lagoon grouped with *U. linza* from England, in the type area (Kent, England fide Hayden *et al.*, 2003) and China, together with samples identified as *Ulva procera* (K. Ahlner) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope *et* J.R. Waaland by Kirkendale *et al.* (2013), from Canada and Australia (0.00-1.14%, 1-8 bp sequence divergence) (Fig. 2).

Ulva linza is a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), widely distributed in all oceans (Guiry & Guiry, 2017). Several publications on *Ulva* spp. phylogenies considered *U. procera* as a separate species from *U. linza* (Heesch *et al.*, 2009;

Saunders & Kucera, 2010; Kirkendale *et al.*, 2013), even if, according to Brodie *et al.* (2007), ITS sequence data had demonstrated that it is conspecific with typical *U. linza*. However, *U. linza* (including *U. procera*) has a high phenotypic plasticity induced by the environment and bloom-forming isolates in the Yellow Sea were similar in morphology to *U. prolifera* O.F. Müller and *U. procera* (Kang *et al.*, 2014). According to Kirkendale *et al.* (2013), there are actually 3–5 genetic groups in the *"linza-prolifera-procera"* cluster, with a morphology ranging from blades to tubes, which makes impossible to use morphology for identifications. A detailed study on type material of the three taxa is needed to define this cluster.

***Ulva fasciata* Delile.** Distance analysis showed that specimens from Venice lagoon and lake Ganzirri in Cape Peloro lagoon grouped with *Ulva fasciata* from Australia (Fig. 2). No sequence divergence was observed within Mediterranean isolates, while one base-pair difference (0.14%) was observed with just one Australian sample. All samples collected in the present work came from habitat with anthropogenic impact and showed a high degree of morphological variability (Figs 11-14).

Ulva fasciata has been reported as a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), widely distributed in all oceans (Guiry & Guiry, 2017). According to Kirkendale *et al.* (2013) the complete lack of sequence diversity within Australia made introduction plausible, however global genetic reference pool was lacking and habitat data did not suggest NIS. Similarly, no genetic diversity was observed among Italian isolates. Its status as a NIS or a native species is doubtful, pending a larger sampling in the Mediterranean and a comparison with type material (type locality: Alexandria, Egypt).

***Ulva laetevirens* Areschoug.** Distance analysis showed that Tunisian specimens are conspecific with *U. laetevirens* from Australia (type area, Port Phillip, South Australia) and other Mediterranean sites, including Venice lagoon, Ragusa and Torre Faro, Italy, as well as with samples from Atlantic North America and China (1-3 bp, 0.00-0.40%) (Fig. 2). In the same genetic species grouped sequences identified as *Ulva rigida* C. Agardh from Venice (data not shown, 100% sequence identity with other Italian specimens). Samples collected in the present work were found in various habitats, both undisturbed and anthropized (Figs 15-20).

U. laetevirens is commonly reported in the Mediterranean Sea, in a variety of sites both exposed and sheltered, mainly oligotrophic (Cormaci *et al.*, 2014), and also found in Atlantic North America, China and Japan (Kirkendale *et al.*, 2013; unpublished in Genbank). Synonymy of *U. laetevirens* with *U. rigida* was discussed by Phillips (1988), which noted that specimens described by Bliding (1968) were misidentified, and she concluded that *U. rigida sensu* Bliding *non* C. Agardh should be referred to *U. laetevirens*. However, Phillips (1988) included erroneously *U. australis* among synonyms of *U. rigida* (Kraft *et al.*, 2010) and, as a consequence, it is possible that she did not analyze the genuine *U. rigida* C. Agardh. Since Bliding's study, many investigations conducted in Europe have recorded the presence of *U. rigida sensu* Bliding, presumably also molecular articles (Hayden *et al.*, 2003; Loughnane *et al.*, 2008), therefore, accessions in GenBank bearing the name *U. rigida* are likely to actually represent *U. laetevirens*. Molecular sequences for *U. laetevirens* from the type region (Port Phillip Bay, Australia) was reported in Kraft *et al.* (2010), while a morphological and molecular study of *U. rigida sensu* C. Agardh from the type region (Cadiz, Spain) is not available. At present, the synonymy between *U. rigida* and *U. laetevirens*, is most likely but cannot be asserted.

In Venice lagoon, Sfriso (2010) reported the coexistence of *U. rigida* and *U. laetevirens* as two distinct species on the basis of subtle anatomical differences. The present finding of *U. laetevirens* in Tunisia is unlikely to be a new report, as it most likely corresponds to *Ulva rigida* already recorded by Ben Maiz *et al.* (1987). Based on genetic data compared to those of Kirkendale *et al.* (2013), it might be considered as a NIS introduced from Australia to the Mediterranean, but it is preferable to consider it as a cryptogenic species *sensu* Carlton (1996), pending upon clarification of its relationship with *U. rigida* and the identification of its native region.

***Ulva australis* Areschoug.** Distance analysis showed that Venetian specimens grouped (0 bp sequence divergence) with other isolates from Venice lagoon and from Australia (type area, Port Adelaide, South Australia), Canada, Korea and China (0.00-0.29%, 1-2 bp) (Fig. 2), confirming the presence of the NIS *Ulva australis* in Venice lagoon, already reported by Manghisi *et al.* (2011).

Its native range includes Northeast Asia, Australia and New Zealand (Heesch *et al.*, 2009; Kraft *et al.*, 2010; Hanyuda *et al.*, 2016) and it is also reported as a NIS in most oceans, introduced by aquaculture or maritime activities, as a fouling species or in ballast waters of trans-ocean shipping (as *U. pertusa*, Hanyuda *et al.*, 2016). In the Mediterranean Sea, *Ulva australis* has been recorded also in Thau lagoon, France (as *U. pertusa*, Verlaque *et al.*, 2002), and Malaga, Spain (Couceiro *et al.*, 2011). In most instances, molecular data were decisive in appropriate taxonomic identification of this species, which was often previously misidentified by morphological data alone (Aguilar-Rosas *et al.*, 2008; Couceiro *et al.*, 2011; Hanyuda *et al.*, 2016). Therefore, its presence in the Mediterranean might even be considerably wider than presently known.

***Ulva ohnoi* Hiraoka *et* Shimada.** Distance analysis showed that the Tunisian specimen grouped with *U. ohnoi* from Cape Peloro lagoon, Italy (0 bp sequence divergence), and from Australia, China, North and South America (0.00-0.54%, 0-4 bp) (Fig. 2).

The new record of the Japanese bloom-forming *U. ohnoi* in Gabes, Tunisia, in a highly impacted area, close to commercial and fishing ports, chemical and oil industries, and tourist pathways (Figs 21-22) is noteworthy. The species was previously recorded in lake Ganzirri, Cape Peloro lagoon, northeastern Sicily, Italy, by Armeli *et al.* (2014), first report in the field in the Mediterranean and in Europe. The species was also found in Atlantic Spain (Gallardo *et* al. 2016), Gulf of Mexico and Florida (Melton *et al.*, 2016a), Venezuela (Melton *et al.*, 2016b), Iran (Pirian *et* al. 2016), India (Kazi *et* al. 2016), Japan (Hiraoka *et* al. 2004, Kirkendale *et* al. 2013), Korea (Bae 2010), Australia (Kirkendale *et* al. 2013), and Hawaiian Islands (O'Kelly *et* al. 2010).

In the Mediterranean, *U. ohnoi* had been previously identified in the algal fouling biomass on ships arriving at the commercial harbour at Sète, Mediterranean coast of France, a medium size port (Mineur *et al.*, 2007) and, similarly, in ballast water from ships arriving to Naples harbour, Italy, coming from Singapore, which had loaded water in Egypt and Lebanon (Flagella *et al.*, 2010). However, as stated by the latter authors themselves, colonization by this species had not been detected in the area (Flagella *et al.*, 2010). The Tunisian sample was found in impacted habitats, as well as those found in Cape Peloro lagoon, and the most likely mean of introduction is the maritime traffic considering that it is relevant both in Gabes harbour and in the Straits of Messina. Further studies are needed to investigate the actual distribution of this species in the Mediterranean, especially considering that all reports worldwide have been made by the use of DNA tools and that the species has been often misidentified under a mix of taxon names (Mineur *et al.*, 2007; O'Kelly *et al.*, 2010; Melton *et al.*, 2016a). Basing on present knowledge, this taxon is commonly regarded as a NIS, having a disjoint distribution and being recorded in highly disturbed sites. However, its presence in the Mediterranean, as well as in other sites, might be underestimated and its actual distribution, as well as its native range, be far different from what presently known.

Conclusions. The Mediterranean Sea has a long history of bioinvasions with a number of records higher than other European seas (Galil *et al.*, 2016). Many species of *Ulva* are often considered as widespread due to natural dispersal (cosmopolitan species), anthropogenic transport (NIS), or both (Kirkendale *et al.*, 2013). *Ulva* species have high dispersal potential due to their peculiar features, including good fouling capacity, dispersal efficiency and fast growth. It is recognized that species of *Ulva* can metabolize different environmental sources of nitrogen producing large biomass and, consequently, green tides (Melton *et al.*, 2016a). Therefore, they have a high potential for introduction and invasiveness if they arrive to altered environments with high availability of nutrients.

Criteria specific to distinguish between NIS and cosmopolitan species have been proposed by Heesch *et al.* (2009) and Kirkendale *et al.* (2013). A taxon is likely to be a NIS if it is found in highly modified environments and/or in close vicinity to potential vectors, such as frequent

maritime traffic, aquaculture sites and artificial modifications of natural barriers (i.e. Suez Canal). However, this is not strict and native species might also thrive in sites with high anthropogenic impacts, as well as introduced species can occur widely, including in undisturbed sites. In addition, NIS usually exhibit higher genetic similarity in comparison to samples from native area. Furthermore, it should be noted that most of the macroalgal species found on hulls are considered cosmopolitan and, consequently, at low risk of introduction outside their present range (Mineur *et al.*, 2007). This assumption may be misleading, however, as little information is available about their original native ranges. Marine floristic studies were uncommon for most sites before the twentieth century and an unknown amount of supposed cosmopolitan species may have had restricted ranges prior to their anthropogenic dispersal (Carlton 1996). It is likely that the distribution pattern of at least some *Ulva* species has been confused by repetitive human-mediated introductions over centuries of ship travelling.

The actual picture of the taxonomy of *Ulva* spp. in the Mediterranean as a whole is far to be clarified and the present data on Tunisian collections aim to be a step towards its clarification. At the best of our knowledge, this is the first study on the Tunisian green algal flora using DNA barcoding methods. The current taxonomic, biogeographical and ecological data gaps can be filled only by cooperative work and standardized methodologies (Galil *et al.*, 2016; Zenetos *et al.*, 2017). DNA barcoding demonstrated as a fast and effective tool for the genetic labelling of challenging taxa, such as Ulvacean species, especially in those areas that are subject to anthropogenic disturbances and quick changes of biodiversity. Data collected in such perspective are also valuable to increase the BOLD system catalogue, amplifying the biodiversity knowledge linked to geographical information, which becomes freely available for the scientific community. DNA barcodes are permanent labels assigned to specimens regardless any subsequent taxonomic or nomenclature variation. An effective monitoring of the biodiversity changes by means of a quick and accurate protocol, such as DNA barcoding, is essential to provide the basis for a correct environmental management.

Acknowledgements.

Dr. Gary Saunders and his colleagues at the Center for Environmental & Molecular Algal Research, University of New Brunswick, are kindly acknowledged for processing specimens within the ALGA (Algal Life Global Audit) project under the iBOL initiative WG1.8 Marine Bio-surveillance. Two anonymous referees are kindly acknowledged for their valuable contributions to improve the manuscript. This study was supported by grants to R.M. (Emmag Erasmus Mundus fellowship) and to G.G. and M.M. (INNOVAQUA PON02_00451_3362185).

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Legends

Fig. 1. Collection sites along the Tunisian coastline.

Fig. 2. Neighbor-Joining tree of *tuf*A sequences generated in the present work (coloured in bold type) and of representative Ulvaceae downloaded from Genbank (TA = type area). Scale bar: substitution per site.

Figs 3-8. Specimens of *Ulva compressa*. 3: Torre Faro, Italy (BOLD# GRAPP003-17); 4-5: Tunisia (BOLD# TUGRE007-17, BOLD# TUGRE003-17); 6-8: Venice Lagoon (BOLD# ITGRE013-11, BOLD# ITGRE014-11, BOLD# GREVE002-17). Scale bar: 2 cm.

Fig. 9. *Ulva mediterranea*, Tunisia (BOLD# TUGRE008-17). Fig. 10. *Ulva* torta, Tunisia (BOLD# TUGRE013-17). Scale bar: 2 cm.

Figs 11-14. Specimens of *Ulva fasciata*. 9-12: Cape Peloro lagoon, Italy (BOLD# ITGRE018-11, BOLD# ITGRE016-11, BOLD# ITGRE003-11, BOLD# GRAPP013-17). Scale bar: 2 cm.

Figs 15-20. Specimens of *Ulva laetevirens*. 13-14, 16-17: Torre Faro, Italy (BOLD# GRAPP014-17, BOLD# GRAPP006-17, BOLD# GRAPP008-17, BOLD# GRAPP012-17); 15: Tunisia (BOLD# TUGRE009-17); 18: Venice Lagoon, Italy (BOLD# ITGRE010-11). Scale bar: 2 cm.

Figs 21-22. Specimens of *Ulva ohnoi*. 19: Cape Peloro lagoon, Italy (BOLD# ITGRE019-11); 20: Tunisia (BOLD# TUGRE012-17). Scale bar: 2 cm.

Tab. 1 Species of foliose Ulvaceae identified by DNA barcoding in the Mediterranean Sea in the present paper and in previous literature.

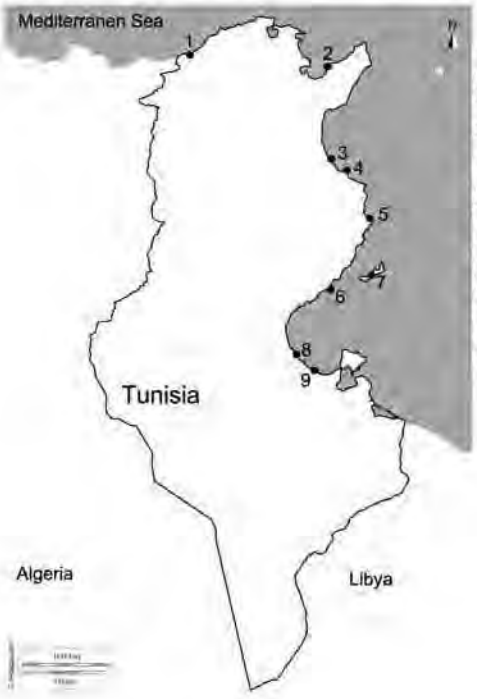
Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment[1]
Ulva australis Areschoug	Venice Lagoon, Italy (as *U. pertusa* Kjellman)	BOLD# ITGRE004-11 BOLD# ITGRE012-11 BOLD# GREVE001-17 GB# HE600186 GB# HE600187 GB# HE600188 GB# HE600189 GB# HE600190	Present study Wolf *et al.*, 2012	Japan (Kjellman, 1897), Northeast Asia (Hanyuda *et al.*, 2016), New Zealand (Heesch *et al.*, 2009), Australia (Kraft *et al.*, 2010), Indonesia, Singapore and the Philippines (Silva *et al.*, 1996), The Netherlands (Stegenga *et al.*, 2007), Spain (Couceiro *et al.*, 2011), Brittany (Coat *et al.*, 1998), Canada and the USA (Saunders & Kucera, 2010), Mexico (Aguilar-Rosas *et al.*, 2008), Yemen, Kenya, Mauritius and Tanzania (Silva *et al.*, 1996)	Thau Lagoon, France (Verlaque *et al.*, 2002), Venice Lagoon, Italy (Manghisi *et al.*, 2011), Malaga (Couceiro *et al.*, 2011)	*U. pertusa* Kjellman is a synonym based on genetic data (Couceiro *et al.*, 2011; Kirkendale *et al.*, 2013)	Likely, from North-eastern Asia; genetic data compared to Kirkendale *et al.* (2013) and Hanyuda *et al.* (2016); anthropized habitat
Ulva californica Wille	Venice Lagoon, Italy	GB# HE600173 GB# HE600174 GB# HE600175 GB# HE600176	Wolf *et al.*, 2012	Indopacific, Britain, Ireland, Senegal, Guadeloupe, Antarctic and the subantarctic islands (Guiry & Guiry, 2017)	Venice Lagoon (Wolf *et al.*,2012)		Likely; genetic sampling insufficient to assess the Mediterranean status; anthropized habitat
Ulva compressa Linnaeus	Kerkennah, Tunisia	BOLD# TUGRE007-17	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci *et al.*, 2014)	Consistent with use in Kirkendale *et al.* (2013)	Unlikely; various habitats
	Korbous, Tunisia	BOLD# TUGRE003-17 BOLD# TUGRE004-17	Present study				
	Sousse, Tunisia	BOLD# TUGRE005-17 BOLD# TUGRE006-17	Present study				
	Tabarka, Tunisia	BOLD# TUGRE001-17 BOLD# TUGRE002-17	Present study				
	Venice Lagoon, Italy	BOLD# ITGRE013-11 BOLD# ITGRE014-11 BOLD# GREVE002-17	Present study				
	Lido of Venice, Italy	GB# HE600184 GB# HE600185	Wolf *et al.*, 2012				
	Torre Faro, Messina, Italy	BOLD# GRAPP002-17 BOLD# GRAPP003-17	Present study				

[1] Based on criteria by Heesch *et al.* (2009) and Kirkendale *et al.* (2013).

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment[1]
Ulva fasciata Delile	Venice Lagoon, Italy	BOLD# ITGRE015-11	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci *et al.*, 2014)	Consistent with usage in Kraft *et al.* (2010), comparison with type necessary	Doubtful; genetic data compared to Kirkendale *et al.* (2013); anthropized habitat
	Cape Peloro Lagoon, Italy	BOLD# ITGRE003-11 BOLD# ITGRE016-11 BOLD# ITGRE017-11 BOLD# ITGRE018-11	Bertuccio *et al.*, 2013/2014				
		BOLD# GRAPP013-17	Present study				
Ulva mediterranea Alongi, Cormaci et G. Furnari	Zarat, Tunisia	BOLD# TUGRE008-17	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci *et al.*, 2014)	Consistent with usage in Hiraoka *et al.* (2017); genetic data compared also to Kirkendale *et al.* (2013)	Unlikely. Anthropized habitats
	Venice Lagoon, Italy	GB# HE600177	Wolf *et al.*, 2012				
Ulva laetevirens Areschoug	Monastir, Tunisia	BOLD# TUGRE009-17	Present study	Australia, Atlantic North America, China and Japan (Kirkendal *et al.*, 2013, unpublished in Genbank)	Common (Cormaci *et al.*, 2014); reported in Tunisia as *Ulva rigida* C. Agardh, see Cormaci *et al.*, 2014	Consistent with use in Kraft *et al.* (2010); synonymy with *Ulva rigida* C. Agardh likely but not asserted (see text).	Doubtful, possible introduction from Australia; genetic data compared to Kirkendale et al. (2013); various habitats
	Sousse, Tunisia	BOLD# TUGRE011-17					
	Tabarka, Tunisia	BOLD# TUGRE010-17					
	Venice Lagoon, Italy	BOLD# ITGRE010-11 BOLD# ITGRE011-11					
	Lido of Venice, Italy	GB# HE600178 GB# HE600179 GB# HE600180 GB# HE600181 GB# HE600182	Wolf *et al.*, 2012				
	Torre Faro, Messina, Italy	BOLD# ITGRE009-11 BOLD# GRAPP014-17 BOLD# GRAPP004-17 BOLD# GRAPP005-17 BOLD# GRAPP006-17 BOLD# GRAPP007-17 BOLD# GRAPP008-17 BOLD# GRAPP009-17 BOLD# GRAPP010-17 BOLD# GRAPP011-17 BOLD# GRAPP012-17	Present study				
	Ragusa, Italy	BOLD# ITGRE009-11 BOLD# ITGRE002-11	Present study				

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment[1]
Ulva linza Linneus	Venice Lagoon, Italy	GB# ITGRE005-11	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci *et al.*, 2014)	= *Ulva "procera"* (K. Ahlner) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope et J.R. Waaland *sensu* Kirkendale *et al.* (2013)	Unlikely; anthropized habitat
Ulva ohnoi Hiraoka et Shimada	Gabes, Tunisia	BOLD# TUGRE012-17	Present study	Spain (South Atlantic) (Gallardo *et al.*, 2016), Venezuela (Melton *et al.*, 2016), India (Kazi *et al.*, 2016), Iran (Pirian *et al.*, 2016), Japan (Hiraoka *et al.*, 2004, Kirkendale *et al.*, 2013), Korea (Bae, 2010), Australia (Kirkendale *et al.*, 2013), Hawaiian Islands (O'Kelly *et al.*, 2010)	Cape Peloro Lagoon (Armeli *et al.*, 2014); not previously reported in Tunisia		Likely, from Asia; anthropized habitats
	Cape Peloro Lagoon, Italy	BOLD# ITGRE019-11	Present study				
		BOLD# ITGRE019-11					
Ulva torta (Mertens) Trevisan	Chaffar, Sfax, Tunisia	BOLD# TUGRE013-17	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci *et al.*, 2014)	Consistent with use in Kirkendale *et al.* (2013)	Unlikely; undisturbed habitat
Ulva sp.	Cape Peloro Lagoon, Italy	BOLD# ITGRE020-11	Bertuccio *et al.*, 2013/2014	Australia, North America, South Korea (Guiry & Guiry, 2017)	Common (Guiry & Guiry, 2017); Italy (Furnari *et al.*, 2003)	= *Ulva flexuosa* subsp. *pilifera sensu* Bertuccio *et al.* (2013/2014); based on results of Mares *et al.* (2011)	Doubtful
		BOLD# ITGRE021-11					
		BOLD# ITGRE023-11					

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment[1]
Umbraulva dangeardii Wynne et Furnari	Torre Faro, Messina, Italy	BOLD# ITGRE007-11 BOLD# ITGRE008-11	Present study	Europe, Atlantic Islands, Africa, South-west Asia, Australia and New Zealand (Guiry & Guiry, 2017)	Common (Guiry & Guiry, 2017); Italy (Furnari *et al.*, 2003)	=*Umbraulva olivascens* (P.J.L. Dangeard) G. Furnari	Unlikely; anthropized habitat



	Site	Latitude	Longitude
1	Tabarka	36.9579 N	8.7554 E
2	Korbous	36.8279 N	10.5691 E
3	Sousse	35.8335 N	10.6417 E
4	Monastir	35.7737 N	10.8377 E
5	Chebba	35.2421 N	11.1392 E
6	Chaffar, Sfax	34.5334 N	10.5841 E
7	Kerkennah	34.6622 N	11.1222 E
8	Gabes	33.8918 N	10.1188 E
9	Zarat	33.6901 N	10.3819 E

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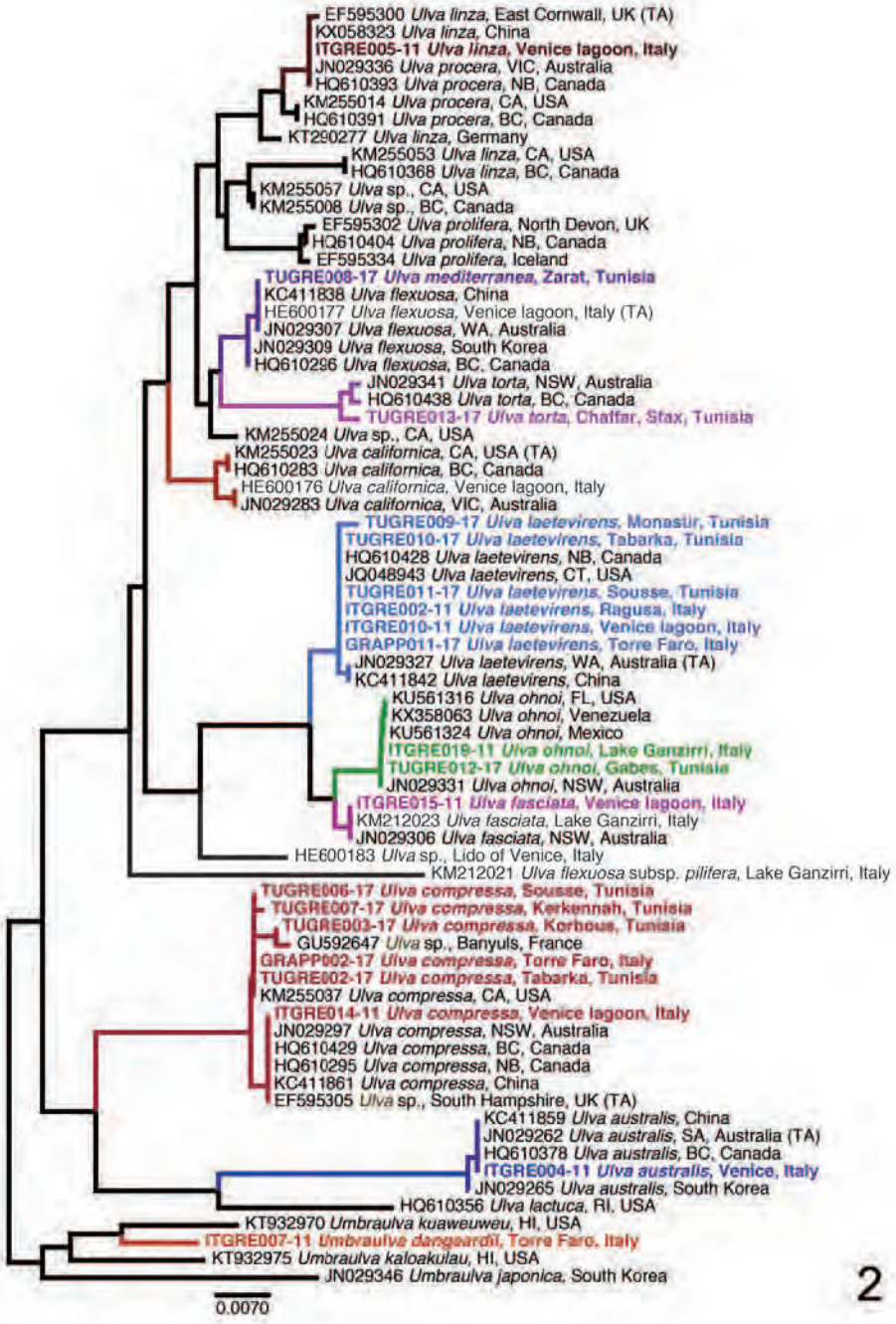
EF595300 Ulva linza, East Cornwall, UK (TA)
KX058323 Ulva linza, China
ITGRE005-11 Ulva linza, Venice lagoon, Italy
JN029336 Ulva procera, VIC, Australia
HQ610393 Ulva procera, NB, Canada
KM255014 Ulva procera, CA, USA
HQ610391 Ulva procera, BC, Canada
KT290277 Ulva linza, Germany
KM255053 Ulva linza, CA, USA
HQ610368 Ulva linza, BC, Canada
KM255057 Ulva sp., CA, USA
KM255008 Ulva sp., BC, Canada
EF595302 Ulva prolifera, North Devon, UK
HQ610404 Ulva prolifera, NB, Canada
EF595334 Ulva prolifera, Iceland
TUGRE008-17 Ulva mediterranea, Zarat, Tunisia
KC411838 Ulva flexuosa, China
HE600177 Ulva flexuosa, Venice lagoon, Italy (TA)
JN029307 Ulva flexuosa, WA, Australia
JN029309 Ulva flexuosa, South Korea
HQ610296 Ulva flexuosa, BC, Canada
JN029341 Ulva torta, NSW, Australia
HQ610438 Ulva torta, BC, Canada
TUGRE013-17 Ulva torta, Chaffar, Sfax, Tunisia
KM255024 Ulva sp., CA, USA
KM255023 Ulva californica, CA, USA (TA)
HQ610283 Ulva californica, BC, Canada
HE600176 Ulva californica, Venice lagoon, Italy
JN029283 Ulva californica, VIC, Australia
TUGRE009-17 Ulva laetevirens, Monastir, Tunisia
TUGRE010-17 Ulva laetevirens, Tabarka, Tunisia
HQ610428 Ulva laetevirens, NB, Canada
JQ048943 Ulva laetevirens, CT, USA
TUGRE011-17 Ulva laetevirens, Sousse, Tunisia
ITGRE002-11 Ulva laetevirens, Ragusa, Italy
ITGRE010-11 Ulva laetevirens, Venice lagoon, Italy
GRAPP011-17 Ulva laetevirens, Torre Faro, Italy
JN029327 Ulva laetevirens, WA, Australia (TA)
KC411842 Ulva laetevirens, China
KU561316 Ulva ohnoi, FL, USA
KX358063 Ulva ohnoi, Venezuela
KU561324 Ulva ohnoi, Mexico
ITGRE019-11 Ulva ohnoi, Lake Ganzirri, Italy
TUGRE012-17 Ulva ohnoi, Gabes, Tunisia
JN029331 Ulva ohnoi, NSW, Australia
ITGRE015-11 Ulva fasciata, Venice lagoon, Italy
KM212023 Ulva fasciata, Lake Ganzirri, Italy
JN029306 Ulva fasciata, NSW, Australia
HE600183 Ulva sp., Lido of Venice, Italy
KM212021 Ulva flexuosa subsp. pilifera, Lake Ganzirri, Italy
TUGRE006-17 Ulva compressa, Sousse, Tunisia
TUGRE007-17 Ulva compressa, Kerkennah, Tunisia
TUGRE003-17 Ulva compressa, Korbous, Tunisia
GU592647 Ulva sp., Banyuls, France
GRAPP002-17 Ulva compressa, Torre Faro, Italy
TUGRE002-17 Ulva compressa, Tabarka, Tunisia
KM255037 Ulva compressa, CA, USA
ITGRE014-11 Ulva compressa, Venice lagoon, Italy
JN029297 Ulva compressa, NSW, Australia
HQ610429 Ulva compressa, BC, Canada
HQ610295 Ulva compressa, NB, Canada
KC411861 Ulva compressa, China
EF595305 Ulva sp., South Hampshire, UK (TA)
KC411859 Ulva australis, China
JN029262 Ulva australis, SA, Australia (TA)
HQ610378 Ulva australis, BC, Canada
ITGRE004-11 Ulva australis, Venice, Italy
JN029265 Ulva australis, South Korea
HQ610356 Ulva lactuca, RI, USA
KT932970 Umbraulva kuaweuweu, HI, USA
ITGRE007-11 Umbraulva dangeardii, Torre Faro, Italy
KT932975 Umbraulva kaloakulau, HI, USA
JN029346 Umbraulva japonica, South Korea
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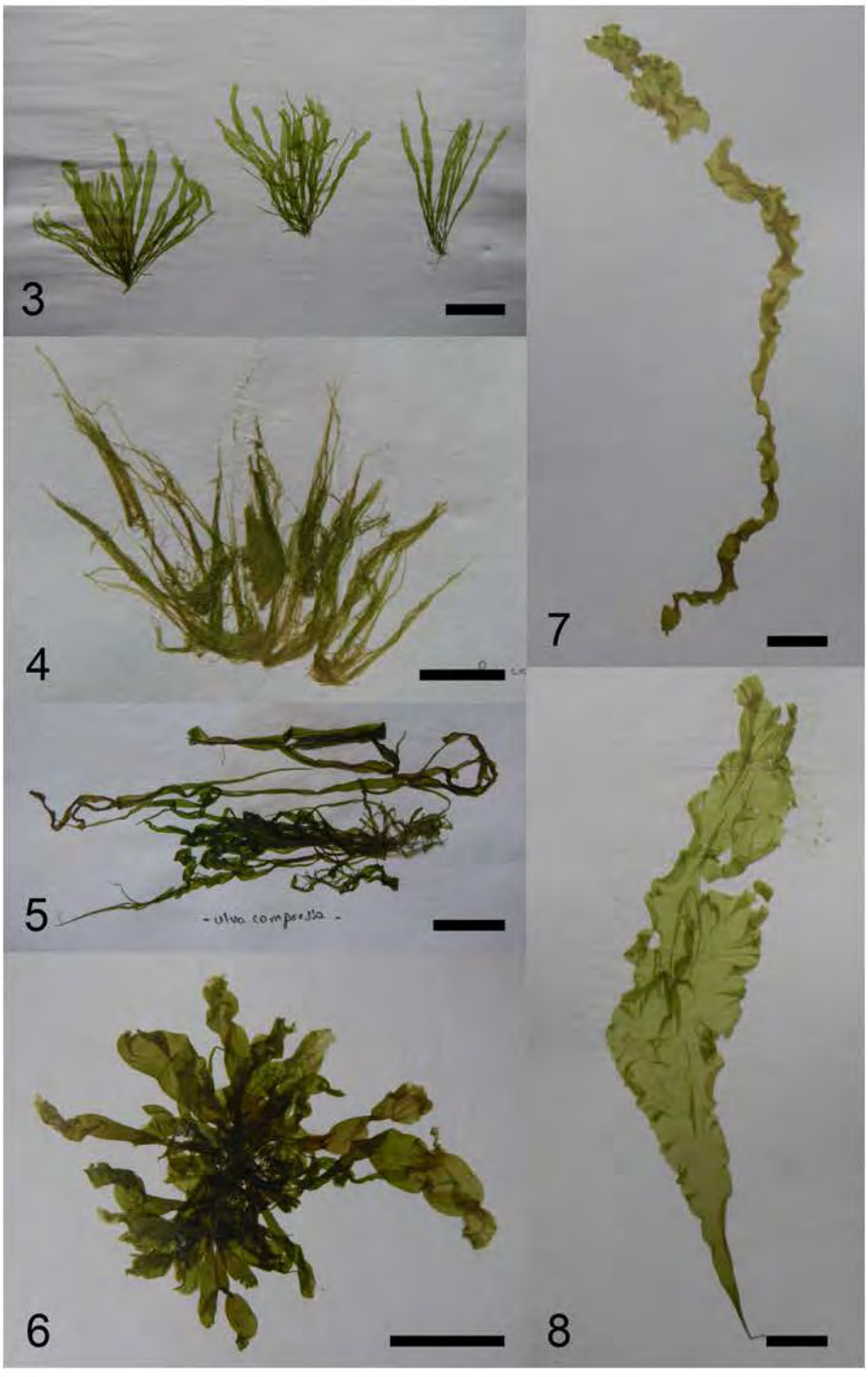
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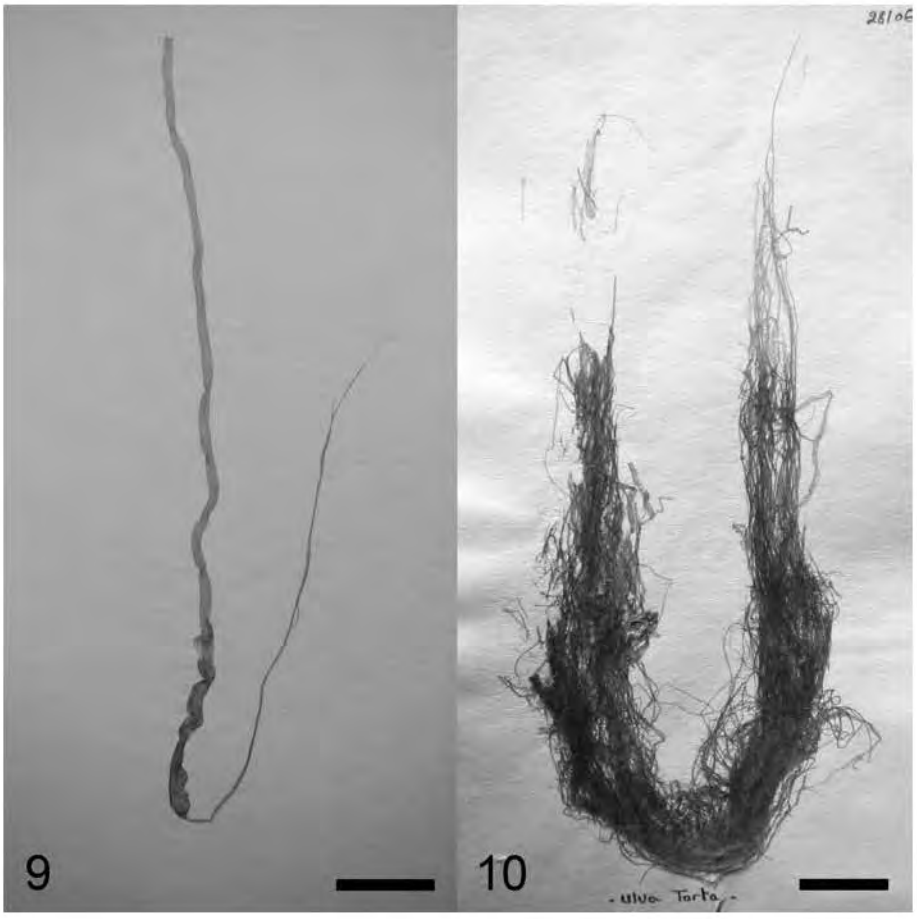
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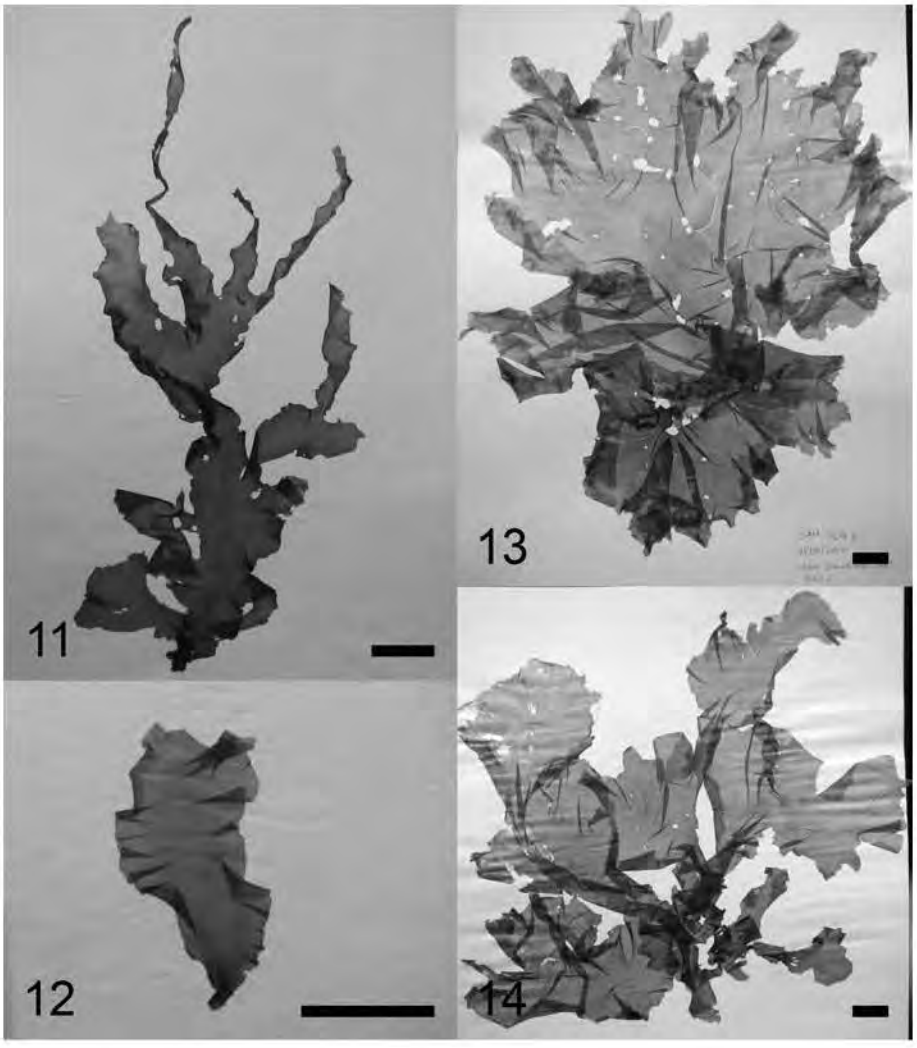
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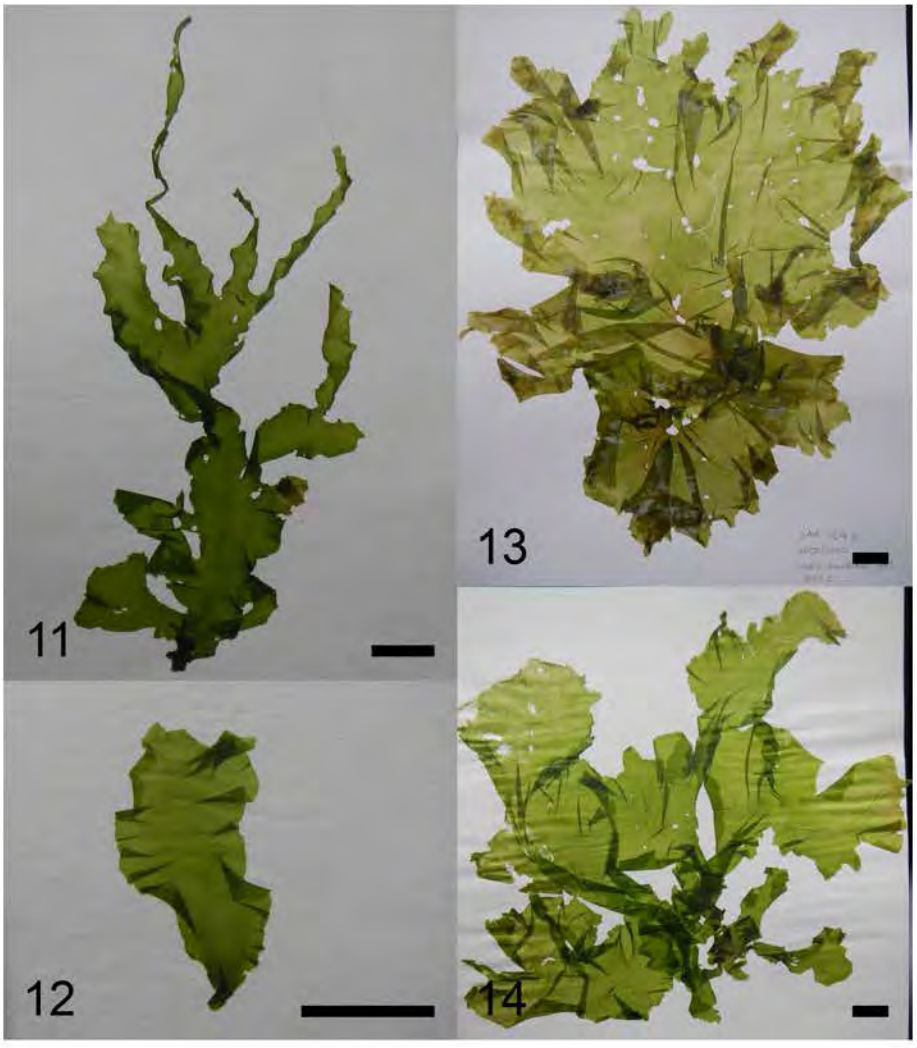
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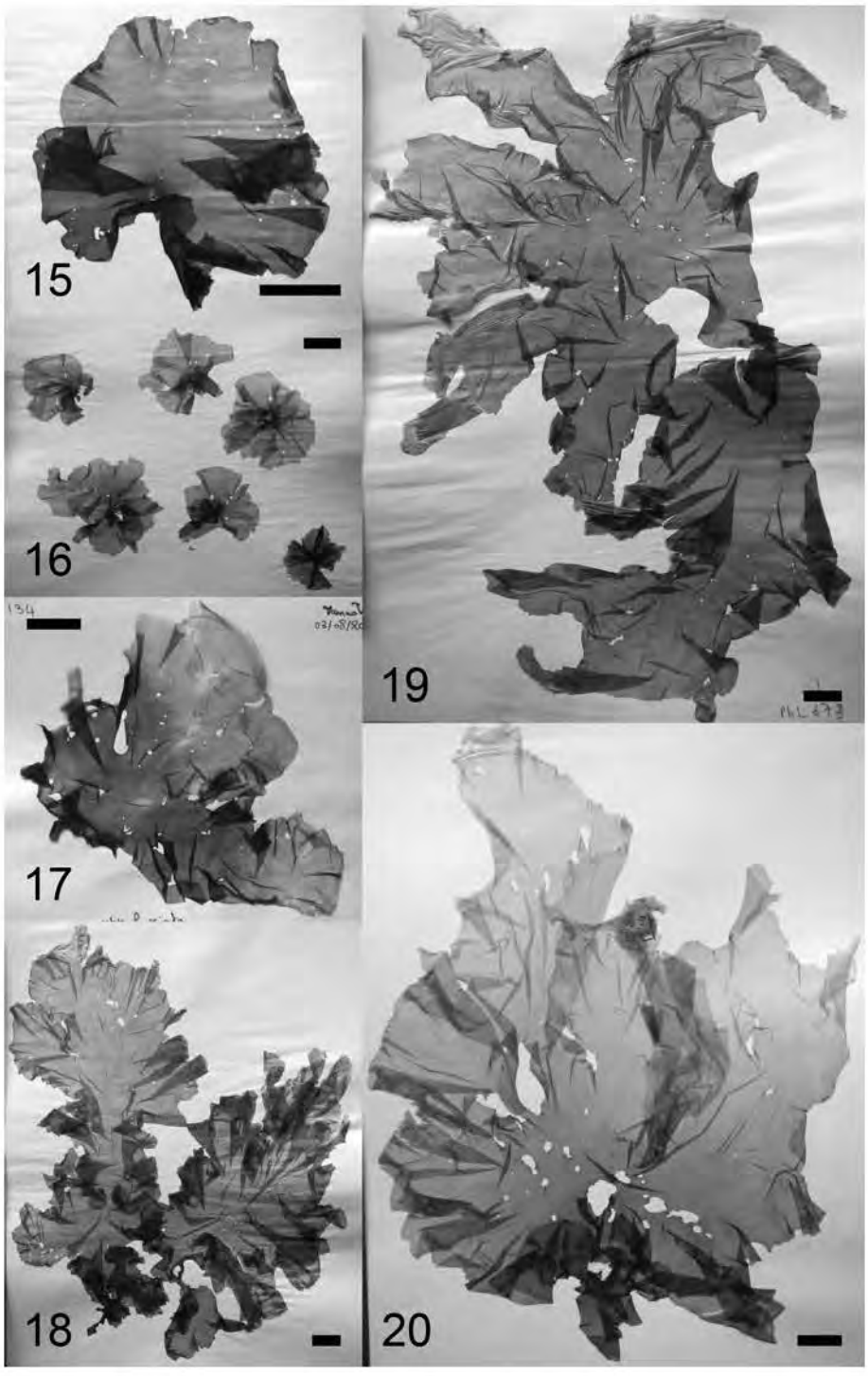
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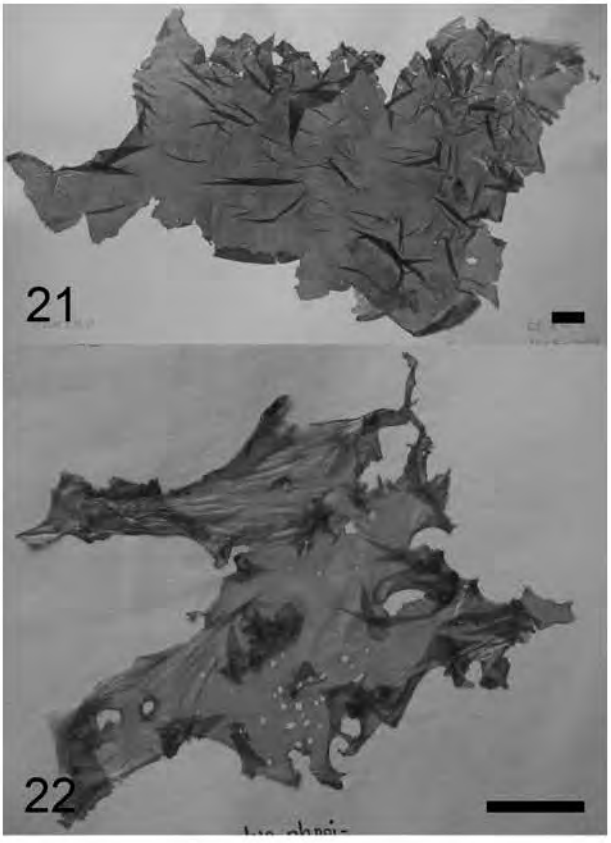
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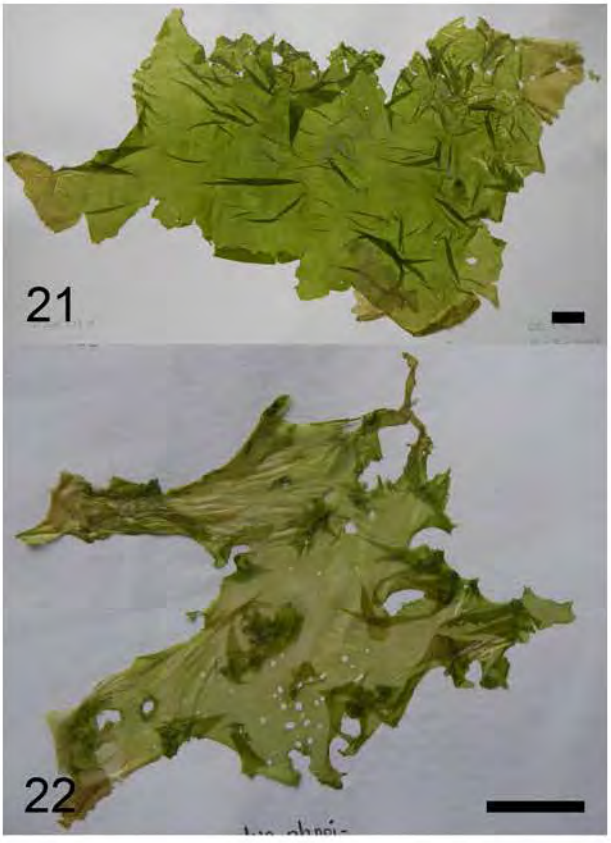
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6. Conclusions

Marine biodiversity includes a huge genetic variability within the species and their populations and it assessment is still a challenging task, particularly regarding the phyla reputed as difficult in their identification such as algae. In fact, seaweeds or macroalgae are important aquatic organisms for understanding ecosystem processes, conservation, and water quality. Unfortunately, algal biodiversity can be impacted upon negatively by factors such as global warming, increased environmental stress arising from fisheries and aquaculture activities, and by accidental introductions of invasive species. For this reason, it is important to catalogue the macroalgal biodiversity of these environments to assist in their conservation and utilization. Identification of algae is a challenging task, which requires experience, skills and access to the literature due to simply morphologies, phenotypic plasticity and convergentevolution. In the past three decades, molecular techniques have been used to resolve many taxonomic problems and to re-assess the global diversity of seaweeds. In these framework, DNA barcoding (Hebert *et al.,* 2003) rely as a new methodfor species recognition and discovery by the use of a short fragment of DNA from a standardized region of the genome.

Within a program of census of macroalgal species along Tunisian coasts, the target of this thesis was to carry on an effective survey of the macroalgal diversity of Tunisia, which allowed to reveal cryptic species, allochthonous introductions and to identify problematic taxa.

As opposed to morphological identifications, molecular methods are better tools for a first approach towards screening and uniting biological specimens in genetic groups as a first step to assigning them to species and genera in a strategy best termed molecular-assisted alpha taxonomy (MAAT) (Filloramo & Saunders, 2016). This methodology is agreeable in organisms with simple morphologies, with a high degree of phenotypic plasticity or convergence, and heteromorphic life histories, like marine macroalgae.

In the present thesis, the occurrence of two new alien species, *Hypnea cervicornis* J. Agardh and *Spermothamnion cymosum* (Harvey) De Toni is reported, the latter considered as doubtful for the Mediterranean Sea, collected in Tabarka, close to a tourist harbour, and confirm the presence of another alien species, *Hypnea "cornuta"* (Kützing) J. Agardh, highlighting cryptic diversity among the Mediterranean reports of this taxon, collected at Gabes and Zarat, close to commercial and fishing harbours.

Furthermore, five genetic species of *Ulva* were recognized along Tunisian coasts, namely the native species *Ulva compressa* Linnaeus, *Ulva flexuosa* Wulfen, and *Ulva torta* (Mertens) Trevisan, and the non indigenous species (NIS) *Ulva laetevirens* Areschoug and *Ulva ohnoi* Hiraoka *et* Shimada, both newly reported for Tunisia.

It is widely acknowledged that taxonomic identification within most algal genera by means of dichotomous keys based on morphological characters, is a challenging task because of the paucity of available characters and their significant plasticity in response to environmental factors (Heesch *et al.*, 2009; Hofmann *et al.*, 2010; Couceiro *et al.*, 2011). As a consequence, DNA data have been progressively used as an easier alternative for species recognition. The main limit of MAAT, however, is that type sequences, generated from type specimens, or at least topotype material, should be used as a reference to define species or clarify species concepts (Heesch *et al.*, 2009; Hofmann *et al.*, 2010). Nevertheless, these data are rarely available, particularly when type specimens are very old, or lost, and/or type localities are unknown or vague, as is, for instance, the case with most of the *Ulva* type specimens described by Linnaeus (1753) (Womersley, 1984), and the names used in sequence data retrieved from GenBank need to be used with caution (Heesch *et al.*, 2009).

At the best of our knowledge, this is the first study dealing with macroalgal taxonomy in Tunisia using a DNA approach.

This kind of paper is also useful to add records to the BOLD system catalogue, amplifying the biodiversity knowledge linked to geographical information, and making them freely available for the scientific community. Its added value in comparison to a classic floristic list is that DNA barcodes are permanent labels assigned to specimens regardless any subsequent taxonomic or nomenclature variation.

An effective monitoring of the biodiversity changes by means of a quick and accurate tool, such as DNA barcoding, is essential to provide the basis for a correct environmental management.

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Appendix 1. List of Specimens

A.1.1. Project: TUGRE - Greens of Tunisia (phylum Chlorophyta)

BOLD Process ID	Sample ID	Class	Order	Family	Species	Collection Date	Site	Lat	Lon
TUGRE001-17	RM0190S	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	04-Apr-2015	Tabarka	36.9579	8.7554
TUGRE007-17	RM0067	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	01-Jun-2014	Kerkennah	34.6622	11.1222
TUGRE006-17	RM0296	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	26-Mar-2016	Sousse	35.8335	10.6417
TUGRE005-17	RM00S1	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	10-Aug-2015	Sousse	35.8335	10.6417
TUGRE004-17	RM0229	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	08-Apr-2015	Korbous	36.8279	10.5691
TUGRE003-17	RM0228	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	08-Apr-2015	Korbous	36.8279	10.5691
TUGRE002-17	RM0203	Ulvophyceae	Ulvales	Ulvaceae	*Ulva compressa*	04-Apr-2015	Tabarka	36.9579	8.7554
TUGRE008-17	RM000A	Ulvophyceae	Ulvales	Ulvaceae	*Ulva flexuosa*	12-Mar-2014	Zarat	33.6901	10.3819
TUGRE011-17	RM0270	Ulvophyceae	Ulvales	Ulvaceae	*Ulva laetevirens*	12-Apr-2015	Sousse	35.8335	10.6417
TUGRE010-17	RM0190E	Ulvophyceae	Ulvales	Ulvaceae	*Ulva laetevirens*	04-Apr-2015	Tabarka	36.9579	8.7554
TUGRE009-17	RM0134	Ulvophyceae	Ulvales	Ulvaceae	*Ulva laetevirens*	03-Aug-2014	Monastir	35.7737	10.8377
TUGRE012-17	RM0279	Ulvophyceae	Ulvales	Ulvaceae	*Ulva ohnoi*	10-Aug-2015	Gabes	33.9181	10.1188
TUGRE013-17	RM0089	Ulvophyceae	Ulvales	Ulvaceae	*Ulva torta*	28-Jun-2014	Chaffar. Sfax	34.5334	10.5841

A.1.2. Project: TURED - Reds of Tunisia (phylum Rhodophyta)

BOLD Process ID	Sample ID	Class	Order	Family	Species	Collection Date	Site	Lat	Lon
TURED001-17	RM0254	Florideophyceae	Corallinales	Corallinaceae	*Amphiroa beauvoisii*	10-Apr-2015	Monastir	35.7737	10.8377
TURED002-17	RM0050	Florideophyceae	Corallinales	Corallinaceae	*Amphiroa cryptarthrodia*	27-Apr-2014	Port of Chebba	35.2308	11.1629
TURED003-17	RM0119	Florideophyceae	Corallinales	Corallinaceae	*Amphiroa rigida*	30-Jul-2014	Tabarka	36.9579	8.7554
TURED004-17	RM0282	Florideophyceae	Rhodymeniales	Champiaceae	*Champia sp.*	10-Aug-2015	Gabes	33.9181	10.1188
TURED005-17	RM0233	Florideophyceae	Gigartinales	Gigartinaceae	*Chondracanthus acicularis*	08-Apr-2015	Korbous	36.8279	10.5691
TURED007-17	RM0261	Florideophyceae	Corallinales	Corallinaceae	*Corallina caespitosa*	12-Apr-2015	Sousse	35.8335	10.6417
TURED006-17	RM0100	Florideophyceae	Corallinales	Corallinaceae	*Corallina caespitosa*	01-Aug-2014	Sousse	35.8335	10.6417
TURED008-17	RM0253	Florideophyceae	Corallinales	Corallinaceae	*Corallina caespitosa*	10-Apr-2015	Monastir	35.7737	10.8377
TURED010-17	RM0001	Florideophyceae	Ceramiales	Rhodomelaceae	*Digenea simplex*	05-Feb-2014	Ksour Essef	35.419	11.0406
TURED009-17	RM0231	Florideophyceae	Corallinales	Corallinaceae	*Ellisolandia sp.*	08-Apr-2015	Korbous	36.8279	10.5691
TURED011-17	RM0223	Florideophyceae	Rhodymeniales	Champiaceae	*Gastroclonium sp.*	08-Apr-2015	Korbous	36.8279	10.5691
TURED012-17	RM0054	Florideophyceae	Halymeniales	Halymeniaceae	*Grateloupia filicina*	27-Apr-2014	Port of Chebba	35.2308	11.1629
TURED015-17	RM0062	Florideophyceae	Halymeniales	Halymeniaceae	*Halymenia floresii*	01-Jun-2014	Kerkennah	34.6622	11.1222
TURED016-17	RM0281	Florideophyceae	Halymeniales	Halymeniaceae	*Halymenia floresii*	10-Aug-2015	Gabes	33.9181	10.1188
TURED022-17	RM0218	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cervicornis*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED017-17	RM0155	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cornuta*	10-Aug-2014	Gabes	33.9181	10.1188
TURED018-17	RM0288	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cornuta*	10-Aug-2015	Gabes	33.9181	10.1188
TURED019-17	RM0289	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cornuta*	10-Aug-2015	Gabes	33.9181	10.1188
TURED020-17	RM0292	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cornuta*	10-Aug-2015	Gabes	33.9181	10.1188
TURED021-17	RM0167	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea cornuta*	15-Aug-2014	Zarat	33.6901	10.3819
TURED026-17	RM0271	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea musciformis*	12-Apr-2015	Sousse	35.8335	10.6417
TURED023-17	RM0007	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea musciformis*	01-Mar-2014	Le Kram	36.8305	10.3188
TURED024-17	RM0097	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea musciformis*	01-Aug-2014	Sousse	35.8335	10.6417
TURED025-17	RM0098	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea musciformis*	01-Aug-2014	Sousse	35.8335	10.6417
TURED027-17	RM0295	Florideophyceae	Gigartinales	Cystocloniaceae	*Hypnea musciformis*	13-Aug-2015	Sousse	35.8335	10.6417
TURED028-17	RM0074	Florideophyceae	Corallinales	Corallinaceae	*Jania adhaerens*	22-Jun-2014	Chebba	35.2421	11.1392
TURED029-17	RM0169	Florideophyceae	Corallinales	Corallinaceae	*Jania adhaerens*	30-Aug-2014	Zarzis	33.5159	11.1178

BOLD Process ID	Sample ID	Class	Order	Family	Species	Collection Date	Site	Lat	Lon
TURED013-17	RM0202	Florideophyceae	Corallinales	Corallinaceae	*Jania subulata*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED014-17	RM0232	Florideophyceae	Corallinales	Corallinaceae	*Jania subulata*	08-Apr-2015	Korbous	36.8279	10.5691
TURED033-17	RM0081	Florideophyceae	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	22-Jun-2014	Chebba	35.2421	11.1392
TURED034-17	RM0140	Florideophyceae	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	03-Aug-2014	Monastir	35.7737	10.8377
TURED031-17	RM0070	Florideophyceae	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	01-Jun-2014	Kerkennah	34.6622	11.1222
TURED032-17	RM0051	Florideophyceae	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	27-Apr-2014	Port of Chebba	35.2308	11.1629
TURED030-17	RM0033	Florideophyceae	Ceramiales	Rhodomelaceae	*Palisada tenerrima*	20-Apr-2014	Kerkennah	34.6622	11.1222
TURED039-17	RM0052	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Peyssonnelia rubra*	27-Apr-2014	Chebba	35.2421	11.1392
TURED035-17	RM0116	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Peyssonnelia rubra*	30-Jul-2014	Tabarka	36.9579	8.7554
TURED036-17	RM0123	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Peyssonnelia squamaria*	30-Jul-2014	Tabarka	36.9579	8.7554
TURED037-17	RM0180	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Peyssonnelia squamaria*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED038-17	RM0137	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Peyssonnelia squamaria*	03-Aug-2014	Monastir	35.7737	10.8377
TURED040-17	RM0184	Florideophyceae	Gigartinales	Phyllophoraceae	*Phyllophora sp.*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED045-17	RM0198	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED041-17	RM0005	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	01-Mar-2014	Le Kram	36.8305	10.3188
TURED042-17	RM0043	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	25-Apr-2014	Sidi Bou Said	36.8664	10.3501
TURED043-17	RM0099	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	01-Aug-2014	Sousse	35.8335	10.6417
TURED044-17	RM0125	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	30-Jul-2014	Tabarka	36.9579	8.7554
TURED046-17	RM0139	Florideophyceae	Gelidiales	Pterocladiaceae	*Pterocladiella capillacea*	03-Aug-2014	Monastir	35.7737	10.8377
TURED047-17	RM0115	Florideophyceae	Peyssonneliales	Peyssonneliaceae	*Ramicrusta sp.*	30-Jul-2014	Tabarka	36.9579	8.7554
TURED048-17	RM0165	Florideophyceae	Rhodymeniales	Rhodymeniaceae	*Rhodymenia ardissonei*	15-Aug-2014	Zarat	33.6901	10.3819
TURED049-17	RM0230	Florideophyceae	Gigartinales	Phyllophoraceae	*Schottera nicaeensis*	08-Apr-2015	Korbous	36.8279	10.5691
TURED050-17	RM0236	Florideophyceae	Gigartinales	Phyllophoraceae	*Schottera nicaeensis*	08-Apr-2015	Korbous	36.8279	10.5691
TURED054-17	RM0209	Florideophyceae	Ceramiales	Wrangeliaceae	*Spermothamnion cymosum*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED055-17	LLG5051	Florideophyceae	Ceramiales	Wrangeliaceae	*Spermothamnion cymosum*	08-Jul-2014	Finistère	48.7377	-4.0421
TURED052-17	RM0182	Florideophyceae	Gigartinales	Sphaerococcaceae	*Sphaerococcus coronopifolius*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED053-17	RM0212	Florideophyceae	Gigartinales	Sphaerococcaceae	*Sphaerococcus coronopifolius*	04-Apr-2015	Tabarka	36.9579	8.7554
TURED051-17	RM0113	Florideophyceae	Gigartinales	Sphaerococcaceae	*Sphaerococcus coronopifolius*	30-Jul-2014	Tabarka	36.9579	8.7554

Appendix 2. DNA protocols

A.2.1. DNA extraction recipes

DNA Extraction Buffer (Reds)[14]

		Final concentrations:
0.543 g	Tris base	-
0.870 g	Tris-HCl	0.1 M Tris-HCl pH 8.0
1.86 g	Na_2 EDTA·$2H_2O$	0.05 M
1.165 g	NaCl	0.2 M
24.54 g	K Acetate	2.5 M

Add < 100 ml ddH_2O.
Adjust pH to 8.0 with (about 0.2 g of NaOH pellets).
Adjust final volume to 100 ml.
Sterilize (autoclave or filter). Store at room temperature.

2x CTAB buffer
10 ml of 1 M Tris-HCl, pH 8.0
28 ml of 5 M NaCl
4 ml of 0.5 M EDTA
2 g of CTAB (Cetyltrimethyl ammonium bromide)
Adjust final volume to 100 ml
Sterilize (filter). Store at room temperature.

If needed, add 1% polyvinylpyrrolidone (PVP) to an aliquot just prior to starting extraction. Once it has been added the shelf life of the buffer is only 2-3 days.

1 M Tris-HCl pH 8.00
Dissolve 1.211 g of Tris base in 8 ml of distilled H2O. Adjust pH to 8.0 adding 0.42 ml of fuming HCl. Let the solution cool to room temperature before definitive pH adjustment Adjust final volume to 10 ml with distilled H2O.
Sterilize (autoclave or filter).

0.5 M Na_2EDTA
Add 1.861 g of Na_2EDTA·$2H_2O$ to 8 ml of distilled H_2O. Stir vigorously on a magnetic stirrer, adjust pH to 8.0 with NaOH (about 0.2 g of NaOH pellets).
Sterilize (autoclave or filter).
NOTE: Na_2EDTA will not go into solution until pH is adjusted to approximately 8.0 by the addition of NaOH.

[14] SAUNDERS G.W., 1993 - Gel purification of red algal genomic DNA: an inexpensive and rapid method for the isolation of polymerase chain reaction-friendly DNA. *Journal of Phycology* 29: 251-254.

5 M NaCl
Disolve 29.21 g of NaCl in 80 ml of distilled H2O. Adjust volume to 100 ml with distilled H2O.
Aliquot and sterilize (autoclave or filter).

10% Tween 20
Mix 1 ml of Tween 20 and 9 ml of sterile ddH_2O. Store at room temperature.

Proteinase K (20 mg/ml)
Add to 100 mg of Proteinase K (powder stored at -20°C)
5 ml of sterile ddH_2O.
Shake to completely mix. Dispense into 200 µl aliquots in microtubes and store at -20°C.

10% PVP
Disolve 10 g of PVP in sterile (autoclaved) H_2O up to 100 ml.

SDS 10%
Dissolve 10 g of SDS in 90 of sterile distilled H_2O. Warm to 68 °C to dissolve. Adjust pH to 7.2 with a few drops of fuming HCl. Adjust volume to 100 ml. Aliquot. Do not sterilize.
NOTE: SDS precipitates at room temperature. Warm to 60°C before use.

Chloroform-isoamyil alcohol (24:1)
Mix 24 volumes of chloroform with 1 volume of isoamyil alcohol.
Work in a fume hood. Store at room temperature.

A.2.2. Polymerase Chain Reaction recipes

2.5 mM (each) dNTPs
Mix:

dNTPs stock (10 mM each)	50 ul
sterile ddH_2O	150 ul

Store at –20 °C. Prepare a few of such 200 ul aliquots.

Primers
Dilute primers according to producer instruction up to 100 uM (stock).
Prepare PCR working aliquots (10 um):

Stock primer (100 uM)	5 ul
sterile ddH_2O	45 ul

Store at –20 °C.

10% PVP
Dissolve 10 g of PVP in sterile (autoclaved) H_2O up to 100 ml.
Filter sterilize aliquots of 1 ml to use in PCR.

A.2.3. Agarose gel electrophoresis recipes

Ethidium bromide (10 mg ml^{-1})
Add 100 mg of ethidium bromide to 10 ml of distilled H2O. Store at 4 °C in the dark for 1-2 days to dissolve.
Store at 4 °C in the dark.

Gel loading buffer 6X
Type III (Sambrook et al.1989, table B.13)
100 µl 5% bromophenol blue
100 µl 5 % xylene cyanol FF
600 µl glycerol
1.2 ml sterile distilled H_2O

5% bromophenol blue
Dissolve 0.5 g of bromophenol blue in 10 ml of sterile distilled H_2O.

Xilene cyanol FF 5 %
Dissolve 0.5 g of xylene cyanol FF in 10 ml of sterile distilled H_2O.

TAE 50X
121 g Tris base
28.55 ml glacial acetic acid
50 ml 0.5 M Na_2EDTA (pH 8.0)
Adjust volume to 500 ml with distilled H_2O. Store at room temperature.

TAE 1X EtBr free: dilute 1:50 with distilled water.
(**TAE 1X with EtBr:** add 40 µl of EtBr (stock solution: 10 mg ml^{-1}) per litre of **TAE 1X**).

0.5 M Na_2EDTA
Add 1.861 g of $Na_2EDTA \cdot 2H_2O$ to 8 ml of distilled H_2O. Stir vigorously on a magnetic stirrer, adjust pH to 8.0 with NaOH (about 0.2 g of NaOH pellets).
Sterilize (autoclave or filter).
Note: Na_2EDTA will not go into solution until pH is adjusted to approximately 8.0 by the addition of NaOH.