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DNA barcoding of select freshwater and marine red algae (Rhodophyta)

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Abstract – The first COI sequences from European material of *Batrachospermum helminthosum* and *B. gelatinosum* are presented and compared with equivalent data from North America. The variation between the continents was 53 bp (8%) and 10 bp (1.5%), respectively for the two species, suggesting cryptic diversity in at least the former species. *Polysiphonia hemisphaerica* from Norway and *P. boldii* from Texas differed by 8 bp (1.2%). A hybrid tetrasporophyte produced from a cross between these two species revealed COI sequence identical with that of the female parent, demonstrating uniparental inheritance of mitochondria. *Ptilota serrata* and *P. gunneri*, which can be difficult to distinguish morphologically, were 38 bp (5.7%) different with no intraspecific variation. Sequences for a species temporarily labeled as *P. serrata* sp.1 from eastern Canada in The Barcode of Life Data Systems (BOLD), were identical with that for *P. gunneri* from Norway. The morphologically similar species *Plumaria plumosa* is represented by triploid parasporangial plants and sexually reproducing plants that are geographically separated, with only the asexual phase at the northern part of the range. The two phases had COI sequences that differed by 4 bp. Sequences of the invasive species *Gracilaria vermiculophylla* from France and the Russian Far East (Vladivostok) were identical and similar to the reported invasive haplotype from elsewhere. In addition, COI sequences obtained for "*Heterosiphonia*" *japonica* and *Antithamnion hubbsii* from introduced populations in Norway are presented.

COI / DNA barcoding / red algae / species identification / Rhodophyta / *Batrachospermum* / *Ptilota* / *Plumaria* / *Polysiphonia hemisphaerica* / *Polysiphonia boldii* / *Gracilaria vermiculophylla* / "*Heterosiphonia*" *japonica* / *Antithamnion hubbsii*

Résumé – Pour la première fois, des séquences de *Batrachospermum helminthosum* et *B. gelatinosum* en provenance d'Europe sont présentées et comparées avec les mêmes taxa américains. La distance génétique observée entre les deux continents est de 53 bp (8%) pour B. helminthosum et 10 bp (1,5%) pour *B. gelatinosum*, ce qui suggère la présence de diversité cryptique dans le premier taxon. *Polysiphonia hemisphaerica* de Norvège et *P. boldii* du *Texas* différent de 8 bp (1.2%). Un hybride tétrasporophytique résultant du croisement des deux espèces précédentes présente une séquence de COI identique avec celle du parent maternel, ce qui démontre l'hérédité maternelle des mitochondries. *Ptilota serrata* et *P. gunneri*, deux espèces qui peuvent être difficiles à distinguer sur des bases morphologiques présentent une distance génétique de 38 bp (5,7 %) et pas de divergence intraspécifique. Les séquences d'un taxon annoté comme *P. serrata* sp.1 dans BOLD en provenance de la côte Est du Canada sont identiques à *P. gunneri* de Norvège. Une espèce morphologiquement proche, *Plumaria plumosa*, est représentée par des spécimens parasporangiaux triploides et des spécimens se reproduisant sexuellement qui sont géographiquement séparés et dont la phase sexuée se localise au nord de la distribution de l'espèce. Les deux phases ont des séquences qui divergent de 4 bp. Des séquences de l'espèce invasive

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Gracilaria vermiculophylla en provenance de France et du Grand Est Russe (Vladivostok) sont identiques et similaires à l'haplotype invasif caractérisé ailleurs. Par ailleurs, les séquences de COI acquises pour "*Heterosiphonia*" *japonica* et *Antithamnion hubbsii* pour des populations introduites en Norvège sont présentées.

COI / code-barres ADN / algues rouges / identification spécifique / Rhodophyta / *Batrachospermum* / *Ptilota* / *Plumaria* / *Polysiphonia hemisphaerica* / *Polysiphonia boldii* / *Gracilaria vermiculophylla* / "*Heterosiphonia*" *japonica* / *Antithamnion hubbsii*

INTRODUCTION

DNA barcoding based on a standardized sequence of the mitochondrial cytochrome oxidase subunit I gene (COI), has proven to be extremely useful for species-level identification. For red algae, the potential of DNA barcoding as a taxonomic tool was first demonstrated by Saunders (2005) who designed novel primers. Later works by Robba *et al.* (2006), House *et al.* (2008), Sherwood *et al.* (2008), Saunders (2009), Clarkston & Saunders (2010), Le Gall & Saunders (2010), have also assessed the use of this gene region for identification of red algae covering a broad spectrum of orders. Combined with analyses of vegetative and reproductive morphology, DNA barcoding has clarified taxonomic questions and uncovered cryptic species (Le Gall & Saunders 2010). The number of COI sequence records in GenBank and through The Barcode of Life Data System (BOLD, www.barcodinglife.org) is rapidly accumulating. However, there is a nearly unlimited need for increasing taxon sampling and the number of geographical isolates to assess the range of variation within species. For freshwater red algae in the order Batrachospermales, molecular data have proven to be a powerful tool in clarifying phylogenetic relationships among taxa at higher levels (e.g., Entwisle *et al.,* 2009) and at species level (e.g., House *et al.,* 2008; Sherwood *et al.,* 2008). Most species within this order were originally described from Europe in classical studies by Sirodot (1884), Kylin (1912) and others, and were based entirely on vegetative and reproductive morphology; molecular data from European isolates are however still highly underrepresented.

In this study I present the first COI sequences for the freshwater red algae *Batrachospermum helminthosum* Bory de Saint-Vincent and *B. gelatinosum* (Linnaeus) De Candolle from European material (Norway) in order to make comparisons with equivalent data for species going under the same names North America.

Starting in the early 1970s, I have undertaken culture studies of red algae aimed at clarifying species limits by using crossability tests and by comparisons made of different isolates grown in a "common garden" and under various culture conditions. Most culture isolates belong to the order Ceramiales and have been maintained for up to 40 years. In recent years DNA sequences (*rbc*L, Rubisco spacer, cox 2-3 spacer) have been acquired for species of *Ceramium* (Gabrielsen *et al.*, 2003; Skage *et al.,* 2005), *Caulacanthus* (Rueness & Rueness, 2000; Zuccarello *et al.,* 2002), and *Gracilaria* (Rueness, 2005). Since 2006, many COI sequences have been obtained from cultured and field-collected samples.

In this study, COI sequence data were used to assess the relationship between the closely related species *Polysiphonia hemisphaerica* Areschoug and *P. boldii* Wynne *et* Edwards, including a hybrid tetrasporophyte produced from a cross between these two taxa (Rueness, 1973). *P. hemisphaerica* has only been recorded from warm-water embayments in Norway and Sweden, whereas *P. boldii* is only known from the Texas coast of Gulf of Mexico (Wynne & Edwards, 1970). Both species have six periaxial cells and the relationship with other species with six periaxial cells, such as *Lophosiphonia obscura* (C. Agardh) Falkenberg, needs to be studied.

Ptilota serrata Kützing and *P. gunneri* Silva, Maggs *et* Irvine are two species with overlapping distributions that can be difficult to discriminate using morphological characters. Hence, COI sequences were compared. *Plumaria plumosa* is a species that may resemble *Ptilota* species morphologically, though they are rarely confused. Unlike most Ceramiales, *Plumaria plumosa* has a well-defined triploid phase (Drew, 1939; Whittick, 1977) that reproduces by mitotic paraspores (Rueness, 1968). In Europe, the triploid phase and the haploid and diploid gametangial and tetrasporangial phases show a distinct geographical distribution with the triploid parasporophytes towards the northern limits. Along the Norwegian coast only parasporophytes are present. Similarly, in the western Atlantic where *P. plumosa* is distributed from New Jersey to Newfoundland, only the parasporangial phase is found from the northern portion of the range in Newfoundland (Whittick, 1977). COI sequences were acquired for parasporangial thalli from Norway and Scotland, and for a gametangial thallus from France.

COI sequences are also presented for three species that have recently been introduced along European coasts. When sufficient DNA barcodes become available from both native and introduced populations, COI may help to understand the possible origin and spread of invasive species. Sequences are presented for *Gracilaria vermiculophylla* (Ohmi) Papenfuss (Rueness, 2005), "*Heterosiphonia" japonica* Yendo (Lein, 1999) and *Antithamnion hubbsii* Dawson (as *Antithamnion nipponicum* Yamada *et* Inagaki) (Rueness *et al.,* 2007) and compared with other available COI sequences from the same species.

MATERIAL AND METHODS

Specimens for this study were obtained from unialgal cultures and field collections. Details of collection data, vouchers and GenBank accession numbers for COI sequences are listed in Table 1. The two freshwater red algae, *Batrachospermum helminthosum* and *B. gelatinosum,* were collected from streams near Oslo, Norway. Specimens for microscopic analyses were fixed in 4% formaldehyde solution, with unfixed subsamples dried on herbarium paper as a voucher and another in a vial with silica gel for later DNA extraction. The marine red algae, with the exception of samples of *Gracilaria vermiculophylla* from Vladivostok and *Ptilota gunneri* from Norway, were grown as unialgal cultures in an enriched seawater medium and under culture conditions similar to those reported in Rueness (2005). Cultures were maintained at 12°C and 17°C excepting *Ptilota serrata*, which was grown at 3°C. Isolates were maintained by clonal reproduction (apical cuttings); some of the isolates have been kept for more than 40 years.

DNA was extracted from silica-dried or live algae using the DNeasy™ Plant Mini Kit (Qiagen, Hilden, Germany). The COI region was amplified using the primer combination GazF1 and GazR1 as designed by Saunders (2005) and

Table 1. Collection data, voucher information for taxa analyzed, and GenBank accession numbers for COI sequences

Taxon	*Collection details*	*Voucher information*	*GenBank accession no.*
Batrachospermum gelatinosum	Norway, Åroselva, county of Buskerud, 21 May 2010, *coll. E.-A. Lindstrøm & M. R. Kile*	Herbarium specimen, University of Oslo (O)	HQ412539
Batrachospermum helminthosum	Norway, Askerelva, county of Akershus, female plant, 28 July 2009, *coll. J. Rueness*	Herbarium specimen University of Oslo (O)	HQ412542
Batrachospermum helminthosum	Norway, Askerelva, county of Akershus, male plant, 28 July 2009, *coll. J. Rueness*	Herbarium specimen, University of Oslo (O)	HQ412541
Batrachospermum helminthosum	Norway, Askerelva, county of Akershus, vegetative specimen, 28 July 2009, *coll. J. Rueness*	herbarium specimen, University of Oslo (O)	HQ412540
Batrachospermum helminthosum	Norway, Leiravassdraget, Mikkelsbekk, county of Akershus, 14 Sep 2009, *coll. E.-A. Lindstrøm & M. R. Kile*	Herbarium specimen, University of Oslo (O)	HQ412543
Polysiphonia hemisphaerica	Norway, Oslofjord, see Rueness (1971, 1973)	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412544
Polysiphonia boldii	USA, Texas, Port Aransas, see Wynne & Edwards (1970)	Cultured isolate, originally received from Dr M.J. Wynne	HQ412545
P. hemisphaerica ♀ × *P. boldii* ♂	Tetrasporophyte grown from hybrid carpospores	Cultured isolate and herbarium specimen, University of Oslo (O)	Identical with HQ412545
Ptilota serrata	Norway, Spitzbergen, station 262, 30 Aug 2005, *coll. J. Gitmark*	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412546
Ptilota serrata	Norway, Balsfjord, county of Troms, 9 Nov 2007, *coll. G.G. Nervold*	Herbarium specimen, University of Oslo (O)	HQ412547
Ptilota gunneri a)	Norway, Finnøy, county of Møre and Romsdal, April 2006, *coll. J. Rueness*	Herbarium specimen, University of Oslo (O)	HQ412548
Ptilota gunneri b)	Norway, Finnøy, county of Møre and Romsdal, April 2006, *coll. J. Rueness*	Herbarium specimen, University of Oslo (O)	HQ412548
Plumaria plumosa	Norway, Oslofjord, parasporic, see Rueness (1968)	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412549
Plumaria plumosa	Scotland, St. Andrews, parasporic, *coll. A.D. Boney*	Cultured isolate and herbarium specimen (O), University of Oslo (O)	HQ412550
Plumaria plumosa	France, Roscoff, female plant, *coll. J. Rueness*	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412551

Table 1. Collection data, voucher information for taxa analyzed, and GenBank accession numbers for COI sequences *(cont'd)*

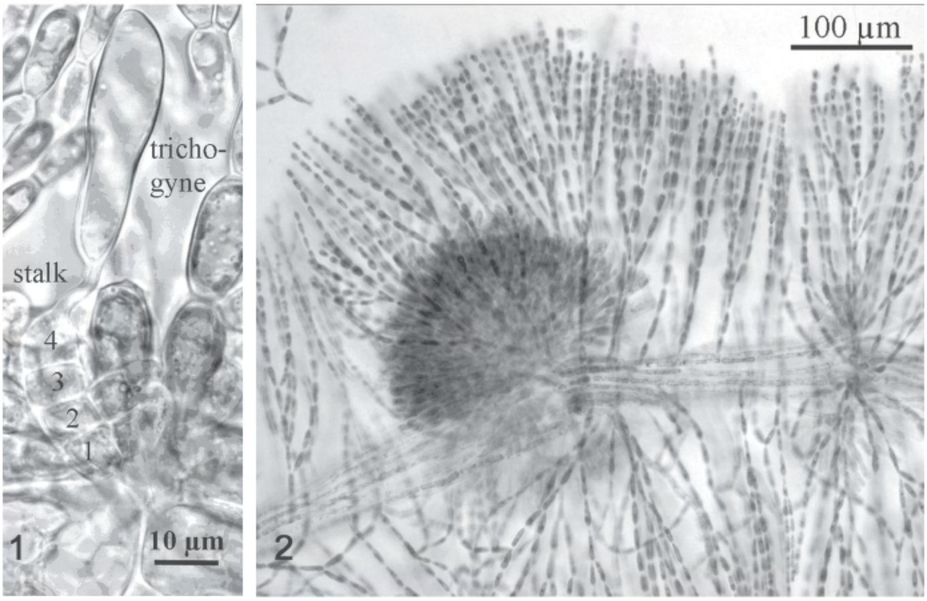
Taxon	*Collection details*	*Voucher information*	*GenBank accession no.*
Gracilaria vermiculophylla	France, Belon estuary, see Rueness (2005)	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412552
Gracilaria vermiculophylla	Russia, Peter the Great Bay, Vladivostok, May 2007, *coll. I. M.Yakovleva*	Herbarium specimen, University of Oslo (O)	HQ412553
"Heterosiphonia" japonica	Norway, near Marine Station, University of Bergen, county of Hordaland, 20 June 2000, *coll. J. Rueness* (see Bjærke & Rueness, 2004)	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412554
Antithamnion hubbsii	Norway, Store Kalsøy, county of Hordaland. 11 Sep 2007, *coll. J. Rueness*, see Rueness *et al.* (2007)	Cultured isolate and herbarium specimen, University of Oslo (O)	HQ412555

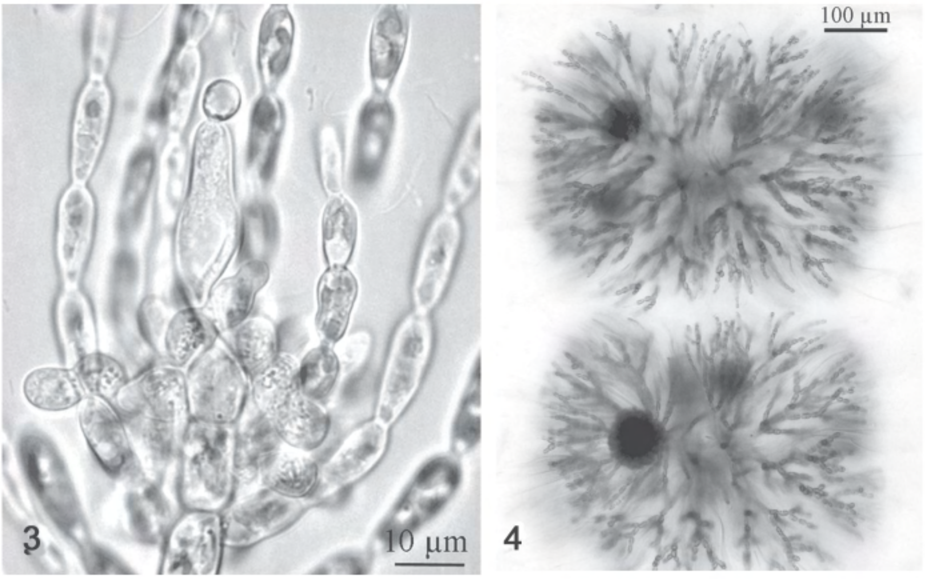
the PCR amplification was done in an Eppendorf Mastercycler® ep following the protocol outlined in Saunders (2005). All PCR products were purified using Wizard®SV Gel and PCR Clean-UP System (Promega, Madison, WI, USA) before bidirectional sequencing using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences in both directions were assembled and manually edited using the software BioEdit v.7.04 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA).

RESULTS AND DISCUSSION

Freshwater red algae

Batrachospermum helminthosum, originally described from Brittany in France, is one of the best studied species in the genus with respect to intraspecific genetic variation (House *et al.*, 2008 and papers cited therein). Three individuals from one site in Norway revealed identical COI sequences and a fourth specimen from a river 100 km distant from the first site was identical for the 602 bp available for comparison. No other COI sequences (or other DNA sequences) are available for European samples of this taxon. On the other hand, House *et al.*, (2008) analyzed COI from a total of 82 individuals covering a total of 15 stream segments throughout the range in North America. The COI data revealed 16 haplotypes in a complex pattern and differed from 0 to 44 bp of the 664 bp (up to 6.6% sequence variation). As compared with the results obtained in the present study of Norwegian individuals, the closest American haplotype differed by as much as 53 bp (8%). In conclusion, this is a taxon with putative "intraspecific" COI divergence values substantially higher than those reported for other red algal species, and with apparently no morphological differences using standard taxonomic characters for the genus *Batrachospermum*. Morphological characteristics of the female reproductive structures are shown in Figs 1-2. The trichogyne shown in Fig. 1 is some-





Figs 1-2. *Batrachospermum helminthosum*. **1.** 4-celled carpogonium branch arising from periaxial cell. Carpogonium with stalk formation between trichogyne and carpogonium base. **2.** Post fertilization development of single, axial carposporophyte.

Figs 3-4. *Batrachospermum gelatinosum*. **3.** Fertilized carpogonium with attached spermatium. **4**. Axis with whorl branches containing several spherical carposporophyte at various distances from axis.

what more inflated than that typically illustrated for *B. helminthosum.* Detailed morphological analyses and more sequence data from Europe, and in particular from the type locality in France, are required.

Batrachospermum gelatinosum is the type species of the genus and was originally described from northern Europe. It is a commonly reported freshwater red alga from streams in the northern hemisphere. Plants are monoecious and carpogonia and carposporophytes are illustrated in Figs 3-4. The Norwegian specimen analyzed in this study differed by 10 bp from the closest COI sequence available for the species in GenBank (EU628669, Nova Scotia, Canada). In a recent extensive study of *B. gelatinosum* from North America, House *et al.* (2010) used COI as a DNA marker for a phylogeographic study and included an additional three specimens from Europe (France, England). The Norwegian sample differed by 3 bp (0.45%) from both the France and the UK individuals, whereas specimens from Norway, France and UK differed from the dominant haplotype in North America by 10 bp (1.5%), 8 bp (1.2%) and 7 bp (1.05%), respectively. As in *B. helminthosum*, it is clear that more data from European samples are required to establish the range of intraspecific variation, but it is clear that the genetic variation in *B. gelatinosum* is considerably lower than observed in *B. helminthosum* despite being highly plastic in its morphology.

Marine red algae

Polysiphonia hemisphaerica and *P. boldii*

These two entities have been shown to be very similar in vegetative and reproductive morphology (Wynne & Edwards, 1970; Rueness, 1971), and in a culture study (Rueness, 1973) cross-fertilization resulted in carposporophyte development. Released carpospores germinated into tetrasporophytes with aborted tetrasporangial formation. This hybrid tetrasporophyte, produced from a carpospore of the cross: *P. hemisphaerica* (♀) × *P. boldii* (♂) has been maintained in culture by vegetative reproduction.

Comparison of COI sequences from *P. hemisphaerica* and *P. boldii* showed that they were only 8 bp different or 1.2%. The hybrid tetrasporophyte was identical in COI sequence to *P. hemisphaerica* suggesting that mitochondria are inherited uniparentally from the female parent. The taxonomic status of the two entities remains to be resolved.

Ptilota and *related genera*

Ptilota gunneri and *P. serrata* are the only species represented in the North Atlantic Ocean with *P. serrata* having a more northern and amphiatlantic distribution, whereas *P. gunneri* is limited to European Atlantic coasts from Arctic Norway to Canary Islands. In northern Norway and Spitzbergen, where both species are present, they may often be difficult to separate morphologically. The COI sequences of the two species differed by 38 bp (5.7%). The two individuals of *P. serrata* from North Norway and Spitzbergen were identical, as were approximately 20 sequences from western Atlantic available in the BOLD database. The only COI sequence of *P. serrata* available in GenBank (AY970640) comes from a specimen from the Pacific coast of Canada. This is actually a different species, *P. filicina* J. Agardh, and is correctly referred to as such in BOLD (Gary Saunders, personal communication). Sequences of the two specimens of *P. gunneri* from the Norwegian west coast were identical, and did not differ from a specimen from Ireland in GenBank (EU194975). Interestingly, four georaphically widely separated specimens from eastern Canada, temporarily labeled as *P. serrata* species 1 in BOLD, have COI sequences identical with *Ptilota gunneri* from Europe, strongly suggesting that this species in fact is present on western Atlantic coasts.

Plumaria plumosa resembles *Ptilota* species, but is only rarely misidentified as *Ptilota*. Unlike most Ceramiales, *Plumaria plumosa* has a well-defined triploid phase (Drew, 1939; Whittick, 1977) that reproduces by mitotic paraspores (Rueness, 1968). In Europe, the triploid parasporangial phase and the haploid and diploid gametangial and tetrasporangial phases show a distinct geographical distribution with the triploid parasporophytes towards the northern limits. Along the Norwegian coast only parasporophytes are present, and in the western Atlantic where *P. plumosa* is distributed from New Jersey to Newfoundland, a similar situation is reported with only the parasporangial phase found in Newfoundland (Whittick, 1977). COI sequences revealed that parasporangial thalli from Norway and Scotland were identical and differed by 4 bp (0.6%) from the gametangial thallus from France. Several COI sequences of *Plumaria plumosa* from western Atlantic are available in BOLD. They were 99.1% similar, but the differences have not been analyzed in detail.

Introduced marine red algae

Gracilaria vermiculophylla

This alga has been extensively studied in recent years, especially after it was first recognized as an invasive species in Europe and North America (Rueness, 2005; Saunders, 2009). Genetic barcodes have become available from > 300 individuals in native and introduced populations (Skriptsova & Choi, 2009; Yang *et al.,* 2008; Saunders, 2009; Kim *et al.,* 2010). Apparently, introduced populations have identical COI sequences (with a few exceptions distinguished by a single substitution). In native populations many haplotypes are known, with the invasive type only represented in E-Korea, W-Japan and Far-East Russia (Kim *et al.,* 2010). The results of the present study are in full accordance with these findings. The specimens from France and Vladivostok analyzed in this study were identical with the invasive haplotype (haplotype no. 6 in Kim *et al.,* 2010).

"Heterosiphonia" japonica

The generic placement of this species has not been settled, hence the quotation marks. Morphological and molecular studies (Choi, H-G 2001; de Jong *et al.,* 1998) show that the alga seems to be more closely related to *Dasysiphonia* (Lee & West, 1980) than to *Heterosiphonia*, and it was in fact recorded as ?*Dasysiphonia* sp. when first reported from Europe (Stegenga, 1997; Lein, 1999). The conspecificity of the alga found in Europe and *Heterosiphonia japonica* Yendo from Korea, was confirmed by Bjærke & Rueness (unpublished), who compared cox 2-3 spacer sequences from several individuals from Europe (Norway, Atlantic and Mediterranean coasts of France, and the Galician coast of Spain; GenBank accession nos. AY624317-25) with specimens from Korea (GenBank accession numbers AY624326-30). Sequences were identical over the 318 bp aligned. ITS2 sequences further corroborated these results (GenBank accession numbers AY624336 - 42). COI sequences acquired in the present study from a specimen from the west coast of Norway was identical with sequences available in BOLD, and also referred to by Schneider (2010), who recently reported the first finds of the alga from western Atlantic (Rhode Island).

Antithamnion hubbsii

The nomenclatural history of this species was recently clarified by Athanasiadis (2009). The alga has erroneously been referred to as *A. nipponicum* and reported as such as a recent introduction along the North Carolina and Pacific coasts of North America (Cho *et al.,* 2005) and in Europe from the Mediterranean Sea (Verlaque & Riouall, 1989; Curiel *et al.,* 1996, as *A. pectinatum*), the Atlantic coasts of the Iberian Peninsula (Secilla *et al.,* 2007), and Norway (Rueness *et al.,* 2007). The COI sequence presented in this paper is the first made available for this species.

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