Cryptogamie, Algologie, 2010, 31 (4): 423-433

Is routine DNA barcoding an efficient tool to reveal introductions of alien macroalgae? A case study of *Agardhiella subulata* (Solieriaceae, Rhodophyta) in Cape Peloro lagoon (Sicily, Italy)

*Antonio MANGHISI [a], Marina MORABITO [a *], Clara BERTUCCIO [a], Line LE GALL [b], Arnaud COULOUX [c], Corinne CRUAUD [c] & Giuseppa GENOVESE [a]*

[a] *Dept. Life Sciences "M. Malpighi"- Botany, University of Messina, Italy*

[b] *Muséum National d'Histoire Naturelle (MNHN), UMR 7138/USM 603, "Systématique, Adaptation Évolution", Paris, France*

[c] *Genoscope, Centre national de séquençage, Évry, France*

Abstract – A comprehensive assessment of macroalgal biodiversity was conducted along the coasts of the Straits of Messina (Italy) using DNA barcoding. This approach confirmed the introduction of *Agardhiella subulata* in Cape Peloro lagoon (Messina, Italy), an aquatic system in which shellfish importation and stabulation are widely practiced.

Based on a comparison of *rbc*L sequences, a sample from lake Ganzirri was shown to be conspecific with *A. subulata* from North Carolina (USA).

Suspecting that shells of the oyster *Crassostrea gigas* constitute the main vector for introduction of exogenous species, individuals from imported stocks were screened for the presence of epizoic algae, which were then sequenced for the DNA barcode region (5' end of COI gene). A small specimen growing on an oyster shell was assigned with confidence to *A. subulata* based on its COI sequence, a task that would have been virtually impossible based on morphological characters of the immature specimen. Based on the example here, we conclude that monitoring algal diversity using DNA barcoding would clearly be a rapid and efficient approach to highlight the introduction of alien species.

***Agardhiella subulata* / DNA barcoding / alien species / Mediterranean / Rhodophyta / Strait of Messina / transitional environments / COI-5' region / *rbc*L gene**

Résumé – Une étude détaillée de la diversité des algues a été réalisée le long des côtes du Détroit de Messine (Italie) au moyen de code-barres ADN. Cette approche a confirmé l'introduction de *Agardhiella subulata* dans la lagune du Cap Peloro (Messine, Italie), un système aquatique dans lequel les pratiques aquacoles d'importation et de stabulation de coquillages sont courantes. Sur la base de la comparaison de la séquence codant la *rbc*L, il a été démontré qu'un spécimen en provenance du lac Ganzirri était conspécifique de *A. subulata* de Caroline du Nord (USA).

Suspectant que les coquilles d'huîtres constituent le principal vecteur d'introduction d'espèces exogènes, les individus importés ont été minutieusement observés en vu de mettre en évidence la présence d'algues épizoïques qui ont été séquencées pour le marqueur mitochondrial COI. Un spécimen de petite taille croissant sur une coquille d'huître a été identifié comme *A. subulata* au moyen de sa séquence de COI, une tâche qui

* Correspondence and reprints: morabitom@unime.it

se serait avérée quasiment impossible, sur ce spécimen immature, sur la base de caractères morphologiques. Sur la base de cet exemple, nous concluons que le suivi de la biodiversité des algues couplées à une approche de code-barres ADN serait un moyen efficace et rapide pour détecter l'introduction d'espèces exogènes.

***Agardhiella subulata* / code-barres ADN / espèces exogènes / Méditerranée / Rhodophyta / Détroit de Messine / environnements de transition / région COI-5' / gène *rbc*L**

INTRODUCTION

As part of a comprehensive assessment of macroalgal biodiversity along the coasts of the Straits of Messina (Italy) using DNA barcoding, we are completing a survey of the flora of the brackish lakes (transitional water systems) Faro and Ganzirri (Sicily). They form a complex ecosystem (Cape Peloro lagoon), which is under significant anthropogenic pressure. The two brackish lakes are connected by a channel and both of them communicate with the open sea. They are located in the Oriented Nature Reserve of Cape Peloro (Region Sicily, ref. D.A. 21/6/01), which is also a Site of Community Importance (ref. 92/43/EEC) and a Special Area of Conservation (ref. 79/409/EEC).

Being transitional environments, the lakes experience natural stresses (variable salinity and water temperature), have lower diversity, and display abrupt changes in dominant species. Moreover, due to the urbanization of the area, they also suffer from high human induced disturbances through organic enrichment, pollution, and physical habitat alterations. Lake Faro is a site where livestock of adult shellfish (mussels, clams and oysters) imported from farming areas in Spain, France, Netherlands and various Italian localities, are re-immersed directly in the basin for a stabulation period. This step is required to guarantee microbiologic reclamation of the shellfish needed for food quality reasons before trading in local markets.

Since late spring 2007, a population of a previously unreported florideophyte has been observed. Specimens were identified as *Agardhiella subulata* (C. Agardh) Kraft & M.J. Wynne (Solieriaceae) in accordance with morphological, anatomical and reproductive characters (Manghisi *et al.,* 2008).

The type locality of *Agardhiella subulata* is "*In mari Canadensi*" (Agardh, 1822, as *Sphaerococcus subulatus*), which could be almost anywhere along the Atlantic coast of North America (Kraft & Wynne, 1979). It is mainly distributed along the eastern American coast (Yarish *et al.*, 1984), but it is also present in the Indian Ocean (Silva *et al.*, 1996).

It was first reported in Europe from Great Britain in 1973 (Farnham & Irvine, 1979). However, according to Farnham (1980), Eno *et al.* (1997) and Arenas *et al.* (2006), UK reports of *A. subulata* could be misidentifications of the Pacific species *Sarcodiotheca gaudichaudii* (Montagne) P.W. Gabrielson.

According to Verlaque (2001), *A. subulata* was first found in the Mediterranean, in Thau lagoon (Hérault, France), by Ben Maiz in 1984 (Ben Maiz, 1986) who reported it as *Solieria chordalis* (C. Agardh) J. Agardh. In Italy, it was reported in 1987 by Perrone and Cecere (1994) in Mar Piccolo (Taranto, Apulia) and in Pantano d'Arci (Catania, Sicily), where a single specimen was collected by M. Cormaci and G. Furnari in 1990. However, the latter authors did not report the species in Sicily in subsequent publications (Furnari *et al.*, 2003; Cormaci *et al.*, 2004; Furnari, 2010). More recently, *A. subulata* was reported in Yerseke, Nether-

lands (Stegenga, 1999), and in Venice lagoon, Italy (Curiel *et al.*, 2005; Sfriso & La Rocca, 2005).

Even though Perrone and Cecere (1994) did not exclude a Tethyan origin for *A. subulata* in the Mediterranean, it is noteworthy that all European localities where the species is present share the characteristic of housing either oyster aquaculture farms or stabulation sites. Since lake Faro is also a site of shellfish trading and it is in open connection with lake Ganzirri, we aimed to verify whether oyster importation is a likely vector for the introduction of *A. subulata* in Cape Peloro lagoon. We therefore screened for the presence of epizoic algae on oyster shells imported to Messina (Italy) from various European farming sites before they were subjected to stabulation in lake Faro.

We used DNA barcoding (sequencing the 5' region of the mitochondrial encoded *cytochrome c oxidase* I gene (COI-5')) to identify the algal specimens, which was shown to be an effective DNA barcode for red and brown algae (Saunders, 2005, 2008, 2009; Robba *et al.*, 2006; Lane *et al.*, 2007; Kucera & Saunders, 2008; Sherwood *et al.*, 2008; McDevit & Saunders, 2009; Clarkston & Saunders, 2010; Le Gall & Saunders, 2010).

MATERIALS AND METHODS

A variety of morphs of *A. subulata* were collected at various seasons in lake Ganzirri (Cape Peloro lagoon, Sicily, Italy). Samples used for this 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. Additionally, a red alga epizoic on an oyster shell (*Crassostrea gigas* Thunberg) from marketable livestock imported from a Dutch farming site to the Sicilian market was recovered with a portion of the thallus preserved in formalin and the remaining tissue frozen 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).

Anatomical observations were made on hand sections of fresh or formalin preserved thalli stained with 1% aniline blue solution and observed under a Diaplan Leica light microscope 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). The plastidial *rbc*L gene was PCR amplified (Freshwater *et al.*, 1994) and purified as above, to permit comparison to sequences for *A. subulata* in public databases. Sequencing reactions were performed by an external company (MWG Biotech AG, Germany) as well as by Genoscope (www.genoscope.fr, Évry, France). Specimen data and sequences were deposited in the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org).

Table 1. List of specimens of *A. subulata* used in the present study

Specimen ID	*Collection site*	*Latitude*	*Longitude*	*Collection date*	*Collector*	*BOLD process ID*
CB347	Lake Ganzirri, Messina, Italy	N38°15.470'	E15°36.499'	15.I.2010	C. Bertuccio	ITRED005-10
CB362	Lake Ganzirri, Messina, Italy	N38°15.804'	E15°37.576'	15.I.2010	C. Bertuccio	ITRED006-10
SACOM001	Netherlands, Aquiculture (epizoic on *C. gigas*)			25.III.2010	P. Stroscio	ITRED009-10
CB038	Lake Ganzirri, Messina, Italy	N38°15.685'	E15°36.939'	08.IV.2009	C. Bertuccio	ITRED002-10
MS-CB004	Lake Ganzirri, Messina, Italy	N38°15.552'	E15°36.989'	01.IV.2009	C. Bertuccio	ITRED001-10
PhL289	Lake Ganzirri, Messina, Italy	N38°15.470'	E15°36.499'	24.V.2007	G. Genovese	ITRED008-10
CB123	Lake Ganzirri, Messina, Italy	N38°15.470'	E15°36.499'	17.VI.2009	C. Bertuccio	ITRED003-10
HS002	Hardenhoek, Zeeland, Netherlands	N51°28.933'	E4° 3.252'	26.VI.2008	H. Stegenga	ITRED007-10
CB303	Lake Ganzirri, Messina, Italy	N38°15.552'	E15°36.989'	20.XI.2009	C. Bertuccio	ITRED004-10

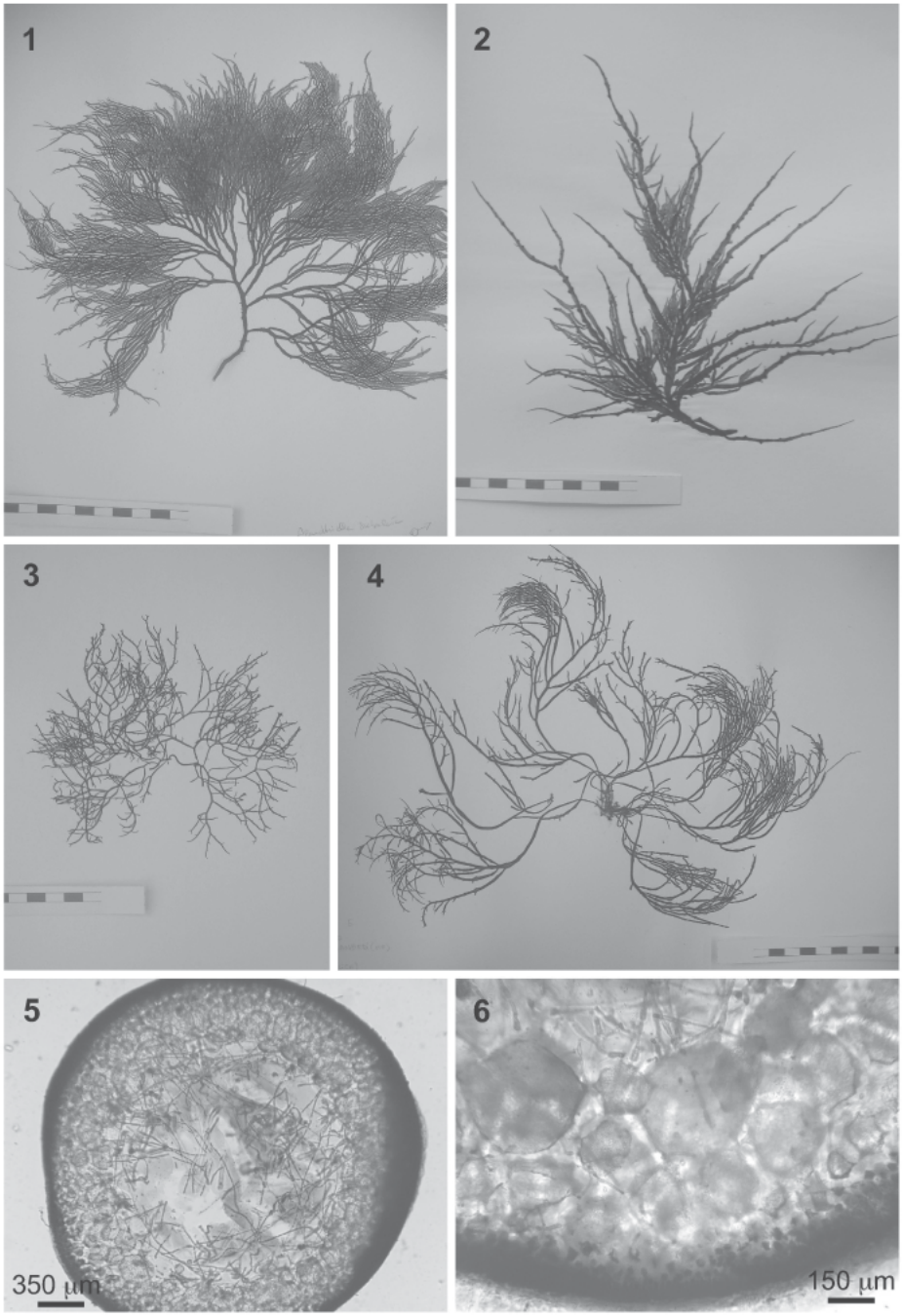
Forward and reverse sequence reads were assembled with the software Chromas Pro (v. 1.41, Technelysium Pty Ltd) and a multiple sequence alignment was constructed in MacClade 4 (Maddison & Maddison, 2000), including sequences of species of Solieriaceae downloaded from GenBank (Benson *et al.*, 2010).

The COI-5' alignment of 22 sequences with 588 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) to define species.

RESULTS

The morphology of plants collected at lake Ganzirri was highly variable. Thalli were erect, 10-40 cm high, reddish-brown in colour (Figs 1-4), growing from a basal disc, with axes terete (0.5-2.5 mm in diameter) and ramified. The branching pattern was typically alternate with axes tapering towards their tips. Colourless deciduous hairs were present on branches of young plants.

Thalli had a multiaxial structure with a pseudo-parenchymatous cortex and a filamentous medulla (Figs 5-6). In mature thalli the outer cortex was composed of a single layer of small, ellipsoidal, pigmented cells. The inner cortex was composed of 4-5 layers of large, spherical to ovoid cells (Fig. 5). The medulla was composed by periclinal axial filaments, interconnecting filaments and rhizoidal filaments, initiated from both axial filaments and innermost cortical cells (Fig. 6, arrow), hardly distinguishable from each other. Fertile male and female gametophytes, with cystocarps, and tetrasporophytes were observed (Figs 1, 2, 4).



Figs 1-6. *Agardhiella subulata* from lake Ganzirri. **1.** Male gametophyte. **2.** Female gametophyte with cystocarps. **3.** Sterile thallus. **4.** Tetrasporophyte. Scale bar: dark blocks = 1 cm. **5.** Cross section of a mature thallus showing the pseudo-parenchymatous cortex and the filamentous medulla. **6.** Cross section showing the cortex and rhizoidal filaments initiated by inner cortical cells (arrow).

The thallus growing epizoically on the marketable oyster (SACOM001) was small, 3.1 cm high, and sterile, sharing habit and anatomical features with specimens from lake Ganzirri (Figs 7-9).

The COI-5' region was successfully sequenced for all studied specimens. The length of the sequences was 664 bp excluding the PCR primers. All sequenced specimens, including the Dutch sample (HS002) and the oyster epizoic (SACOM001), had identical COI-5' sequences (Fig. 10).

Based on a comparison of *rbc*L sequences, a sample from lake Ganzirri (PhL289) was determined to be conspecific with the specimen of *A. subulata* from North Carolina (Federal Basin, New Hanover Co., USA, GenBank U04176; Fredericq *et al.*, 1999), with a distance of 3 bp over a total length of 1374 bp (0.22% divergence).

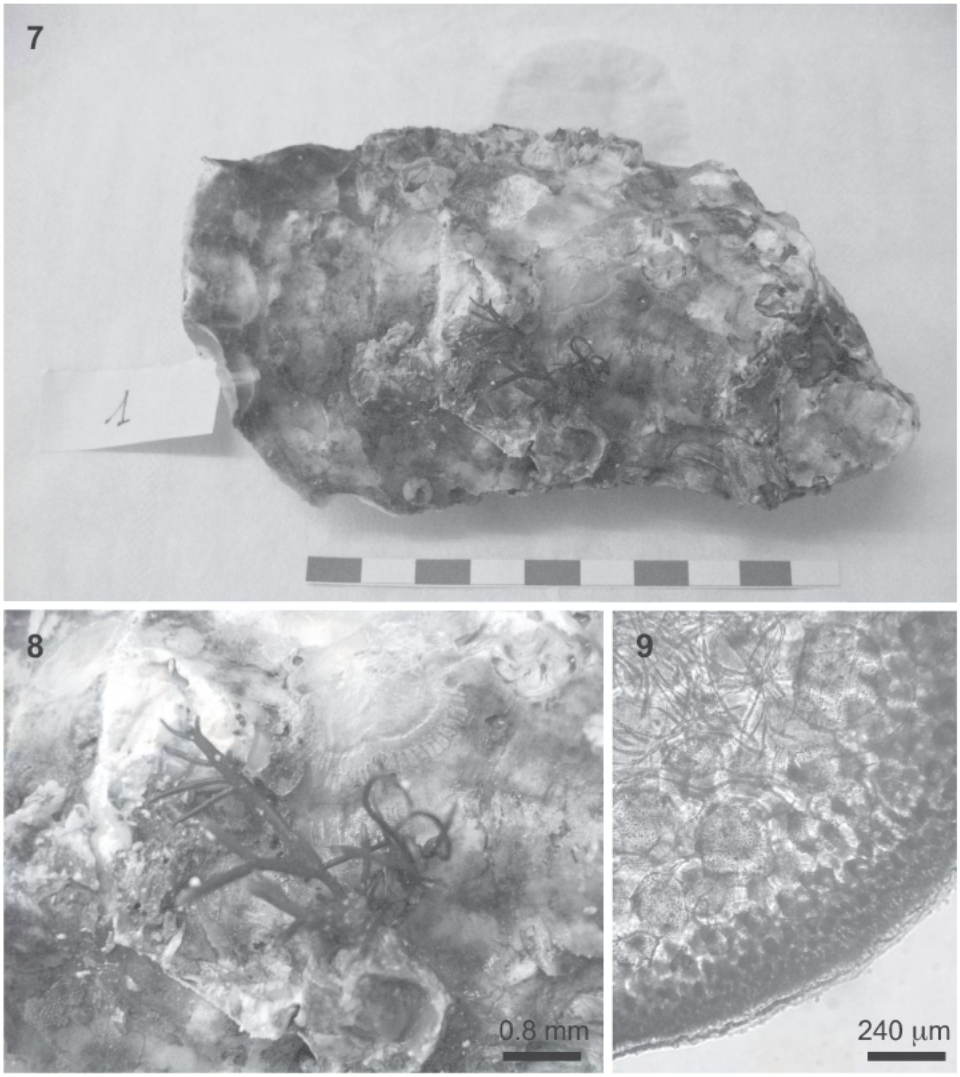
DISCUSSION

Aquaculture is a major vector for the introduction of exogenous species along with fouling on hulls, ballast water, aquarium trading and fishing nets (Verlaque, 2005). When compared to these other vectors, the shellfish trade, especially oysters, is by far the main vehicle of introduction of macroalgae into the Mediterranean and the North East Atlantic (Verlaque *et al.*, 2007). Macroalgal spores and propagules are able to settle on shells and the transplantation of oysters and other molluscs may be responsible for 44% of the macroalgal introductions, both intercontinentally and within Europe, with the North West Pacific as the major donor area (Wallentinus, 2002).

COI-5' sequences allowed us to determine that specimens collected in lake Ganzirri and on an oyster shell from the Netherlands were conspecific, suggesting that oysters were the vector of introduction for that species in Cape Peloro lagoon. We had to sequence a second marker, *rbc*L, to assign our specimens to *A. subulata*. Therefore the lack of a comprehensive data bank of standard sequences hampered identification of our invasive species based on the standard barcode sequence alone. We argue that the generalisation of a standard marker, i.e., the barcode strategy, and the population of the barcode of life data systems (BOLD, www.boldsystems.org) will most likely enhance biodiversity monitoring studies and early discovery of exogenous species.

According to Italian food quality and safety regulations, imported oysters coming from aquaculture facilities must be subjected to a period of immersion in places like lake Faro for a stabulation period. It is likely that many organisms, especially macroalgae, are introduced to regions as a result of this practice. However, despite the presumed importance of oyster transportation in species introductions, only a few studies have assessed the actual role of this vector (Mineur *et al.*, 2007). The difficulty of identifying immature macroalgae to known species is a severe impediment to carry on such studies.

The importation of non-indigenous oysters, such as the Japanese *Crassostrea gigas*, generates a massive and recurring transfer of livestock between aquaculture sites. In the Mediterranean Sea, the majority of sites for shellfish aquaculture are developed in coastal lagoons, such as Cape Peloro, which, being transitional water systems, are highly susceptible to introductions and human induced stressors in general.



Figs 7-9. *Agardhiella subulata* from a Dutch farming site. **7.** Thallus epizoic on *Crassostrea gigas* (scale bar = 10 cm). **8.** Detail of 7. **9.** Cross section of the thallus showing the pseudo-parenchymatous cortex and part of the filamentous medulla.

According to the criteria defined by Ribera & Boudouresque (1995), *A. subulata* should be recognized as an introduced species because: (i) it is new to Cape Peloro lagoon, (ii) its distribution is discontinuous, (iii) it is in the vicinity of a potential source of introduction (i.e. oyster transfer), (iv) it is genetically identical to the Atlantic populations based on COI-5' sequences, and (v) its new station is very localized.

Although we have established that a particular Dutch oyster farm is a source of *A. subulata* for possible introduction into Cape Peloro lagoon, it is highly probable that there have been (and continue to be) multiple introductions

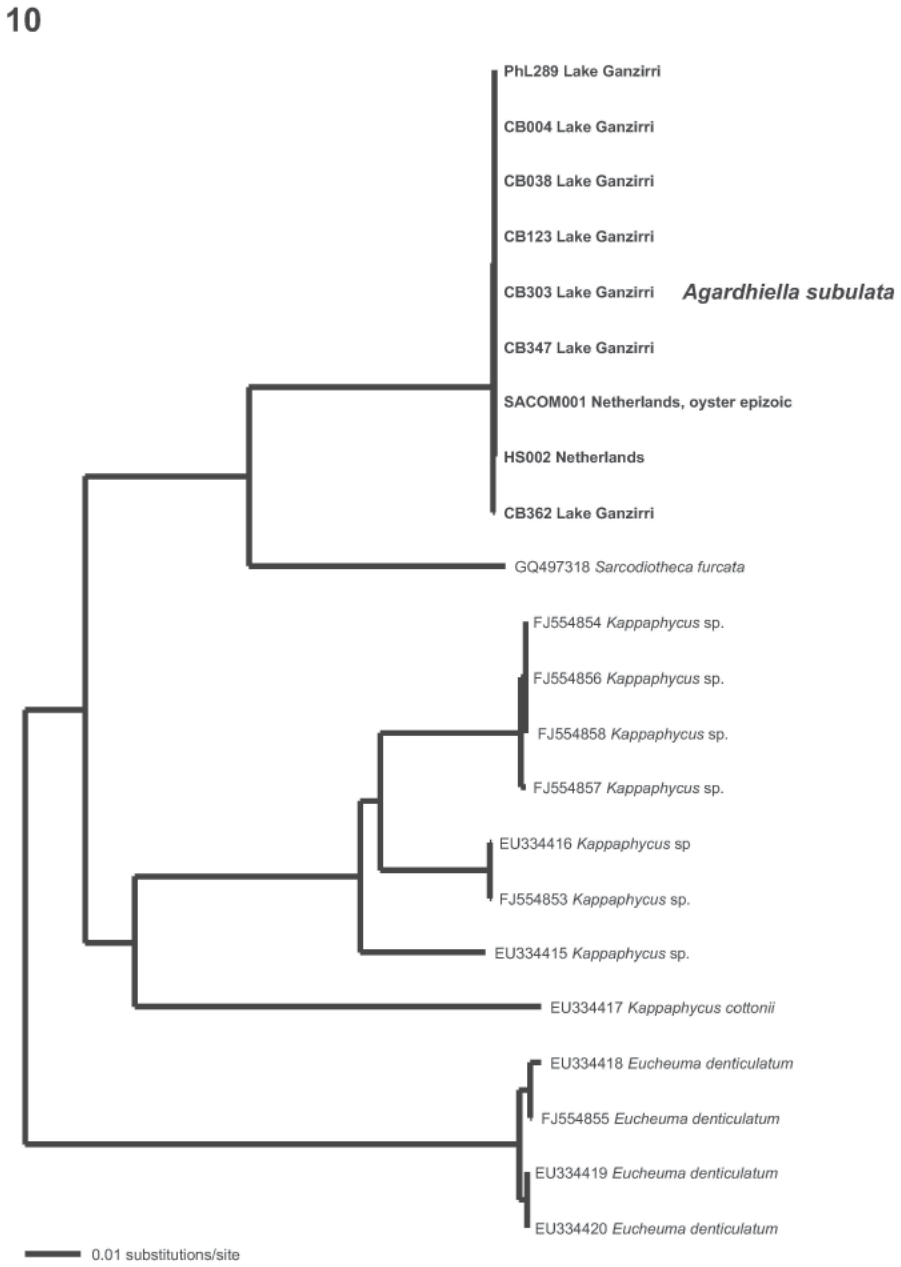


Fig. 10. Neighbor-Joining tree of COI-5' sequences of representative Solieriaceae. The samples of *Agardhiella subulata* are in bold.

from other aquaculture sites such as Thau lagoon, France, and that this species is likely over-introduced (repeated input from one source).

The species was found in Cape Peloro lagoon in 2007 and, since then, all reproductive stages were observed and a large population get established all year round. This species is therefore either in its settlement or naturalization phase according to the criteria of Ribera and Boudouresque (1995). Observations of its presence, abundance and reproductive phenology are in progress to ascertain the phase of invasion.

It is interesting that all nine individuals sequenced for COI-5' are identical, including the juvenile specimen growing on an oyster shell from the Netherlands. Nevertheless, we cannot draw definite conclusions on the genetic structure of populations of *A. subulata* having so few sequences. Acquiring additional COI-5' sequences, especially from its natural range, could be used to define different haplotypes and to localize source populations for the introductions (Rueness, 2010). Collecting more and more sequences of *A. subulata* from different localities, both from natural areas and sites of introductions, could help in tracking its spread and aid in determining which vectors, either by oyster importation or by other means, are most responsible.

In conclusion, we have highlighted that standardised molecular tools for species identification, such as the DNA-barcode, are useful to characterise introduced species at their early stage of settlement.

Acknowledgements. The Authors would like to thank Dr. Herre Stegenga and Mr. Pietro Stroscio who kindly provided Dutch specimens, and Mr. Marco Vicinanza and Mrs. Simona Armeli Minicante for help in sampling at lake Ganzirri. Gary Saunders is thankfully acknowledged for improving the English of this manuscript and for his valuable comments. Two anonymous referees are greatly acknowledged as their inputs significantly improved the manuscripts. This research has benefit of the agreement n_2005/67 between the Genoscope and the Museum National d'Histoire Naturelle on the project 'Macrophylogeny of life' directed by Guillaume Lecointre.

REFERENCES

AGARDH C.A., 1822 — *Species algarum rite cognitae cum synonymis, differentiis specificis et descritionibus succintis. Volumen primum pars posterior.* Lundae, Officina Berlingiana, pp. 169-398.

ARENAS F., BISHOP J.D.D., CARLTON J.T., DYRYNDA P.J., FARNHAM W.F., GONZALEZ D.J., JACOBS M.W., LAMBERT C., LAMBERT G., NIELSEN S.E., PEDERSON J.A., PORTER J.S., WARD S. & WOOD C.A., 2006 — Alien species and other notable records from a rapid assessment survey of marinas on the south coast of England. *Journal of the marine biological association of the United Kingdom* 86(6): 1329-1337.

BEN MAIZ N., 1986 — *Flore algale (Rhodophyta, Phaeophyceae, Chlorophyceae, Bryopsidophyceae) de l'étang de Thau (Hérault).* Thesis Ecologie, Aix-Marseille II, 354 p.

BENSON D.A., KARSCH-MIZRACHI I., LIPMAN D.J., OSTELL J. & SAYERS E.W., 2010 — GenBank. *Nucleic acids research* 38(s1): D46-D51.

CLARKSTON B.E. & SAUNDERS G.W., 2010 — A comparison of two DNA barcode markers for species discrimination in the red algal family Kallymeniaceae (Gigartinales, Florideophyceae), with a description of *Euthora timburtonii* sp. nov. *Botany* 88(2): 119-131.

CORMACI M., FURNARI G., GIACCONE G. & SERIO D., 2004 — Alien macrophytes in the Mediterranean Sea: a review. *In:* Pandalai S.G. (ed.), *Recent Research Developments in Environmental Biology.* I(1). Trivandrum, Research Signpost, pp. 153-202.

CURIEL D., BELLEMO G., CHECCHIN E., DRI C., MIOTTI C. & MARZOCCHI M., 2005 — Segnalazione di nuove macroalghe per la Laguna di Venezia. *Lavori - Società Veneziana scienze naturali* 30: 41-44.

ENO C.N., CLARK R.A. & SANDERSON W.G., 1997 — *Non-native marine species in British waters: a review and directory.* Peterborough, Joint Nature Conservation Committee (JNCC), 136 p.

FARNHAM W.F., 1980 — Studies on aliens in the marine flora of southern England. *In:* PRICE J.H., IRVINE D.E.G. & FARNHAM W.F. (eds), *The Shore Environment.* Vol. 2: Ecosystems. London, Academic Press, pp. 875-914.

FARNHAM W.F. & IRVINE L.M., 1979 — Discovery of members of the red algal family Solieriaceae in the British Isles. *British phycological journal* 14: 123.

FREDERICQ S., FRESHWATER D.W. & HOMMERSAND M.H., 1999 — Observations on the phylogenetic systematics and biogeography of the Solieriaceae (Gigartinales, Rhodophyta) inferred from *rbc*L sequences and morphological evidence. *Hydrobiologia* 399(1): 25-38.

FRESHWATER D.W., FREDERICQ S., BUTLER B.S., HOMMERSAND M.H. & CHASE M.W., 1994 — A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proceedings of the national academy of sciences of the United States of America* 91(15): 7281-7285.

FURNARI G., GIACCONE G., CORMACI M., ALONGI G. & SERIO D., 2003 — Biodiversità marina delle coste italiane: catalogo del macrofitobenthos. *Biologia marina mediterranea* 10(1): 1-482.

FURNARI G., 2010 — Le macroalghe delle coste italiane: aspetti floristici e vegetazionali. *Bollettino accademia Gioenia di scienze naturali* 42(371): 1-16.

KRAFT G.T. & WYNNE M.J., 1979 — An earlier name for the Atlantic North American red alga *Neoagardhiella baileyi* (Solieriaceae, Gigartinales). *Phycologia* 18(4): 325-329.

KUCERA H. & SAUNDERS G.W., 2008 — Assigning morphological variants of *Fucus* (Fucales, Phaeophyceae) in Canadian waters to recognized species using DNA barcoding. *Botany* 86(9): 1065-1079.

LANAVE C., PREPARATA G., SACCONE C. & SERIO G., 1984 — A new method for calculating evolutionary substitution rates. *Journal of molecular evolution* 20(1): 86-93.

LANE C.E., LINDSTROM S. & SAUNDERS G.W., 2007 — A molecular assessment of northeast Pacific *Alaria* species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding. *Molecular phylogenetics and evolution* 44(2): 634-648.

LE GALL L., SAUNDERS G.W., 2010 — DNA barcoding is a powerful tool to uncover algal diversity: a case study of the Phyllophoraceae (Gigartinales, Rhodophyta) in the Canadian flora. *Journal of phycology* 46(3): 374-389.

MADDISON D.R. & MADDISON W.P., 2000 — MacClade 4: Analysis of phylogeny and character evolution. Sunderland, Massachusetts (USA), Sinauer Associates.

MANGHISI A., LOGORELLI F. & MORABITO M., 2008 — *Agardhiella subulata* (C. Agardh) Kraft *et* M.J. Wynne (Solieriaceae, Rhodophyta), a new introduction in lake Ganzirri (Messina, Sicily). *In: 103° Congresso della Società Botanica Italiana.* Reggio Calabria, Italy, p. 75.

MCDEVIT D.C. & SAUNDERS G.W., 2009 — On the utility of DNA barcoding for species differentiation among brown macroalgae (Phaeophyceae) including a novel extraction protocol. *Phycological research* 57(2): 131-141.

MINEUR F., BELSHER T., JOHNSON M.P., MAGGS C.A. & VERLAQUE M., 2007 — Experimental assessment of oyster transfers as a vector for macroalgal introductions. *Biological conservation* 137(2): 237-234.

PERRONE C. & CECERE E., 1994 — Two Solieriacean algae new to the Mediterranean: *Agardhiella subulata* and *Solieria filiformis* (Rhodophyta, Gigartinales). *Journal of phycology* 30(1): 98-108.

RIBERA M.A. & BOUDOURESQUE C.F., 1995 — Introduced marine plants, with special reference to macroalgae: mechanisms and impact. *In:* Round F.E. & Chapman D.J. (eds), *Progress in Phycological Research.* 11. Bristol, Biopress, pp. 187-268.

ROBBA L., RUSSELL S.J., BARKER G.L. & BRODIE J., 2006 — Assessing the use of the mitochondrial *cox1* marker for use in DNA barcoding of red algae (Rhodophyta). *American journal of botany* 93(8): 1101-1108.

RUENESS J., 2010 — DNA barcoding of selected freshwater and marine red algae (Rhodophyta). *In: 2nd Conference of the European Consortium for the Barcode of Life.* Braga, Portugal, p. 27.

SAMBROOK J., FRITCH E.F. & MANIATIS T., 1989 — *Molecular cloning: a laboratory manual.* New York, Cold Spring Harbor Laboratory Press, 1659 p.

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(2): 251-254.

SAUNDERS G.W., 2005 — Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical transactions of the royal society of London Series B: Biological Sciences* 360(1462): 1879-1888.

SAUNDERS G.W., 2008 — A DNA barcode examination of the red algal family Dumontiaceae in Canadian waters reveals substantial cryptic species diversity. 1. The foliose *Dilsea-Neodilsea* complex and *Weeksia*. *Botany* 86(7): 773-789.

SAUNDERS G.W., 2009 — Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. *Molecular ecology resources* 9(s1): 140-150.

SFRISO A. & LA ROCCA B., 2005 — Aggiornamento sulle macroalghe presenti lungo i litorali e sui bassofondali della Laguna di Venezia. *Lavori - Società Veneziana scienze naturali* 40: 45-56.

SHERWOOD A.R., VIS M.L., ENTWISLE T.J., NECCHI Jr O. & PRESTING G.G., 2008 — Contrasting intra versus interspecies DNA sequence variation for representatives of the Batrachospermales (Rhodophyta): insights from a DNA barcoding approach. *Phycological research* 56(4): 269-279.

SILVA P.C., BASSON P.W. & MOE R.L., 1996 — *Catalogue of the benthic marine algae of the Indian Ocean.* Berkeley, University of California Press, 1259 p.

STEGENGA H., 1999 — Het roodwier *Agardhiella subulata* in Nederland. *Zeepaard* 59(2): 54-57.

SWOFFORD D.L., 2002 — PAUP*. Phylogenetic Analisis Using Parsimony (*and Other Methods). Sunderland, Sinauer Associates.

VERLAQUE M., 2001 — Checklist of the macroalgae of Thau Lagoon (Hérault, France), a hot spot of marine species introduction in Europe. *Oceanologica acta* 24(1): 29-49.

VERLAQUE M., 2005 — WP 4.2 Propagule pressure: shellfish industry - Final Report. *In: 5th PCRD European Programme "Aliens". Algal introductions to European shores.* Marseille, 134 p.

VERLAQUE M., BOUDOURESQUE C.F. & MINEUR F., 2007 — Oyster transfers as a vector for marine species introductions: a realistic approach based on macrophytes. *In:* Briand F. & Moschella P. (eds), *Impact of mariculture on coastal ecosystems.* Lisboa, CIESM Workshop Monographs n° 32, pp. 39-47.

WALLENTINUS I., 2002 — Introduced marine algae and vascular plants in European aquatic environments. *In:* Leppäkoski E., Gollasch S. & Olenin S. (eds), *Invasive Aquatic Species of Europe – Distribution, Impacts and Management.* Dordrecht, Kluwer Academic Publishers, pp. 27-52.

YARISH C., BREEMAN A.M. & VAN DEN HOEK C., 1984 — Temperature, light, and photoperiod responses of some northeast American and west European endemic rhodophytes in relation to their geographic distribution. *Helgoländer Meeresuntersuchungen* 38: 273-304.