

RESEARCH ARTICLE

Identifying alien macroalgae through DNA barcoding: the case of *Hypnea cornuta* (Cystocloniaceae, Rhodophyta)

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Abstract

- 1 - Introduction of non-indigenous macroalgae has become a major topic in the last decades. Potential vectors are aquaculture, fouling on hulls, ballast water, aquarium trading, fishing nets.
- 2 - Within a program of census of macroalgal alien species through DNA barcoding we found a population of previously unreported rhodophyte in Cape Peloro Lagoon and nearby marine coasts (north-eastern Sicily, Italy).
- 3 - All collected individuals were identified as the alien species *Hypnea cornuta* based on morphological characters confirmed by the analysis of COI-5' sequences.

Keywords: alien species, COI-5' region, DNA barcoding, *Hypnea cornuta*, Mediterranean Sea, Rhodophyta, Strait of Messina, transitional environments.

Introduction

The Mediterranean Sea is one of the regions most severely affected by the introduction and spread of alien marine species considered one of the main threats to biodiversity at different scales and extent (Hulme *et al.*, 2009). A non-indigenous species is any species whose translocation into an environment outside its native geographical habitat has been either human-mediated (intentionally or accidentally) or produced by active dispersal via natural pathways (Streftaris *et al.*, 2005). The amount of marine non-indigenous macroalgae is increasing world-wide over the past two decades also depending on the raised attention of the scientific community

to the problem and due to the availability of new tools for species identification, such as DNA sequencing or global databases freely accessible on the world-wide-web. Each introduction involves one vector of transfer (Verlaque *et al.*, 2007) and potential vectors of introductions include aquaculture activities, shipping (fouling on hulls, ballast water), trans-oceanic canals, aquarium trade, fishing nets (Por, 1978; Farnham, 1980; Verlaque and Riouall, 1989; Zibrowius, 1992; Farnham, 1994; Carlton and Hodder, 1995; Ribera Siguan and Boudouresque, 1995; Jousson *et al.*, 1998; Olsen *et al.*, 1998; Ceccherelli and Piazzini, 2001; Magri *et al.*, 2001; Ribera Siguan, 2002; Minchin and Gollasch,

2003; Streftaris *et al.*, 2005; Verlaque and Boudouresque, 2005; Schaffelke *et al.*, 2006; Booth *et al.*, 2007; Verlaque *et al.*, 2007).

As for macrophytes, shellfish transfer is considered to be the most important vector of introduction (Eno *et al.*, 1997; Maggs and Stegenga, 1999; Verlaque, 2001; Ribera Siguan, 2002; Verlaque *et al.*, 2007; Manghisi *et al.*, 2010).

Historically, deballasting from ships and the transportation on the hulls of ships of fixed (fouling) or non-fixed (clinging) species are certainly the most ancient vectors of species introduction. Furthermore, the opening of the Suez Canal in 1869, linking two basins separated for several million years, the Mediterranean and the Red Sea, has led to the introduction of hundreds of Lesseptian immigrants.

In macroalgae the discrimination of taxa is complicated by the high degree of morphological variation within individual species, which may be chiefly influenced by environmental factors occurring in specific habitats. Progress in molecular systematics has led to increasing use of multiple and newly developed markers for identifying species with widespread distributions or without clear-cut morphological diagnostic criteria. Recently, DNA barcoding demonstrated as a quick and efficient tool for the accurate identification of alien species and for the evaluation of their vectors (McDevit and Saunders, 2009; Manghisi *et al.*, 2010; Saunders and Kucera, 2010).

Within a program of census of macroalgal alien species through DNA barcoding along Sicilian coasts, we found a population of *Hypnea cornuta* (Kützing) J. Agardh, previously unreported in Cape Peloro lagoon. In this area, two brackish lakes form a complex ecosystem of great interest because it is under significant anthropogenic pressure. They are located in the Oriented Nature Reserve of Cape Peloro (Region Sicily, ref. D.A. 21/06/01), which is also a

Site of Community Importance (ref. 92/43/EEC) and a Special Area of Conservation (ref. 79/409/EEC).

Materials and Methods

Specimens of *Hypnea cornuta* were collected monthly, between April 2009 and September 2010, at six sites along the coasts of Lake Ganzirri (38° 11'N, 15°33'E) and at the mouth of a channel linking the lake to the open sea, at Torre Faro, in north-eastern Sicily (Messina, Italy). The samples, used in the present study with voucher numbers and collection information are listed in Table 1. For each sample, a voucher specimen was prepared by pressing a single individual on an herbarium sheet with a subsample preserved in 4% formalin in seawater and another dried in silica gel and stored at -20 °C.

All specimens are deposited in the Phycological Herbarium of the Department of Life Sciences "M. Malpighi" of the University of Messina, Italy (MS, <http://sweetgum.nybg.org/ih/>).

Anatomical observations were made on hand sections of fresh or formalin preserved thalli stained with 1% aniline blue solution and observed on a light microscope Diaplan Leica equipped with a LEICA DFC 500 camera (Leica Microsystems, Italy).

DNA was isolated from frozen or silica gel preserved thalli that were ground in liquid nitrogen and treated with a modified Proteinase K protocol (Saunders, 1993) instead of the final agarose gel cleaning procedure, the DNA was purified with the Wizard® DNA clean up System (Promega, Italy).

The COI-5' region was PCR amplified using the primers GazF1 and GazR1 (Saunders, 2005) and gel purified by electrophoresis in a 0.8% agarose gel with subsequent recovery by centrifugation through a home-made dimethyldichlorosilane (DMCS)-treated glass wool column (Saunders, 1993) and subsequent ethanol precipitation (Sambrook *et al.*, 1989).

Table 1 - Algal samples used in the present study with voucher numbers and collection information.

Species	Specimen Id.	Collection Site	Latitude	Longitude	Collection Date
<i>H. cornuta</i>	CB051	Lake Ganzirri	N38°15.470'	E15°36.499'	18/05/2009
	CB053	Lake Ganzirri	N38°15.470'	E15°36.499'	18/05/2009
	CB125	Lake Ganzirri	N38°15.470'	E15°36.499'	17/06/2009
	CB151	Lake Ganzirri	N38°15.686'	E15°37.372'	17/06/2009
	CB197	Lake Ganzirri	N38°15.470'	E15°36.499'	14/07/2009
	CB213	Lake Ganzirri	N38°15.552'	E15°36.989'	14/07/2009
	CB221	Lake Ganzirri	N38°15.804'	E15°37.576'	16/07/2009
	CB237	Lake Ganzirri	N38°15.804'	E15°37.576'	16/07/2009
	CB287	Lake Ganzirri	N38°15.804'	E15°37.576'	13/10/2009
	VF054	Torre Faro	N38°15.709'	E15°35.829'	12/10/2009
	VF055	Torre Faro	N38°15.709'	E15°35.829'	12/10/2009
<i>H. musciformis</i>	CB115	Lake Ganzirri	N38°15.685'	E15°36.939'	20/05/2009

Sequencing reactions were performed by Genoscope (www.genoscope.fr, Evry, France). Specimen data and sequences 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 a multiple sequence alignment was constructed in MacClade 4 (Maddison and Maddison, 2000), including sequences of *Hypnea musciformis* (Wulfen) J.V. Lamouroux (unpublished data) and other *Hypnea* species downloaded from GenBank (Benson *et al.*, 2010, searched on October 15th 2010).

The COI-5' alignment of 96 sequences with 612 nucleotide positions was subjected to distance analysis with a Neighbor-Joining algorithm under a general time reversible model of nucleotide substitution (GTR, Lanave *et al.*, 1984) as performed in PAUP* 4b10 for the Macintosh (Swofford, 2002) in order to assign specimens to genetic species groups.

Results

Hypnea cornuta in Lake Ganzirri exhibited the habit of free-floating plants, while in the open sea it throve attached on rocks within -50 cm.

Plants collected in Lake Ganzirri and at Torre Faro, brownish-red to greenish in colour, formed rather large, richly ramified bushes up to about 20 cm high. Thalli had elongated terete axes, soft in texture and sometimes fragile, with filiform branches, and were characterized by sparse stellate propagules, found scattered upon the filaments (figure 1a, b). The small, stellate spiny propagules had 3-5 rays and were peltately fixed to the branches (figure 1d-f). Thalli had uniaxial structure; cross sections showed rather large medullary cells going smaller towards the periphery (figure 1c). The cortex consisted of a single layer of cells which had a very thick (25-30 μ) polysaccharide outer matrix. The COI-5' region was successfully sequenced for all specimens collected that were resolved in a cluster assignable to *Hypnea cornuta*, distinct from the native species *H. musciformis*. The obtained neighbour joining tree is presented in figure 2 to provide a visual display of COI-5' variation within and between species.

Discussion

Individuals collected in Lake Ganzirri and at Torre Faro were identified as the alien species *Hypnea cornuta* based on morphological

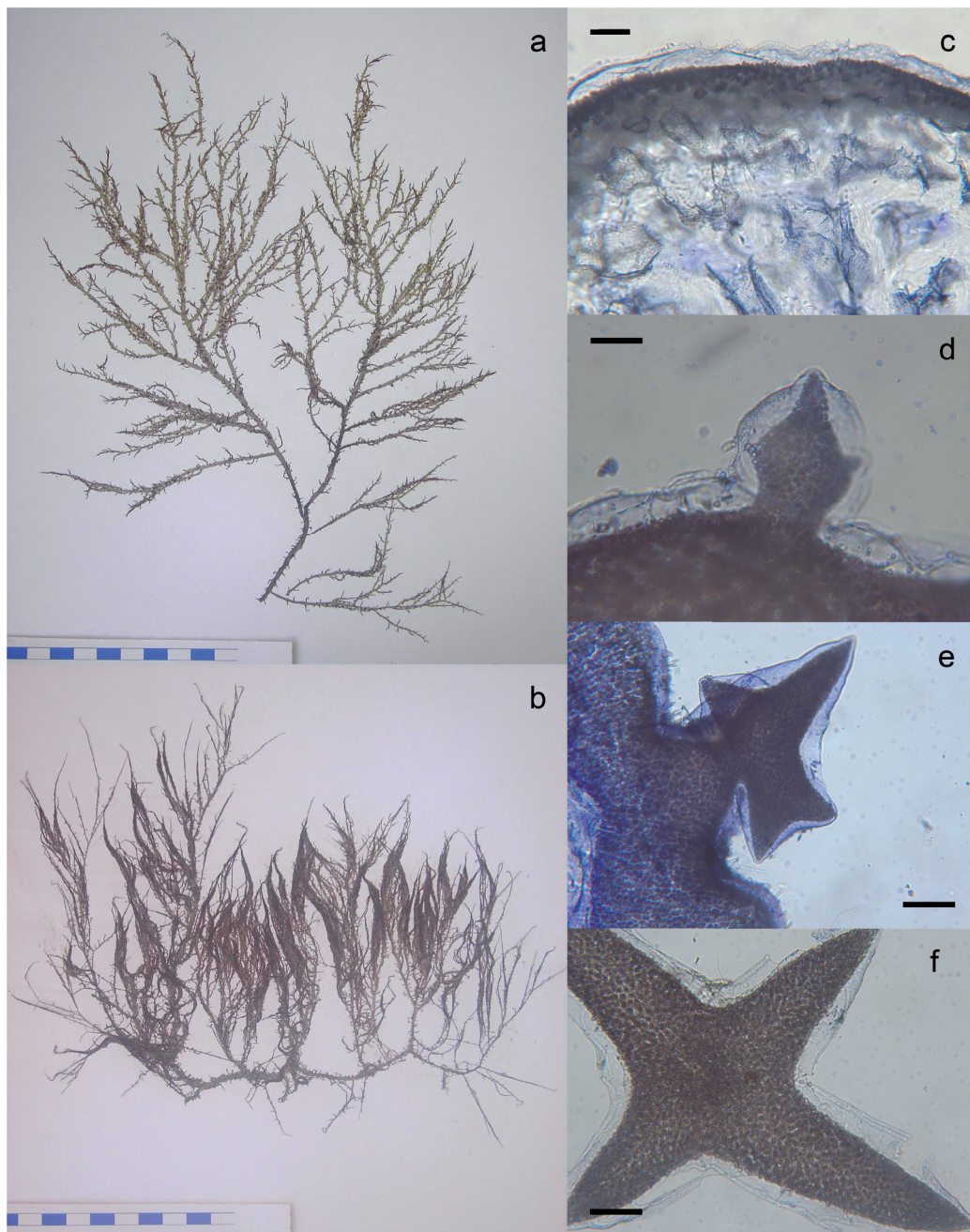


Figure 1. *Hypnea cornuta* from Cape Peloro lagoon. a-b: herbarium specimens (blue bars: 1 cm); c: cross section of the thallus (scale bar: 20 μ m); d-f: stellate propaguli (scale bars: 50 and 100 μ m respectively).

and anatomical characters confirmed by the analysis of COI-5' sequences. In the genus *Hypnea* stellate propagules occur in two species, *H. cornuta* and *H. stellulifera* (J.Agardh) Yamagishi et Masuda, and they are a useful taxonomic character at the species

level. However, the two species can be easily distinguished because *H. cornuta* has rather elongated axes, with filiform branches and sparse stellate propagules, while *H. stellulifera* has short, thick, rigid axes that produce thick adventitious

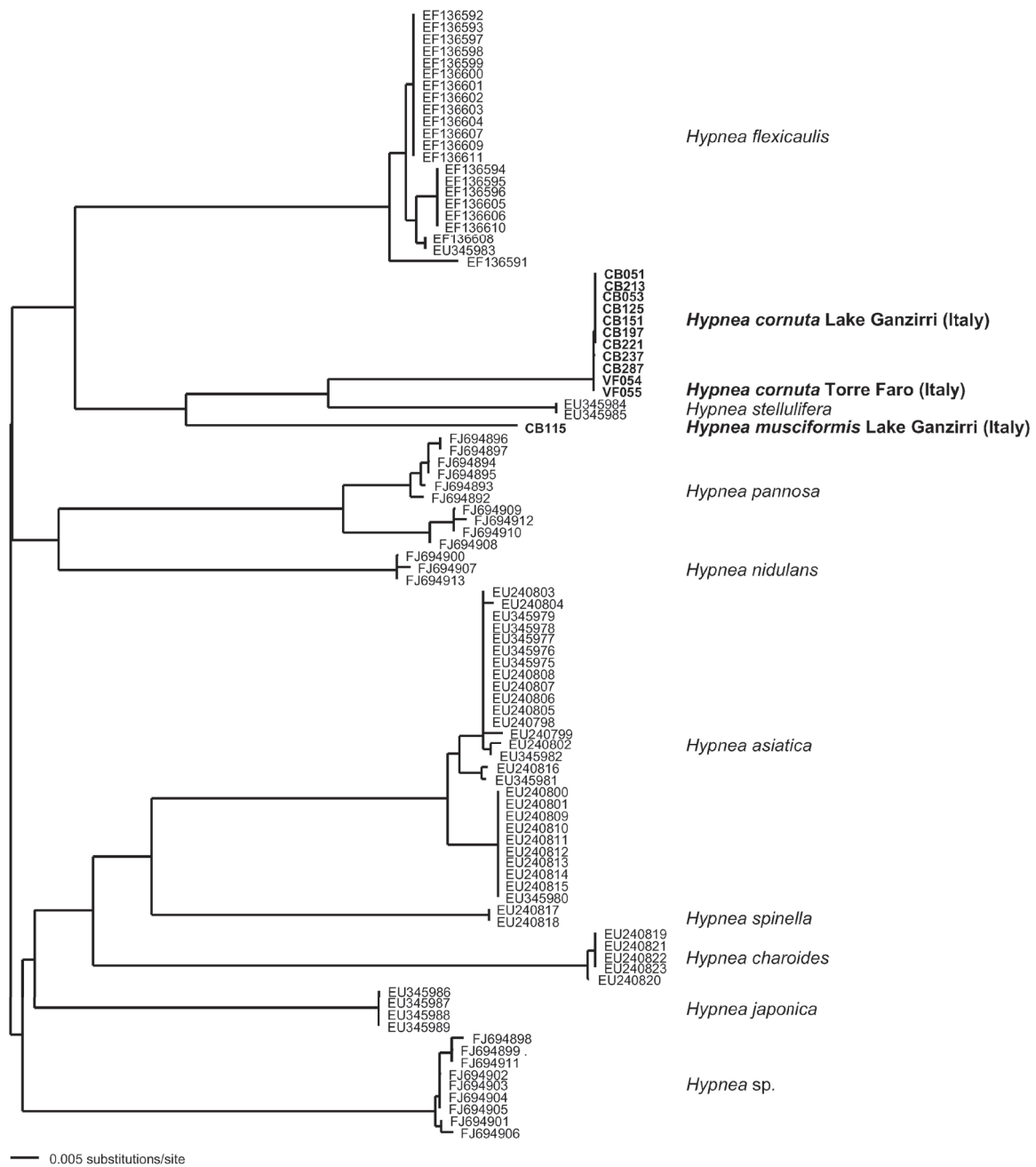


Figure 2. Neighbor joining tree (for assignment of specimens to genetic species, not presented as a robust phylogeny) from COI-5' sequences of species of *Hypnea*. The sequences generated in the present study are indicated with their voucher identification codes (in bold) and the other sequences are indicated with their GenBank accession numbers.

branchlets and abundant stellate propagules (Yamagishi *et al.*, 2003). Moreover, *H. stellulifera* is endemic to tropical Asia.

Hypnea cornuta was described from Guinea (eastern Atlantic Ocean) (Kutzing, 1849) as *Chondroclonium cornutum*. Later, J. Agardh

(1851) transferred it to the genus *Hypnea* J.V. Lamouroux. The species has been reported from various localities in the world, both in the Atlantic and in the Indo-pacific basins, from temperate to tropical areas (Guiry and Guiry, 2010). The first Mediterranean report of the species was from Rhodes Island, Greece, as *H. valentiae* (Turner) Montagne, shortly after the opening of the Suez Canal in 1869 (Reinbold, 1898). Successively, *H. cornuta* has been reported from Egypt in 1948 (Aleem, 1948) and Israel since 1964 (Nemlich and Danin, 1964). In 2000, it has been discovered in the Mar Piccolo of Taranto (southern Italy) (Cecere *et al.*, 2004). In the present paper we report *H. cornuta* in Cape Peloro lagoon and along the shores of the Straits of Messina (Italy).

Hypnea cornuta accomplishes the following criteria for recognition of an alien species (Ribera Siguan and Boudouresque, 1995): it has been previously unknown in Sicily, it has a geographical discontinuity, it is in proximity of a potential source of introduction (oyster transfer, shipping across the Strait of Messina, Lessepsian immigration), it constitutes in situ self-sustaining populations.

With regard to the means of introduction of *Hypnea cornuta* in Cape Peloro Lagoon and along the seashores of the Strait of Messina, the transfer of oysters for aquaculture should be considered, in analogy with *Agardhiella subulata*, another alien florideophyte recently introduced in the lagoon (Manghisi *et al.*, 2010). Furthermore its occurrence might be due to a direct migration from the Red Sea through the Suez Canal (Lessepsian migrant), expanding its distribution. An alternative hypothesis is that the species might be a secondary introduction from the eastern Mediterranean basin (Egypt, Israel, Rhodes Island), maybe carried by ship traffic, as hull fouling or in ballast water. Its present disjointed distribution seems to point to the second hypothesis as the most likely.

In conclusion we demonstrated that the

specimens collected in Cape Peloro lagoon were *Hypnea cornuta* and that in routine alien species monitoring DNA barcoding is a fast-and-friendly tool for the prompt identification of macroalgal taxa even for non-trained taxonomists.

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