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# Barcoding of cryptic stages of marine brown algae isolated from incubated substratum reveals high diversity in Acinetosporaceae (Ectocarpales, Phaeophyceae)[1]

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**Abstract** – To identify cryptic stages of marine brown macroalgae present in the "bank of microscopic forms", we incubated natural substrata of different geographical origins and isolated emerging Phaeophyceae into clonal cultures. A total of 431 clones were subsequently identified by barcoding using 5'-COI. A proportion of 98% of the isolates belonged to the Ectocarpales. The distribution of pairwise genetic distances revealed a K2P divergence of 1.8% as species-level cut-off. Using this threshold, the samples were ascribed to 83 different species, 39 (47%) of which were identified through reference sequences or morphology. In the Ectocarpaceae, 16 lineages of *Ectocarpus* fulfilled the barcode criterion for different species, while three putative new species were detected. In the Chordariaceae, numerous microthalli were microstages of known macroscopic taxa. A separate cluster contained *Hecatonema maculans* and other microscopic species. Taxa traditionally classified in Acinetosporaceae were split in two species-rich groups containing *Pylaiella* and *Hincksia* in one and *Acinetospora* in the other. *Feldmannia* species were present in both clusters. The present study shows that the germling emergence method is suited to reveal the diversity of hidden life-history stages, albeit with a bias towards early successional species.

***Acinetospora* / biogeography / brown algae / cytochrome c oxidase 1 / cox1 / COI / cryptic stages / diversity / DNA barcoding / *Ectocarpus subulatus* Kützing / *Feldmannia* / germling emergence / *Hincksia* / identification / phylogeny**

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1. Dedicated to Dieter G. Müller on the occasion of his 80th birthday.

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## INTRODUCTION

Natural substrata in the marine phytobenthic zone receive a "rain" of algal reproductive cells, which after settling form the so-called "bank of algal microscopic forms" (Hoffmann & Santelices, 1991). Different "crypticisms" complicate the identification of the members of this biota. Such algae may be microscopic species (cryptic size), they may represent the microscopic form of well-known macroalgae (cryptic stage), they may hardly present discernible morphological characters (cryptic morphology), or they may include different species of similar morphology (cryptic species).

The study of macroalgal microstages *in situ* is difficult, and knowledge on this part of the life history comes mainly from culture studies in the laboratory. Following the discovery of microscopic gametophytes in kelp (Sauvageau, 1915), such stages were detected in many species through the isolation of cultures from meiospores produced on the macroscopic sporophytes (e.g. Sauvageau, 1917), or inversely by following the development of zygotes after gamete fusions between zoids produced on macroscopic gametophytes (e.g. Tatewaki 1966). Although such studies provided a general knowledge of the alternation of generations in many species, the ecological role of the "bank of microscopic forms" as supplier of recruits is little studied (e.g. Chapman, 1984; see also references in Robuchon *et al.*, 2014).

Incubation of natural substratum in a nutrient-rich and herbivore-free (Lotze *et al.*, 2001) environment and subsequent isolation of developing germlings appears the logical approach to studying the natural diversity of the microscopic stages. In the present article we refer to this technique as "germling emergence method" in analogy to the "seedling emergence method" for land plants (Roberts, 1981). The approach has, though, rarely been used for marine macroalgae. Ramirez & Müller (1991) and Müller & Ramírez (1994) isolated small brown algae from incubated substrata (scrapings and millimetre-sized fragments of larger algae, small pebbles and sand grains) collected at Easter Island and the Juan Fernandez Archipelago off Chile. They morphologically identified eight strains appearing in culture, which all represented new records, showing the potential of the approach to increase floristic knowledge of smaller taxa escaping standard methods of phytogeographic surveys.

Combining germling emergence with DNA barcoding allows identifying isolates more rapidly if reference sequences are present. Zuccarello *et al*. (2011) and West *et al*. (2012) used several substratum-grown isolates for their molecular-morphological revision of small red algae of the Erythropeltidales. Robuchon *et al.* (2014) identified juvenile kelp sporophytes emerging in culture from cryptic stages present on field-collected substratum. By a similar approach (germling emergence followed by molecular identification using specific primers), Couceiro *et al*. (unpublished) have characterised cryptic stages of *Ectocarpus* spp.

The aim of the present paper was to use the germling emergence method in combination with 5'-COI barcoding to identify the diversity of small brown algae that is hidden in the bank of microscopic forms. The species obtained belonged mainly to the Ectocarpales and included numerous representatives of little-studied groups such as Acinetosporaceae.

## MATERIALS AND METHODS

### Field material

Collections were realised at historically well-studied localities (Roscoff, Northeast Atlantic Ocean (Cardinal, 1964), and Naples, western Mediterranean (Cormaci *et al.*, 2012)) as well as several localities in the less intensely studied Eastern Mediterranean (Tsiamis *et al.*, 2013), including Cyprus where a first comprehensive checklist has appeared only recently (Tsiamis *et al.*, 2014; Table 1). Also, a small number of samples came from Jeju Island, Korea. Abiotic natural substrata, such as rock fragments, pebbles and old shells, were collected on the shore at low tide, by snorkelling in near-surface habitats, or by SCUBA diving. In the latter cases, to avoid contamination with spores from surface water, the collecting tubes (15 or 50 ml FALCON tubes) were filled with sterile seawater in advance of the dive and not opened before reaching the sampled depth.

### Incubation of substratum

After transport or posting of the samples to the laboratory of the first author, the substrata were placed in 90 mm Petri dishes filled with 35 ml culture medium (Provasoli-enriched natural autoclaved sea water, Starr & Zeikus, 1993) and incubated at 15ºC in natural light at a north-facing window. Preliminary experiments showed that with this dish size nutrients were not depleted too fast, and the cultures were amenable to examination under a stereomicroscope. They also revealed that the absence of gastropod or polychaete grazers in the samples was crucial for the emergence of macroalgal germlings. Decreasing the surface of the incubated substratum to about 0.5 $cm^2$ was found an efficient way to exclude these grazers. Small copepods, in contrast, rather played a positive role, as they prevented diatom proliferation. There was thus no need to add germanium dioxide, which might have affected growth of brown algae (Wang, 1993).

### Isolation of strains

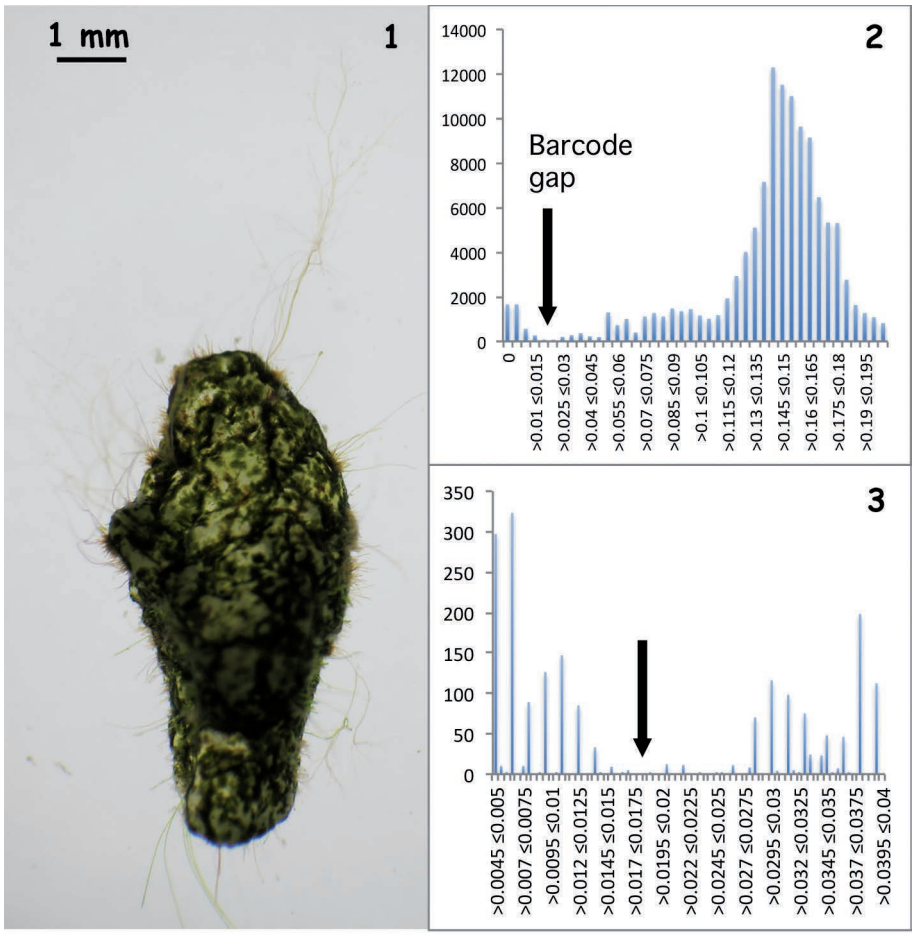
Clonal strains of filamentous brown algae were isolated after 1-3 months by cutting and pipetting fragments of emerging algae (Fig. 1) under the stereomicroscope; green and red algae were not sampled. From each dish we isolated 1-5 individuals, taking care to sample isolates exhibiting different morphologies, for example, erect filaments with ribbon-shaped versus discoid plastids, prostrate algae of different filament densities and cell shapes, or microcrusts. Duplicate isolates from the same dish revealed by identical sequences (see below) were excluded from further analyses because they possibly originated by direct reproduction during cultivation and not from different original individuals. Additional clones of ectocarpoid algae were isolated directly from field material. In several of these, as in a few isolates from emerging germlings, identification was possible based on morphological characters. Clones isolated from incubated substratum and those directly from field material, either macroscopic (≥5mm) or microscopic (<5mm), are together henceforth referred to as "environmental isolates" (categories Sub, MA, Mi, respectively, in Table 2 (Table 2 available on-line as supplementary material, doi/10.7872/crya.v36.iss1.2015.S1).

Table 1. Collecting information

| *Site/date Nº* | *Geographic region* | *Country* | *Region* | *City* | *Locality* | *Sites* | *Zone/depth (m) if subtidal* | *Exposure* | *Sampled substrata* | *Collection dates* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | NE Atlantic | France | Brittany | Roscoff | Santec, Perharidy | Roc'h Ar Bleiz | Lowermost intertidal | Mid-Exposed | Rock | 21-03-11 |
| 2 | NE Atlantic | France | Brittany | Roscoff | Santec, Perharidy | Roc'h Ar Bleiz | Lowermost intertidal | Mid-Exposed | Rock | 29-05-11 |
| 3 | NE Atlantic | France | Brittany | Roscoff | Santec, Perharidy | Roc'h Ar Bleiz | Lowermost intertidal | Mid-Exposed | Rock | 01-09-11 |
| 4 | NE Atlantic | France | Brittany | Roscoff | Santec, Perharidy | Roc'h Ar Bleiz | Lowermost intertidal | Mid-Exposed | Rock | 08-03-12 |
| 5 | NE Atlantic | France | Brittany | Roscoff | Santec, Perharidy | Sites #1, 3 and 7 in Couceiro *et al.* (unpublished) | High to low intertidal | Protected | Pebbles and shells, macroalgae | 2010 to 13 (various dates) |
| 6 | NE Atlantic | France | Brittany | Plougonvelin | Traezh Hir | Rocky shore | Uppermost subtidal | Protected | Macroalgae | 22-08-13 |
| 7 | NE Atlantic | France | Brittany | Plougastell-Daoulas | Le Caro | Gravel beach | Upper subtidal | Protected | Macroalgae | 05-07-12 |
| 8 | NE Atlantic | France | Brittany | | Terenez | High intertidal, rim of salt marsh | High intertidal | Protected | Pebbles and macroalgae | 13-07-11 |
| 9 | West Mediterranean | Italy | Campania | Ischia | Ischia | Castello Aragonese and Laboratory | Uppermost subtidal | Mid-Exposed | Pebbles, shells, rock, macroalgae | 20-03-12 |
| 10 | West Mediterranean | Italy | Campania | Naples | Naples | Porto di Megellina and Castel dell'ovo | Uppermost subtidal | Protected | Pebbles, shells, ropes, macroalgae | 14-03-12 |
| 11 | East Mediterranean | Greece | Saronikos Gulf | Peiraias | Peiraiki | Rocky shore | Water surface | Mid-Exposed, eutrophicated | Macroalgae | 11-04-11 |
| 12 | East Mediterranean | Greece | Saronikos Gulf | Athens | Agios Kosmas | Beach | Water surface | Protected, eutrophicated | Drifting wood, macroalgae | 11-04-11 |
| 13 | East Mediterranean | Greece | Saronikos Gulf | Anavyssos | Sounio | Rocky shore | 1 m | Mid-Exposed | Small pebbles | 11-04-11 |
| 14 | East Mediterranean | Greece | Saronikos Gulf | Athens | Kavouri | Rocky shore | 1 m | Mid-Exposed | Rock | 12-04-11 |
| 15 | East Mediterranean | Greece | Saronikos Gulf | Saronida | Koudounes islet | Rocky shore | 10 m | NA | Sediment in Posidonia bed | 12-04-11 |
| 16 | East Mediterranean | Greece | Evoikos Gulf | Porto Rafti | Harbour | Harbour | Water surface | Protected and mid-exposed | Rock, rope | 13-04-11 |
| 17 | East Mediterranean | Greece | Evoikos Gulf | Porto Rafti | Aulaki | Rocky shore | 2 m | Protected | Small pebbles | 13-04-11 |

Table 1. Collecting information *(continued)*

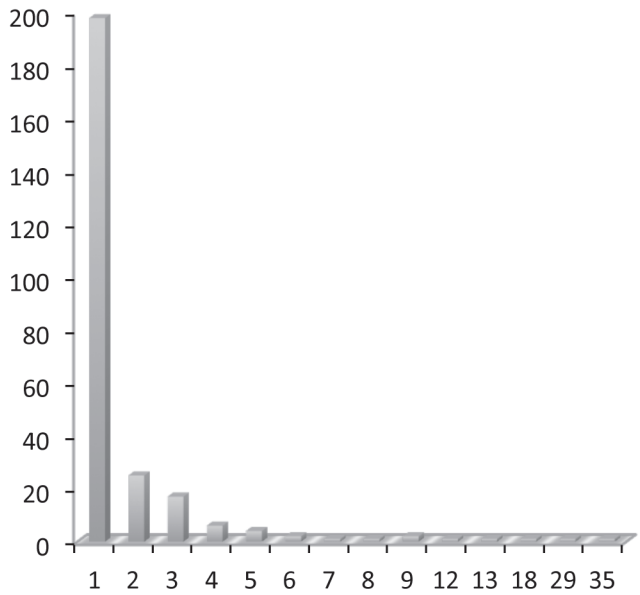
| *Site/date N°* | *Geographic region* | *Country* | *Region* | *City* | *Locality* | *Sites* | *Zone/depth (m) if subtidal* | *Exposure* | *Sampled substrata* | *Collection dates* |
|---|---|---|---|---|---|---|---|---|---|---|
| 18 | East Mediterranean | Greece | Saronikos Gulf | Lagonisi | Pothitos islet | Rocky shore | 25 m (cave) | NA | Small pebbles and shells | 13-04-11 |
| 19 | East Mediterranean | Greece | Saronikos Gulf | Lagonisi | Pothitos islet | Rocky shore | 31 m | NA | Small pebbles and shells | 13-04-11 |
| 20 | East Mediterranean | Greece | Korinthiakos Gulf | Korinthos | Harbour | Harbour | Uppermost subtidal | Protected | Rope | 14-04-11 |
| 21 | East Mediterranean | Greece | Korinthiakos Gulf | Korinthos | Mavra Litharia | Rock, Posidonia | < 5 m | Mid-Exposed | Rock, Patella, macroalgae, Posidonia | 15-04-11 |
| 22 | East Mediterranean | Greece | Korinthiakos Gulf | Aigio | Tsolis | boulders and stones | 15 m | NA | Pebbles and shells, macroalgae | 15-04-11 |
| 23 | East Mediterranean | Greece | Korinthiakos Gulf | Aigio | Tsolis | boulders and stones | 37 m | NA | Pebbles and shells, macroalgae | 15-04-11 |
| 24 | East Mediterranean | Greece | Evoia Island | Kalamos | Kalamos | Rocky shore | 10 m | NA | Cladosiphon mediterraneus | 28-04-12 |
| 25 | East Mediterranean | Greece | Kavala Gulf | Nea Peramos | Vrasida | Rocky shore | < 3 m | Mid-exposed | ND | 30-04-12 |
| 26 | East Mediterranean | Greece | Kavala Gulf | Nea Peramos | Irakleitsa harbour | Harbour | 1 m | Protected harbour, eutrophicated | Sphacelaria tribuloides | 02-05-12 |
| 27 | East Mediterranean | Greece | Ierissos Gulf | Nea Rhoda | Nea Rhoda | Posidonia bed | 20 m | NA | Posidonia oceanica | 03-05-12 |
| 28 | East Mediterranean | Greece | Ierissos Gulf | Nea Rhoda | Nea Rhoda | Rocky shore | 22 m | NA | muddy seabed | 03-05-12 |
| 29 | East Mediterranean | Greece | Dodecanese | Nisyros | Pahia Ammos | Gravel and rocky shore | 2m | Mid-exposed | sand and algal filaments | 24-08-12 |
| 30 | East Mediterranean | Cyprus | Famagusta Bay | Famagusta | off Salamis, north of Famagusta | Posidonia bed | 26 m | NA | sand and sea shells | 30-03-12 |
| 31 | East Mediterranean | Cyprus | Famagusta Bay | Famagusta | off Salamis, north of Famagusta | Posidonia bed | 18 m | NA | coarse sand and seashells | 01-04-12 |
| 32 | East Mediterranean | Cyprus | Chapel Bay | Cape Greco | Agioi Anargyroi | Rocky shore | 25.2 m | NA | substratum in seagrass meadow | 06-04-12 |
| 33 | NW Pacific | S. Korea | Jeju Island | Seogwipo City | Joung Mun | Sandy patches between kelp | 17 m | NA | sand | 06-10-11 |



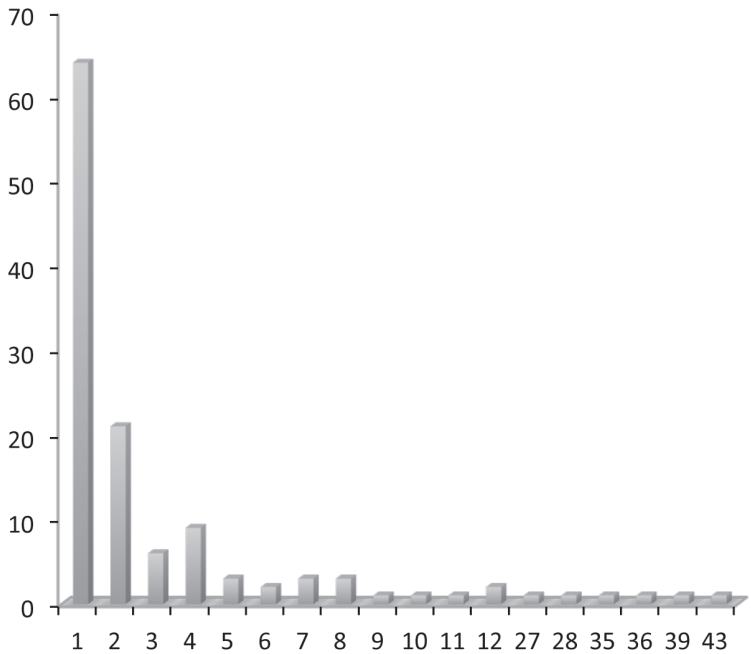

Figs 1-3. **1.** Pebble with emerging filamentous algae after seven weeks in culture. **2.** Distribution (y-axis) of the total of 124750 pairwise genetic K2P distances (PWDs) of 5'-COI (568 bp) of 500 sequences of brown algae, in intervals of 0.5% (x-axis). Position of the barcode gap indicated by an arrow, with 4162 intraspecific (1.5%) and 120544 interspecific distances (>2.5%). A total of 44 PWDs lay in the interval between 1.5 and 2.5%. The largest 4337 PWDs (>20.5%) are not shown. **3.** Distribution (y-axis) of the 2027 PWDs between 0.045% and 4.05%, in intervals of 0.05% (x-axis). Based on these data, the species-limit cut-off was placed at 1.8% (arrow).

## Barcoding

For identification by DNA barcoding we chose partial cytochrome c oxidase I (5'-COI), a marker recently much used in larger red and brown algae (e.g., Saunders, 2005; Le Gall & Saunders, 2010; Mattio & Payri, 2010; Kim *et al.*, 2010; Saunders & McDevit, 2012, 2013; Yang *et al.*, 2014). COI sequences of reference taxa (category RG in Table 2) are scarce for Ectocarpales because previous molecular phylogenies employed rather nrITS, Rubisco spacer (Stache-after Crain *et al.*, 1997) or *rbc*L (Siemer *et al.*, 1998). The most comprehensive phylogenetic work dedicated to the Ectocarpales (Silberfeld *et al.*, 2011) included




Supplementary Fig. 1. Sampling of 263 different 5'-COI sequences from 500 brown algae. A majority of 198 sequences was sampled once.




Supplementary Fig. 2. Sampling frequency of 118 species of brown algae raised from incubated substratum. A majority of 64 species were sampled once.

34 taxa, however it provided 5'-COI only for 17 of these. In addition, morphological identification of Ectocarpales is difficult, for example, of 1466 COI sequences of Ectocarpales publicly available in the Barcode of Life Databases (BOLD) on 27 December 2014, 75% were referred to as "undetermined Ectocarpales", and another 4% determined only to genus level. The samples determined to species level belonged to just 25 species, mostly with comparatively large and easily identifiable fronds. We therefore sequenced 5'-COI of 45 clonal cultures available from previous collections, representing 39 well-identified species of the Ectocarpales, many of which had been used in life-history studies. In addition, some of the environmental isolates, which we could identify morphologically, served as 14 additional reference strains (category RN in Table 2 available on line, doi/10.7872/crya.v36.iss1.2015.S1). They included several type species of higher taxa described in the past. For Ectocarpaceae, the reference taxa included representatives of all lineages so far known (Stache-Crain *et al*., 1997; Peters *et al*., 2010a, b). In *Ectocarpus* and *Kuckuckia*, available (Stache-Crain *et al*., 1997; Peters *et al*., 2010a, b) or newly generated ITS1 and Rubisco spacer sequences (Peters, unpublished data) allowed classification in the lineages defined by Stache-Crain *et al*. (1997). In this work, we append their lineage designations to the species names in *Ectocarpus* (e.g. *E. fasciculatus*5b, *Ectocarpus* sp.2a). If these lineages included more than a single species according to our data (e.g. *Ectocarpus* sp.1b), a further appendix was used to identify the strain origin.

DNA was extracted from a few mg of living algae using the NucleoSpin® 96 Plant Kit. Partial 5'-COI sequences (658bp) were amplified using the primers GAZF2 and GAZR2 (Lane *et al*., 2007). PCR reactions were performed in a total volume of 20 µL containing 0.2 µM of each primer, 150 µM of each dNTP, 2 mM of $MgCl_2$, 1x GoTaq® Flexibuffer, 0.85 units of GoTaq® FlexiDNA polymerase, and 2 µL of 1:30 diluted template DNA. Cycling conditions included an initial denaturation step at 95ºC for 30 s, followed by 35 cycles at 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min, and a final extension at 72ºC for 10 min. PCR products were purified and sequenced with both forward and reverse primers at Genoscope facilities (Evry, France). The sequences were aligned manually with Se-Al™ v2.0a11 (Sequencing Alignment Editor Version 2.0 alpha 11; http://tree.bio.ed.ac.uk/software/seal/) and meticulously checked for correctness by inspecting the chromatogrammes. Pairwise Kimura-2-parameter (K2P) distances between sequences and neighbour-joining (NJ) trees based on them were calculated in PAUP4.0b10. Sequences were deposited in EEDB/GenBank/DDBJ, with accessions from LM994971 to LM995441.

Vouchers (herbarium specimens or permanent microscopic preparations) of 23 field-collected reference taxa and two putatively new species were deposited in the phycological herbarium of the Muséum National d'Histoire Naturelle at Paris (PC). Strains are maintained in the culture collection of the first author.

A species-level cut-off was estimated from the analysis of the distribution of pairwise K2P distances (see Results). In this article a "species" is understood to contain isolates diverging from each other by less than the cut-off value.

## RESULTS

Small algae developed in most cultures of incubated substrata (Fig. 1). For example, only three out of the 60 dishes (5%) incubated in September 2011 showed no brown algae, possibly due to overlooked grazers. In positive dishes, the emergent algae belonged mostly to the green and brown lineages, while red algae were rare. Of the 430 isolated clones of brown algae, 362 emerged from incubated abiotic substratum, while 34 were from microscopic and 34 from macroscopic field individuals (Table 3).

For molecular identification, we used a final alignment of 500 sequences (Table 2), which included 25 reference sequences obtained from public databases or BOLD and 59 newly determined sequences of additional reference taxa, of which 24 belonged to the Ectocarpaceae. Pruning duplicate sequences left 263 different sequences, of which 198 were sampled once. The most frequent sequence (a haplotype of *Hincksia granulosa*) was encountered 35 times (Supplementary Fig. 1).

The 124750 PWDs showed divergence values from 0 (n=1669; sequence identity) to 44%, with a maximum of n=12328 in the interval between 14 and 14.5%. In the distribution of distance intervals (Fig. 2), the "barcode gap" corresponding to the transition from intraspecific to interspecific distances was located between 1.4% PWD and 2.8% PWD (66 pairs). Within this "valley", the first large interval without any hit lay between 1.75 and 1.85% PWD (Fig. 3). We therefore chose a K2P sequence divergence of 1.8% as the species-level cut-off, and using this limit, the 500 sequences were broken down to 118 species, of which 71 were represented by the reference taxa. Of the 83 species present among the environmental isolates, 39 (47%) were identified to species because of sequence similarity with reference taxa or morphology (Table 3). A proportion of 93% of the environmental isolates belonged to the Ectocarpales. Of the 118 species,

Table 3. Summary statistics. Note that some morphologically recognised environmental isolates also served as reference sequences

| *Item* | *Sequences* | *Species* |
|---|---|---|
| All | 500 | 118 |
| Different | 263 | 118 |
| Reference sequences | 84 | 71 |
| Sequences publicly available (RG) | 25 | 24 |
| New reference sequences (RN), total | 59 | 51 |
| Thereof RN no Ectocarpaceae | 35 | 31 |
| Thereof RN Ectocarpaceae | 24 | 20 |
| Environmental isolates | 430 | 83 |
| Macroscopic field thalli (MA) | 34 | 19 |
| Microscopic field thalli (Mi) | 34 | 23 |
| Clones raised from substratum | 362 | 72 |
| Environmental isolates identified | 312 | 39 |
| Environmental isolates not identified | 118 | 44 |

64 were sampled once. The most frequent species isolated was *Hincksia granulosa* (isolated 43 times), followed by *Hecatonema maculans* (n=39), *Ectocarpus* sp.2a (35), *Ectocarpus siliculosus*1a (35), *E. fasciculatus*5b (28), and *Myrionema strangulans* (27) (Supplementary Fig. 2).

NJ phylograms were calculated from all 500 sequences (available upon request from the first author) and from the 263 different sequences (Figs 4-10). The trees must not be interpreted as rigorous phylogenies because of limited resolution of the barcoding sequence. Nevertheless, some of the clusters were in agreement with orders or families revealed in recent multi-gene phylogenies (Silberfeld, 2010, 2011). Within Ectocarpales, the families Scytosiphonaceae, Ectocarpaceae and Chordariaceae formed monophyletic groups (Figs 6-7). A clade close to Chordariaceae included microscopic species, such as *Hecatonema maculans* (Fig. 8). Species classified in Acinetosporaceae according to previous studies formed two separate clades (Figs 9-10).

At the main study site in Brittany, Roc'h Ar Bleiz (=Le Loup), close to Roscoff, sampling was repeated three times, in spring 2011 (22 isolates representing 9 species), autumn 2011 (181 isolates, 25 species), and spring 2012 (39 isolates, 12 species). Of the 30 taxa isolated at this site, only four were not obtained in autumn 2011. Similarly, 63 samples (32 species) from Greece were isolated in spring 2011 and 10 more samples (7 species), from other Greek sites, in spring 2012. A single species of the total of 33 species was not present among the isolates from 2011. In contrast, the two western Mediterranean sites sampled both in spring 2012, were more different from each other: Naples (49 isolates, 17 species) and Ischia (29 isolates, 13 species) shared only five species.

Comparisons between regions (Brittany, Italy representing the western Mediterranean, Greece+Cyprus the eastern Mediterranean, Korea) revealed that Brittany shared ten out of its 39 species with the Mediterranean regions (Fig. 11). The western Mediterranean shared 11 of its 25 species with the Eastern Mediterranean, from where we obtained in total 35 species. Of the six species isolated from Korea, two were also present in Europe, where they were isolated in the Eastern Mediterranean.

## DISCUSSION

The present work for the first time applied the germling emergence method with subsequent barcoding to a large number and diversity of brown algae, and has shown the feasibility of this approach. However, sampling the natural diversity required a considerable effort. The largest collection at one of

Fig. 4. Neighbour-Joining phylogram displaying 5'-COI clustering of 263 different brown algal sequences obtained from 430 clonal cultures raised from environmental samples. A number of 25 public reference sequences (labels in red) and 59 new reference sequences (species names in black) allowed identification of 312 environmental samples (strain designations in black). Strain designations of undetermined samples (n=118) are printed in grey. Designations of eight major clusters, in part agreeing with known higher taxa, are provided. Bootstrap support ≥ 95% from 1000 resamplings is shown by thicker black lines. To facilitate recognition, the roots of the six clusters comprising the Ectocarpales are printed in thick grey lines, note that these do not have strong statistical support. Duplicate (identical) sequences were pruned to calculate this tree. A phylogram of all sequences (duplicates not pruned) is available upon request from the first author. Enlarged sub-sectors of the tree are provided in Figs 5-10. ▶


Tilopteridales
Laminariales
1. Scytosiphon-aceae
2. Ectocarpaceae and Petrospongium
3. Chordariaceae
4. Hecatonema cluster
5A. Pylaiella-Hincksia-Feldmannia cluster
5B. Acinetospora cluster
0.01 substitutions/site

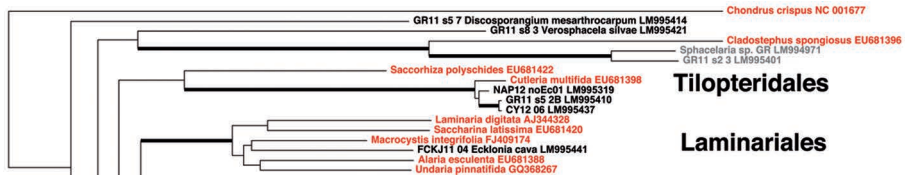



Fig. 5. Detail of Fig. 4 comprising the outgroup (*Chondrus crispus*) and early branching brown algal lineages. See legend to Fig. 4 for explanations.


Fig. 6. Detail of Fig. 4 comprising the clusters corresponding to Scytosiphonaceae, Ectocarpaceae and *Petrospongium*. See legend to Fig. 4 for explanations.

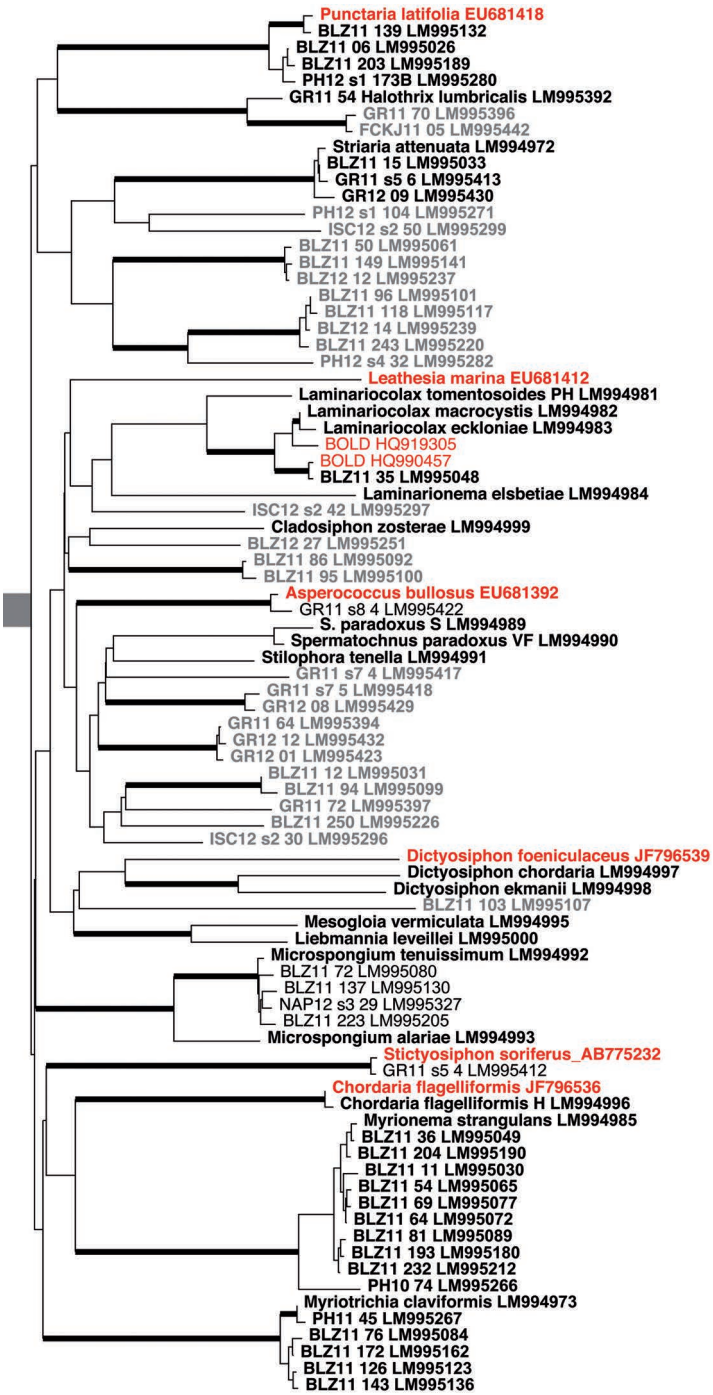



Fig. 7. Detail of Fig. 4 showing a cluster corresponding to Chordariaceae. See legend to Fig. 4 for explanations.

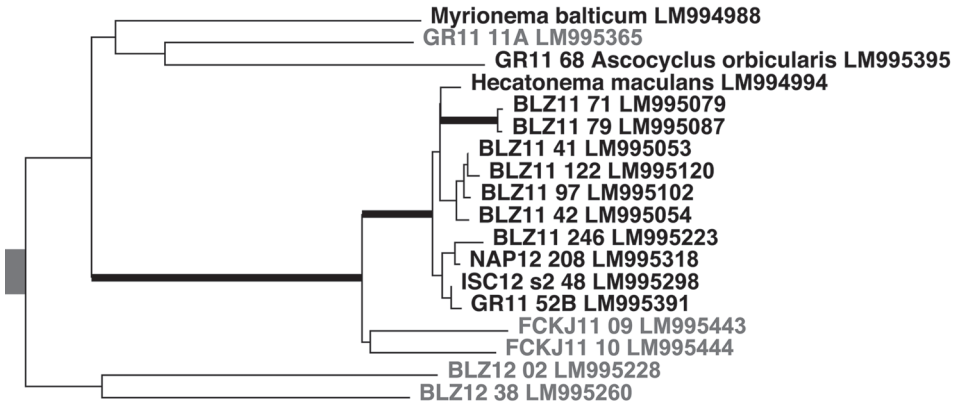



Fig. 8. Detail of Fig. 4 showing a cluster containing microscopic species similar to *Hecatonema maculans* ("*Hecatonema* cluster"). See legend to Fig. 4 for explanations.

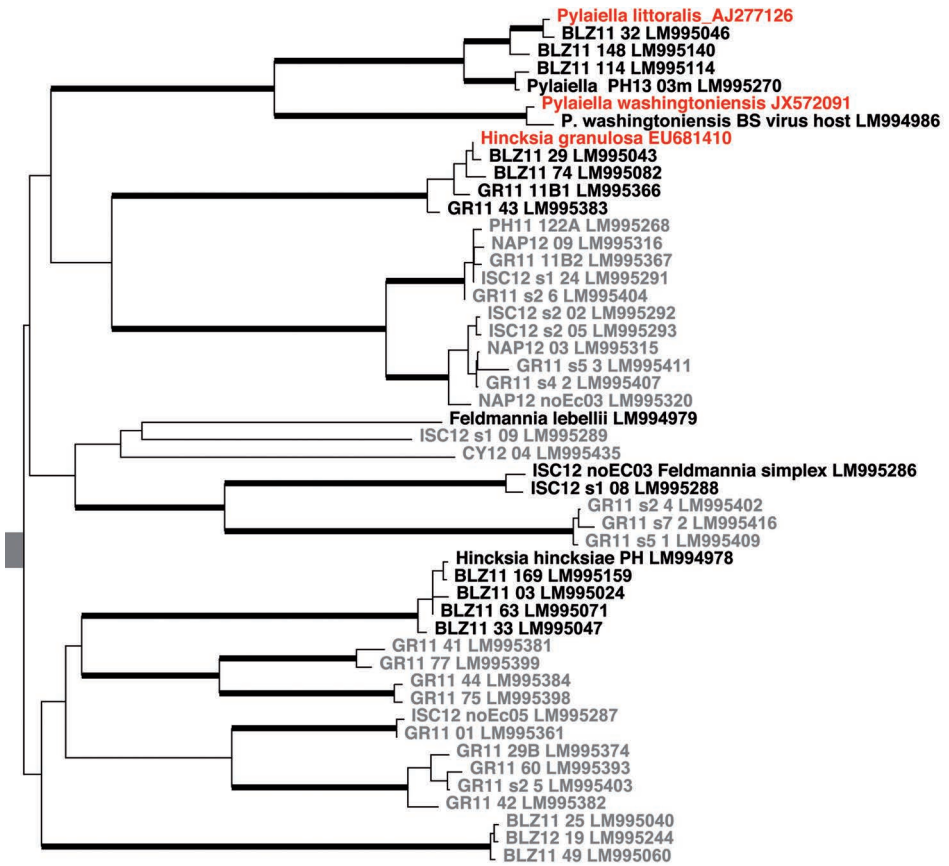



Fig. 9. Detail of Fig. 4 showing a cluster containing species classified in *Pylaiella*, *Hincksia* and *Feldmannia* (PHF group). See legend to Fig. 4 for explanations.

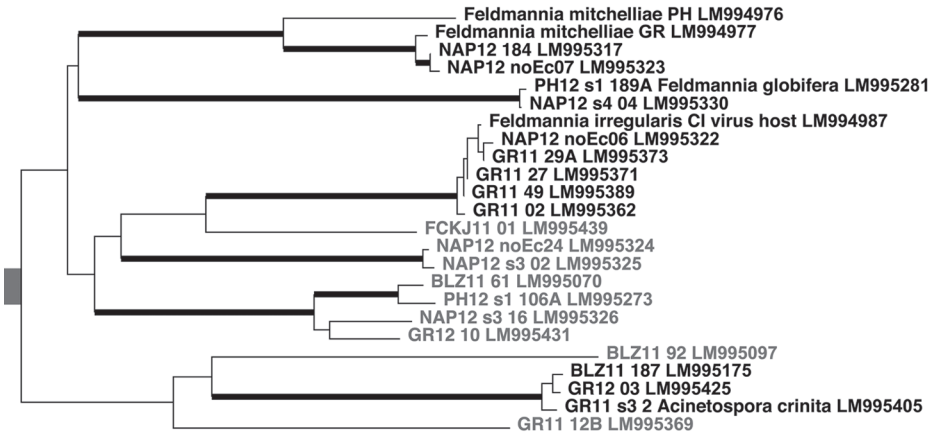



Fig. 10. Detail of Fig. 4 showing a cluster containing species classified in *Acinetospora* and *Feldmannia* ("*Acinetospora* cluster"). See legend to Fig. 4 for explanations.

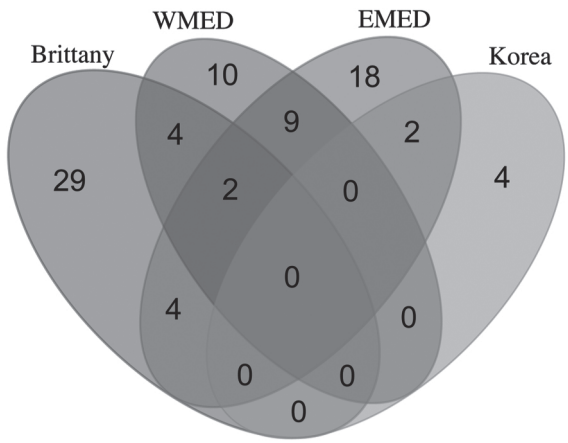



Fig. 11. Distribution of brown algal species isolated from four sampling areas. WMED: West Mediterranean, EMED: East Mediterranean. Diagram produced on-line entering data in http://bioinformatics.psb.ugent.be/webtools/Venn/.

the sites (Roc'h Ar Bleiz) resulted in 28 species from 179 isolates; of these species, 12 (43%) were sampled once. Isolation of 24 and 39 additional clones yielded only one and three further species, respectively. A number of 250 isolates appears thus necessary to obtain a reasonable coverage of the species diversity present on the substratum at a given site.

The isolated taxa did hardly represent the diversity of all cryptic stages present in the field, as there was obviously a bias favouring rapidly growing and proliferating early successional species. By selecting filamentous brown algae we deliberately excluded other life forms, for instance, we did not isolate the green and red algae, germlings of *Giraudyopsis stellifer* Dangeard (Chrysomeridales), Dictyotales, Fucales, sporophytes of *Desmarestia* and most of the kelp

sporophytes; only the samples of kelp sporophytes developing in our dishes of spring 2011 (Robuchon *et al.*, 2014) and a sporophyte from Korea (see below) were raised and identified. In addition, imperfect match of PCR primers may have excluded amplification of DNA of certain lineages. For instance, in six of our eight Greek isolates morphologically identified as Sphacelariales the DNA did not amplify. Improved primers may overcome this problem.

Despite their commonness in the field, the proportion of red algae was insignificant in the raw cultures. For the emergence of Rhodophyta, the culture medium, culture conditions or other factors may have been less suitable. Our method did also not allow distinction between long-term cryptic stages and reproductive cells having settled just before collection of the substrata. On the other hand, the germling emergence method does only reveal the presence of living microstages and thus avoids contamination by fragments of algal fronds or non-surviving propagules, which might compromise metabarcoding approaches using direct isolation of DNA from substratum.

The clones obtained belonged mostly to the Ectocarpales, an order in which reference taxa are limited. Comparison with published as well as new reference sequences obtained from our culture collection of well-identified taxa led to the identification of 47% of the 83 species encountered. We regard this success as encouraging, however it points to the need of comprehensive revisions in difficult groups. Morphological examination of the isolated strains has only started and further scrutiny may result in the identification of additional species.

Our data suggested a species-level cut-off at a K2P divergence of about 1.8% in the Ectocarpales, which is slightly larger than the value of 1.2% inferred for Laminariales and Desmarestiales (Yang *et al.*, 2014). However, this value is arbitrary and should not be mistaken as a strict biological border. Placing the cut-off at 2.7% (the upper end of the "valley" in the distribution of PWD values; Fig. 3) would have rendered only 39 additional values intraspecific, affecting 5 species pairs. Pairs of sequences showing divergences of 1-3% (in our data set n=523 pairs; 0.42% of all 124750 values; 27 species involved) are situated in a transition zone; such taxa may be examples of recent or ongoing speciation possibly still capable of forming fertile hybrids. Additional markers, particularly from the nuclear genome, and populations-size sampling would be needed to examine the genetic exchange in these cases in detail. At larger distances, genetic exchange may cease: for example, *Ectocarpus siliculosus*1a and the genome-sequenced *Ectocarpus* from Peru (lineage 1c), which may produce viable but meiosis-incapable hybrids in the laboratory (Peters *et al.*, 2004), showed a divergence of 3.5%; *E. siliculosus* and *E. crouaniorum*, which in nature may form similar hybrids (Peters *et al.*, 2010a), showed a divergence of 5.4%.

In the following we discuss particular results, in the order (from top to bottom) the taxa appear in Figs 4-10.

**Basal taxa (outgroups)**

There were so far no COI sequences of *Discosporangium mesarthrocarpum* (Discosporangiales) and *Verosphacela silvae* (Onslowiales); their positions in the tree and a number of amino acid substitutions compared to most other brown algae confirmed that these orders form basal branches in brown algal phylogenies (Fig. 5). The two species developed in substratum samples from Greece, obtained by diving at 25 and 15m, respectively, and were identified in culture because of their distinctive characters (Kawai *et al.*, 2007; Alongi *et al.*,

2007). *Verosphacela silvae*, so far only known from the original description based on fixed field material, was isolated for the first time into culture and represents a new record for Greece and the Eastern Mediterranean.

So far there are only two COI sequences from Sphacelariales in the public databases, maybe due to primer mismatches (see above). Like *Cladostephus* and *Sphacelaria radicans* (Dillwyn) C. Agardh (JX572040, not used in our analyses), our sequences of two unidentified species of *Sphacelaria* from Greece (Fig. 5) showed a deletion of 3nt at positions 98-100 of the alignment, which is absent in other brown algae and apparently represents a synapomorphy of the Sphacelariales. Shared amino acid differences to all other taxa (20/219; ~9%) are likewise particular to this order.

Three isolates from the Mediterranean belonged to *Cutleria multifida* (Fig. 5); one from Naples was collected as macroscopic female gametophyte, two (from Greece and Cyprus) developed from substratum. Their COI showed 1.3 and 1.7% genetic divergence to the reference sequence of an isolate from Brittany (Silberfeld *et al*., 2010).

The five reference sequences of Laminariales (Fig. 5) formed a clade, in which a substratum-grown kelp sporophyte from Korea was nested. Its sequence was similar to public data for *Ecklonia radiata* (C. Agardh) J. Agardh (BLAST: 98% identity, AB775229, Kawai *et al.*, 2013) and *Eisenia arborea* Areschoug (BLAST: 97% identity, FJ409145, McDevit & Saunders, 2009). Our isolate belongs to *Ecklonia cava*, which is the only species of *Ecklonia* recorded at Jeju Island (Ga Youn Cho, personal communication). There is no published COI sequence yet for *E. cava*.

### 1. Scytosiphonaceae

The members of the Ectocarpales were contained in six main clusters. The first one, Scytosiphonaceae (Fig. 6, upper cluster), consisted of *Colpomenia peregrina, Scytosiphon lomentaria,* and *Petalonia fascia*. Two substratum-raised cultures from Roscoff were highly similar to reference sequences from the published data for *Colpomenia* and a field gametophyte of *Scytosiphon lomentaria* collected by ourselves at Naples. However, our two Scytosiphon isolates differed by 4.6 and 4.2%, respectively, from another reference sequence of *S. lomentaria* from an individual collected in Brittany (Silberfeld *et al*., 2010), suggesting the presence of cryptic species within the genus *Scytosiphon* in Europe. Three sequences of substratum-raised *P. fascia* from Brittany and a macroscopic field sample from Greece showed between 1.38 and 1.54% divergence to the reference sequence of *P. fascia* collected by Silberfeld *et al*. (2010) in Normandy.

### 2. Ectocarpaceae and *Petrospongium*

A cluster of 24 species (from 151 original sequences; Fig. 6) contained members of the Ectocarpaceae and the published sequence of *Petrospongium berkeleyi*. The little studied Petrospongiaceae was proposed by Silberfeld *et al*. (2011), where it was resolved as sister group to Ectocarpaceae in a multi-gene phylogeny. We generated COI of all known lineages of *Ectocarpus, Kuckuckia*, and of an additional isolate of *Spongonema tomentosum*, confirming the position of *Spongonema* in the Ectocarpaceae found by Silberfeld *et al*. (2011). The pairwise distance data suggested that our sequences of *Ectocarpus* belonged to 16 different species; some species pairs showed genetic distances slightly above our species-level cut-off, these are possibly cases of recent speciation. Other lineages

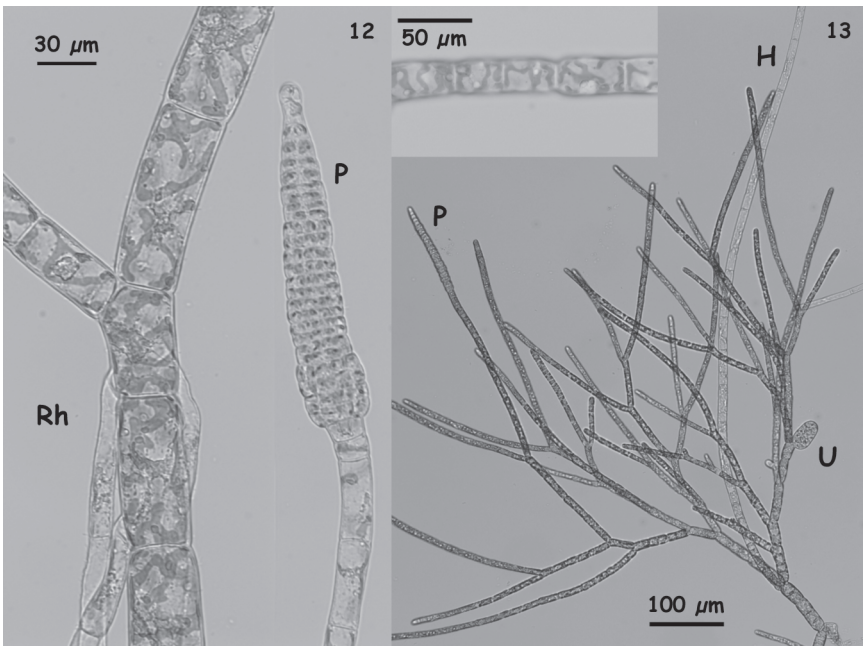
were clearly more separated. We propose to reinstate the species *E. subulatus* Kützing (Kützing, 1843) for individuals referred to as lineage 2d by Stache-Crain *et al.* (1997). Our reference isolate of this species was raised from mud collected in a salt marsh at Pill, Somerset, England. The site is the type locality of *E. amphibius* Harvey (Harvey, 1846), which is a later synonym of *E. subulatus* (Kim, 2010). Two more isolates of *E. subulatus*, with identical COI sequences, were raised from substratum collected in a freshwater-influenced saltmarsh habitat at Terenez, Bay of Morlaix, Brittany. Based on unpublished *rbc*L sequences, Kim (2010) treated individuals of *E. subulatus* from Korea as variation of *E. siliculosus*1a but our COI sequences strongly suggest that this lineage represents an independent species, showing > 6% genetic distance to other *Ectocarpi*, including an autapomorphic non-synonymous substitution L > F. *E. subulatus* is able to tolerate elevated temperatures (Bolton, 1983; isolate from the southernmost *Ectocarpus* population in the Gulf of Mexico at Port Aransas, Texas) and salinity stress (Dittami *et al.*, 2012; isolate from fresh water, Australia). Another *Ectocarpus* isolate from Korea belonged to a lineage (1d) not studied by Stache-Crain *et al.* (1997). This is probably the species Kim (2010) referred to as *E. acutus* Setchell & N.L. Gardner.

Our data included three different lineages of *Kuckuckia*, which were polyphyletic and not markedly separated from *Ectocarpus*. Two of them were also found among clones raised from substratum, however only in Mediterranean samples, in agreement with the principal distribution of *Kuckuckia* in warm-temperate regions (Pedersen, 1989).

*Putative new species* – From substratum collected at Ischia, Italy, we obtained seven independent isolates of a so far unknown lineage of *Ectocarpus*. All isolates of this entity had highly similar sequences (PWD ≤ 0.3%). The distance to the closest lineage of *Ectocarpus* (*E. siliculosus*1a) was 3.1%, suggesting that this *Ectocarpus* from Ischia is a separate, possibly undescribed species. Isolate BLZ11-180 from Roscoff was also a member of the *Ectocarpus-Kuckuckia* cluster but herein different from all other lineages. The plastids were ribbon-shaped (this is the diagnostic character of Ectocarpaceae; Peters & Ramírez, 2001; Silberfeld *et al.*, 2011) and the morphology did not differ from that of *Ectocarpus* (Fig. 12). The three isolates CY12-01, CY12-03 and CY12-07, which possessed a single COI sequence, clustered with Ectocarpaceae and *Petrospongium* on a long branch. They showed ribbon-shaped plastids, dichotomous branching, phaeophycean hairs, terminal slender plurilocular and lateral unilocular sporangia (Fig. 13). BLZ11-180 and the three isolates from Cyprus likely represent two so far undescribed species.

## 3. Chordariaceae

A cluster of 39 species (from 119 sequences, 94 thereof from environmental isolates; Fig. 7) included *Chordaria flagelliformis*, the type species of the Chordariaceae. Reference sequences from 23 species allowed identification of 55 environmental isolates. By DNA barcoding we not only recognised life forms with erect fronds, which would also be amenable to identification by morphology, but also cryptic stages that are part of a heteromorphic life history. Examples among our samples were filamentous microscopic stages of *Myrionema strangulans, Myriotrichia claviformis* and *Striaria attenuata*. These species possess haploid-diploid life histories involving filamentous prostrate microscopic gametophytes (Loiseaux, 1967; Peters 1988, 1991a). *Striaria attenuata* has been isolated previously at Roscoff from a field-collected microstage (Loiseaux 1969, as *Myriotricha* sp.). The sporophytes of *M. strangulans* and *M. claviformis* are




Figs 12-13. **12.** Strain BLZ11-180 from Roscoff, putatively a new species of *Ectocarpus*. Erect filament with ribbon-shaped plastids, rhizoids (Rh), and a plurilocular sporangium (P). **13.** Strain CY12-01 from Cyprus, putatively a new species. Habit, with phaeophycean hair (H), plurilocular (P) and juvenile unilocular (U) sporangium. Insert: Ribbon-shaped plastids in erect filament.

generally found as epiphytes on *Ulva* and *Scytosiphon*, respectively, and are possibly obligate epiphytes on these algae. Molecular sex markers, so far only present for *Ectocarpus* (Ahmed *et al.*, 2014) or cross-fertility experiments would be required to prove that the microstages we isolated are gametophytes.

There are species in Chordariaceae lacking a macroscopic stage. Because their small size and lack of conspicuous morphological characters render their distinction difficult, DNA barcoding is essential for their reliable identification. For example, the sequence of isolate BLZ11-35 resembled that of *Laminariocolax* isolates from Chile (*L. macrocystis*), South Africa (*L. eckloniae)*, and Pacific Canada (unidentified Ectocarpales samples ABMMC12605-10 and MACRO1242-09 in BOLD). Applying the species-limit cut-off at 1.8% PWD, these five samples belong to a single species, probably *L. aecidioides* known to be present at Roscoff as common endophyte in *Laminaria hyperborea* (Gunnerus) Foslie (Peters, 2003). Sexuality found in Chilean *L. macrocystis* has not been demonstrated for Atlantic or North Pacific *L. aecidioides*. However, unilocular sporangia were described in *L. aecidioides* from Greenland (Rosenvinge, 1893), which suggests that it might occur more generally in this common kelp endophyte. Another microscopic species, *Microspongium tenuissimum*, which is likewise not easy to identify morphologically, was known from previous works as endophyte in red algae (Burkhardt & Peters, 1998; Peters, 2003). In the present study, it was raised from abiotic substratum six times in Brittany and once in Italy. At Roscoff it has been reported previously among species appearing in culture (Dangeard, 1970, as

*Streblonema tenuissimum* Hauck). An unidentified ectocarpalean small brown alga in BOLD (MACRO752-07) from Rhode Island, USA, had the same sequence.

In Chordariaceae, a number of 36 clones from 17 species were not identified and possibly represent other microscopic Phaeophyceae or microstages of other species. The eleven reference species not present among the environmental isolates included four taxa the geographical distributions of which do not encompass the sample sites (*Chordaria flagelliformis*, *Dictyosiphon* spp.); the lack of their microstages among the environmental samples is not surprising. The other species (*Cladosiphon zosterae, Laminariocolax tomentosoides, Laminarionema elsbetiae, Leathesia marina, Mesogloia vermiculata, Spermatochnus paradoxus, Stilophora tenella*) are known to occur at or near the study sites (Feldmann, 1954; Peters, unpublished), however we did not find them in the bank of microscopic stages. Their microstages may inhabit environments different from the rock surface, pebbles and shell fragments sampled in our study, or may be too inconspicuous or slow growing to be isolated in our experiments.

**4. *Hecatonema* cluster**

A group of eight species (47 sequences) formed a cluster including a large number of microscopic algae that did not form erect filaments and morphologically resembled *Hecatonema maculans* (= *H. terminale* (Kützing) Kylin; Fig. 8). We confirmed the identity of these isolates by adding the reference sequence of an isolate of *H. maculans* from the area of the type (Maine, NW Atlantic) and the habitat (epiphytic on *Palmaria palmata*; Collins, 1896). Isolates of *H. maculans* were abundant in Brittany and also present in Greece and Italy. Two isolates from Korea differed from *H. maculans* by approximately 3.5% and represent North Pacific members of the same genus. Other identified microscopic isolates in this cluster included the reference taxon *Myrionema balticum* (material from the Western Baltic Sea, close to the type locality) and an isolate from Greece identified in field material as *Ascocyclus orbicularis*, both found as epiphytes on seagrass. None of the taxa from the *Hecatonema* cluster has previously been included in molecular phylogenetic studies. The genetic separation of the cluster from Chordariaceae, if confirmed in multi-gene phylogenies, would justify re-instatement of the family Hecatonemataceae (tribu Hécatonématées in Loiseaux, 1967).

**5. Acinetosporaceae**

The remaining 29 species were forms that based on previous phylogenetic works (Peters & Ramirez, 2001; Silberfeld, 2011) would have been included in Acinetosporaceae, represented in Silberfeld *et al*. (2011) by *Pylaiella littoralis* and *Hincksia granulosa*. Algae in this group possess several discoid plastids per cell and heterotrichy, i.e. they have basal creeping filaments issuing erect, usually monosiphonous filaments; the latter grow by means of intercalary meristematic regions. The life history is of the direct type or involves an alternation of similar generations. Sexuality has been reported for three species, with monoicous gametophytes and anisogamy (Sauvageau 1896a, b; Müller, 1969; Müller & Ramírez, 1994). In our analyses, these algae were placed in two separate clusters.

*5A. Pylaiella-Hincksia-Feldmannia cluster* – A number of 17 species formed a cluster henceforth referred to as "PHF group" (Fig. 9). From Roscoff we isolated *P. littoralis* and its cryptic sibling species (Geoffroy *et al*., 2015), forming a clade together with the North Pacific taxon *P. washingtoniensis*. The latter is

also the host of the virus PlitV-1 (Maier *et al*., 1998), for which there was so far no molecular identification. The PHF group further included *Hincksia hincksiae* (the type species of *Hincksia*; Guiry & Guiry, 2014). The species is morphologically characterised by adaxial series of conical plurilocular sporangia (Cardinal 1964). This feature was also present in our reference material isolated as epiphyte on its common host *Saccorhiza polyschides* at Roscoff. Another *Hincksia*, *H. granulosa*, was the most frequent isolate from Roscoff and also found twice in Greece, which constitutes new records of this species for Greece. However, COI did not place *H. hincksiae* and *H. granulosa* in a single clade. In addition to *Pylaiella* and *Hincksia*, the PHF cluster contained a minute *Feldmannia* found as epiphyte on *Cystoseira* sp. at Plougonvelin, Brittany. This *Feldmannia* may correspond to *F. lebellii*, the type species of *Feldmannia* Hamel (Hamel, 1939). Two isolates from Italy and one from Greece agreed morphologically with *F. simplex*, which is another minute species of *Feldmannia* (Sauvageau, 1933; Cardinal, 1964). The putative *F. lebelii* and *F. simplex* formed a clade together with three undetermined species. In total, the PHF cluster included ten undetermined species.

*5B. Acinetospora cluster* – The cluster of the last 12 species (Fig. 10) included *Acinetospora crinita*, the type species of Acinetosporaceae, characterised by monosporangia in addition to plurilocular and rare unilocular reproductive organs (Sauvageau, 1899). *A. crinita* was not included in molecular phylogenies of the brown algae, except for a very early study on 5S ribosomal RNA (Lim *et al.*, 1986). We isolated *A. crinita* both from Brittany and Greece. The cluster also included three species currently placed in *Feldmannia*. A number of seven isolates of *F. irregularis*, for which we used the available culture of the host of the FirrV-1 virus from the Canary Islands (Müller & Frenzer, 1993) as reference, developed from environmental isolates from Italy and Greece. *Feldmannia globifera*, isolated four times from Roscoff and once from Naples, was morphologically identified in culture because of characteristic globular plurilocular sporangia, born directly on the filaments or on short pedicels branching at right angles from the filaments (Cardinal, 1964). *Feldmannia mitchelliae*, characterised by its symmetrical ovoid plurilocular sporangia, was also a member of the *Acinetospora* cluster. In *F. mitchelliae*, COI revealed cryptic speciation between the Atlantic and the Mediterranean: whereas our two isolates of this taxon from Brittany had an identical COI, our four isolates from Greece and Italy, despite showing a similar morphology, were separated from the Atlantic strains by 5.3-5.7% PWDs. In addition to the five identified species, the *Acinetospora* cluster included seven species yet to determine.

Our study has shown that barcoding of small brown algae obtained by the germling emergence method is practicable to reveal the diversity of cryptic stages of the Ectocarpales, however depending on the availability of reference taxa the identification success differed markedly among taxonomic subgroups (Table 4). COI resolved three of the presently accepted families of the Ectocarpales (Silberfeld *et al*., 2011) as clades. Scytosiphonaceae was confirmed but represented by only a few samples, which were all identified. Maybe the microstages of other members of this family are too rare or slow growing to be well represented among the emergent algae. Ectocarpaceae was also confirmed. Similar to Desmarestiales (Yang *et al*., 2014), this family is comprehensively sampled (Stache-Crain *et al*., 1997, Peters *et al*., 2010a, b; this paper), and exemplifies a group in which COI sequences, combined with a diagnostic morphological character, can reveal undescribed species. A major taxonomic problem in the Ectocarpaceae consists in finding the scientific names

Table 4. Distribution of species among COI clusters in Ectocarpales

| *Family/COI cluster* | *Species* | *Reference sequences* | *Identified species* | *Non-identified species* | *Proportion of identified species* |
|---|---|---|---|---|---|
| All Ectocarpales | 103 | 73 | 61 | 42 | 0.59 |
| Scytosiphonaceae | 3 | 4 | 3 | 0 | 1.00 |
| Ectocarpaceae (incl. *Petrospongium*) | 24 | 26 | 21 | 3 | 0.88 |
| Chordariaceae | 39 | 28 | 22 | 17 | 0.56 |
| *Hecatonema* cluster | 8 | 3 | 3 | 5 | 0.38 |
| *Pylaiella-Hincksia-Feldmannia* cluster | 17 | 7 | 7 | 10 | 0.41 |
| *Acinetospora* cluster | 12 | 5 | 5 | 7 | 0.42 |

corresponding to the different lineages. Chordariaceae, with a number of reference species available, was also confirmed and showed that DNA barcoding permits identification of microscopic taxa and microstages, which would hardly be recognisable morphologically. Other microscopic isolates belonged to a genetically different group of microscopic species, which included *Hecatonema maculans* and other microcrusts so far classified in *Myrionema*, such as *Ascocyclus orbiculare* (=*Myrionema magnusii*) and *Myrionema balticum*. Genetic barcoding of cultures will add to the revision of these difficult taxa. Filamentous algae resembling *Acinetospora, Pylaiella, Hincksia* and *Feldmannia* are less well studied than *Ectocarpus*. Their considerable morphological diversity (Hamel, 1939; Cardinal, 1964) was born out by a large genetic diversity found in the present account, however the scarcity of reference sequences left 60% of the species inferred from COI data unidentified. There will be no progress without thorough and comprehensive taxonomic revision of these forms. Maybe our cultures at closer scrutiny will reveal morphological characters that allow identification of more taxa. Because of the isomorphic life history, characteristic morphological features described from field material are more likely to appear in culture in these algae than in the micoscopic stages of the Chordariaceae.

The new sequences from the present study will be useful references in future surveys of small brown algal diversity. As exemplified above by two endophytic genera in Chordariaceae, comparing our data with those present in BOLD will also help to identify previously collected samples that were only roughly classified taxonomically and therefore suppressed in the public databases.

The germling emergence method coupled to DNA barcoding has obvious advantages for capturing cryptic elements of the macroalgal flora of remote locations, where high access and logistics costs, limited time in the field and laboratory facilities typically complicate algal surveys. In particular and in addition to these aspects, it enhances the potential of scientific diving at such locations (Sayer *et al.*, 2013) considering the ease of collection of substratum samples in sterile tubes by a research diver. Environmental 5'-COI barcoding has previously found to uncover significant diversity in microalgal groups such as dinoflagellates (Stern *et al.*, 2010; the present study has detected a higher proportion of environmental isolates than the study on dinoflagellates) – arguably, culturing and isolation of germlings emerging from substratum samples adds a new tool with added scope and enhanced capability of detecting cryptic

biodiversity to environmental barcoding approaches. Recent examples, albeit with limited numbers of samples, were contributions to marine macroalgal surveys at Adelaide Island, a high-latitude West Antarctic locality (Mystikou *et al*., 2014) and Ascension, a remote tropical island (Tsiamis *et al*., 2015).

Even if efficient for a rapid and large-scale screening of species diversity, the single gene DNA-barcoding approach used in this study has been criticized since it relies on the assumption that a single gene genealogy is representative of the species phylogeny (DeSalle *et al.*, 2005; Chase *et al.*, 2005). Problems linked to a single-gene approach, such as incomplete lineage sorting or introgression, were reviewed by Leliaert *et al.* (2014) for DNA-based species delimitation in algae. In order to improve our knowledge of species diversity and delineation within the Ectocarpales, the strategy would be to increase the gene sampling to two or more, unlinked genes and to combine methods of phylogenetics and population genetics with various data and criteria (referred to as integrative taxonomy, Dayrat, 2005) to selected taxa.

The brown algae of Roscoff are historically well studied (Feldmann, 1954; Cardinal, 1964; Feldmann & Magne, 1964) and evident changes are noticed rapidly because of constant observations. However, DNA barcoding is required for monitoring modifications in the community composition of cryptic stages and species. Data like those presented in this work may help to follow the floristic changes that are expected to happen in the future due to the introduction of Indo-Pacific fouling algae travelling by ships through the Northeast and Northwest passages.

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