Short communication

Roberta Skukan, José M. Rico and Yaisel J. Borrell*

DNA barcoding-based assessment of the invasive and native non-crustose *Codium* species in the central Cantabrian Sea, southern Bay of Biscay

https://doi.org/10.1515/bot-2020-0060
Received September 24, 2020; accepted December 18, 2020; published online January 6, 2021

Abstract: In this work, we identified non-crustose invasive (*Codium fragile* subsp. *fragile*) and native *Codium* spp. (*Codium tomentosum* and *Codium vermilara*) in the central Cantabrian Sea using DNA barcoding (tufA and rbcL genes). We designed a new FCO*tuf*A genetic marker for identifying *Codium* spp. in fresh and herbarium material. The tufA and rbcL sequences revealed three different single haplotypes for each of the species and a lack of intraspecific genetic diversity. The FCO*tuf*A genetic marker revealed one new haplotype of *C. fragile* within the native region (South Korea), suggesting the possibility of higher genetic diversity in the donor region of this invasive species.

Keywords: Cantabrian Sea; DNA barcoding; herbarium samples; non-crustose *Codium* species.

The marine green macroalga *Codium* shows a wide variety of morphological forms (Pedroche 2001), especially in cryptic subspecies of *Codium fragile* (Provan et al. 2008) and some other non-crustose native *Codium* spp., e.g., *Codium duthiae* and *Codium geppiorum* (Verbruggen and Costa 2015). Thus, authors have concluded that the taxonomic assignments for species within this genus should be supported by genetic analysis (Silva 1955; Verbruggen et al. 2007), as in the case of other macroalgae of a cryptic nature (Montes et al. 2016; Steinhagen et al. 2018). Previously, DNA barcoding with chloroplast molecular markers (*rpl*16-*rps*3, *tufa*, and *rbc*L) has been shown to be an effective tool for distinguishing fresh as well as herbarium samples of *Codium* spp. (Provan et al. 2008; Verbruggen et al. 2017).

Eight species from the genus *Codium* from the intertidal zone along the Cantabrian coast have been described based on their morphological features (Cires and Moliner 2010). Among those species, seven were reported as native between 1889 and 1955 (Cires and Moliner 2010), and one, *C. fragile* subsp. *fragile* (henceforth *C. fragile*) (Silva 1955), was recognized as a widely distributed invasive seaweed from Japan and Korea. As with *C. fragile*, other Codium spp. such as *C. fragile* subsp. *atlanticum* (A. D. Cotton) P. C. Silva, *Codium tomentosum* Stackhouse, *Codium vermilara* (Olivi) Delle Chiaje, and *Codium decorticatum* (Woodward) M. Howe have dichotomous branching (non-crustose) thalli, so visual identification in the field may result in incorrect species assignment, and microscopic visualization and DNA barcoding-based assessments are needed.

In this study, we aimed to update the current knowledge on the presence of non-crustose native and invasive *Codium* spp. in the central Cantabrian coast and to develop a new protocol for amplifying short fragments from the discriminative *tuf*A genetic marker to identify problematic herbarium samples or other seaweed material.

Non-crustose *Codium* spp. were collected on the central Cantabrian coast and one additional site on the northern coast of Portugal (Figure 1, Table 1). Samples were preserved in 96% ethanol for morphological identification and in silica gel for molecular work. All collected material was examined under a microscope. The presence of utricules with tips ending in a sharp mucron was used as a morphological trait to differentiate between the native (non-mucronate) and invasive *C. fragile* (mucronate) (Silva 1955) forms. Native non-crustose *Codium* spp. were also

***Corresponding author: Yaisel J. Borrell,** Department of Functional Biology, Genetics, University of Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Spain, E-mail: borrellyaisel@uniovi.es. https://orcid.org/0000-0003-2840-4384
Roberta Skukan, Department of Biology of Organisms and Systems (BOS), University of Oviedo, C/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain; Department of Functional Biology, Genetics, University of Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Spain; and Neoalgae Micro Seaweeds Products, Polígono de Lloreda, C/Carmen Leal Mata 191, 33211 Gíjon, Asturias, Spain
José M. Rico, Department of Biology of Organisms and Systems (BOS), University of Oviedo, C/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain

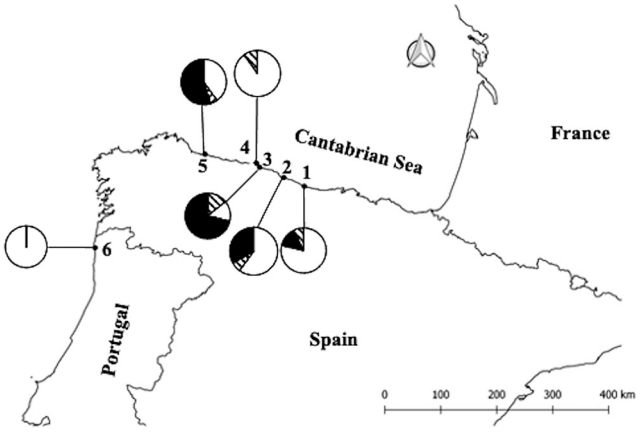

Figure 1: Map of *Codium* sampling locations in northern Spain and Portugal. 1-Vidiago ($N = 15$), 2-de Vega ($N = 14$), 3-Gijón (port) ($N = 7$), 4-Aramar ($N = 40$), 5-Tapia de Casariego ($N = 20$), and 6-Viana do Castelo ($N = 27$). Species identification based on tufA gene shown: black, *C. fragile*; white, *C. tomentosum*; hatched, *C. vermilara*. Species identification based on tufA gene shown: black, *C. fragile*; white, *C. tomentosum*; hatched, *C. vermilara*.

separated based on visual inspection as proposed by Maggs and Kelly (2007) based on the type of branching, shape of branching, and shape of the thallus base.

DNA was extracted using the GeneMATRIX Plant and Fungi Purification Kit (EURx Cat. No. E3595, Roboklon GmbH, Berlin, Germany). PCR products were amplified using the *tuf*A marker (813 bp) from Saunders and Kucera (2010) (forward primer: tufGF4) and Famà et al. (2002) (reverse primer: tufAR) and the partial fragment of the *rbc*L marker (787 bp) following the protocol of Verbruggen et al. (2007). FCO*tuf*A primers (190–200 bp) were designed using the web interface Primer3Plus (Untergasser et al. 2007) and default parameters for the amplification of problematic fresh and herbarium samples (Supplementary Table S1). There were no reference genomes available at the time in GenBank for the species in this study, so *tuf*A sequences from different families within the order Bryopsidales and sequences obtained in this study were used to design forward (FCO*tuf*AFw: 5′-TCTCAATTACAGGAAGAGGTACG-3′) and reverse (FCO*tuf*ARv: 5′-ATCTTTTTGAATGCCACGTAA-3′) primers for identifying *Codium* spp (figshare DOI: 10.6084/m9.figshare.11897196). We used slightly modified PCR conditions from those reported by Famà et al. (2002) by lowering the annealing temperature (45 °C for 1 min). The same PCR conditions were used for the amplification of FCO*tuf*A. For *rbc*L, we optimized the PCR conditions following the Verbruggen et al. (2007) protocol. The samples were sent for purification and sequencing to Macrogen (Amsterdam, Netherlands).

Forward and reverse sequences were aligned to produce consensus sequences using freeware BioEdit (Hall 1999) that were blasted in NCBI (Altschul et al. 1997). Information about the sequences downloaded from GenBank is available in figshare (DOI: https://doi.org/10.6084/m9.figshare.12176574). All alignments were performed with the MUSCLE algorithm in MEGA ver. 10.0.5 (Kumar et al. 2018) using the neighbor-joining (NJ) method with 1000 bootstrap replicates.

Samples from this study showed 100% similarity with previously reported public *C. fragile* subsp. *fragile* and *C. vermilara* sequences from GenBank for both the *tuf*A and *rbc*L markers (Table 1). The *Codium* spp. clustering of Cantabrian samples was well supported, with high

Table 1: The total number of collected *Codium* species and specimens used for GenBank ID and BLAST procedures on plastid haplotypes from North Coast of Spain (region Asturias) and Portugal (location Viana do Castelo).

Place, year and total number of collected specimens (I/N)[a]	Coordinates	Gene (voucher code)	Gene (GenBank ID)	Gene (assignment in GenBank, % of similarity, geographical origin of top hits)
Aramar, February 2016 (0/155)	43°60′99″N, 5° 78′55″W	tufA (FCO-Alg.2287)/ rbcL (FCO-Alg.2298)	tufA (MK569392)/ *rbc*L (MK569395)	tufA (*C. vermilara*, KP685880.1,100%, island Krk, Croatia)/rbcL (*C. vermilara*, EF108092.1,99.83%, island Krk, Croatia)
Port of Gijón, June 2017 (131/48)	43°55′44″N, 5° 69′97″W	FCO-Alg.2230	tufA (MK569393)	*C. fragile* subsp. *fragile* MH427857.1 (100%, Victoria, Australia)
Tapia de Casariego, December 2016 (33/111)	43°57′02″N, 6° 94′97″W	tufA (FCO-Alg.2239)/ rbcL (FCO-Alg.2238)	tufA (MK569394)/ *rbc*L (MK569396)	tufA (*C. tomentosum*, KX855797.1, 100%, southern Australia)/rbcL (*C. fragile* subsp. *fragile*, KJ909148.1, 99.83%, Jeju, South Korea)
Viana do Castelo, March 2016 (0/168)	41°68′78″N, 8° 84′66″W	FCO-Alg.2272	rbcL (MK569397)	No match[b]
De Vega[c] (4/145)	43°47′48″N, 5° 13′45″W	–	–	–
Vidiago[d] (1/15)	44°40′15″N, 4° 65′13″W	–	–	–

[a](I/N): Total number of specimens collected of invasive *Codium fragile* (I), or native *Codium* spp (N). [b]Assigned to *C. tomentosum* with *tufA* gene (see the text). [c,d]Other sampling locations from central Cantabrian Sea, with a total number of *Codium* spp.

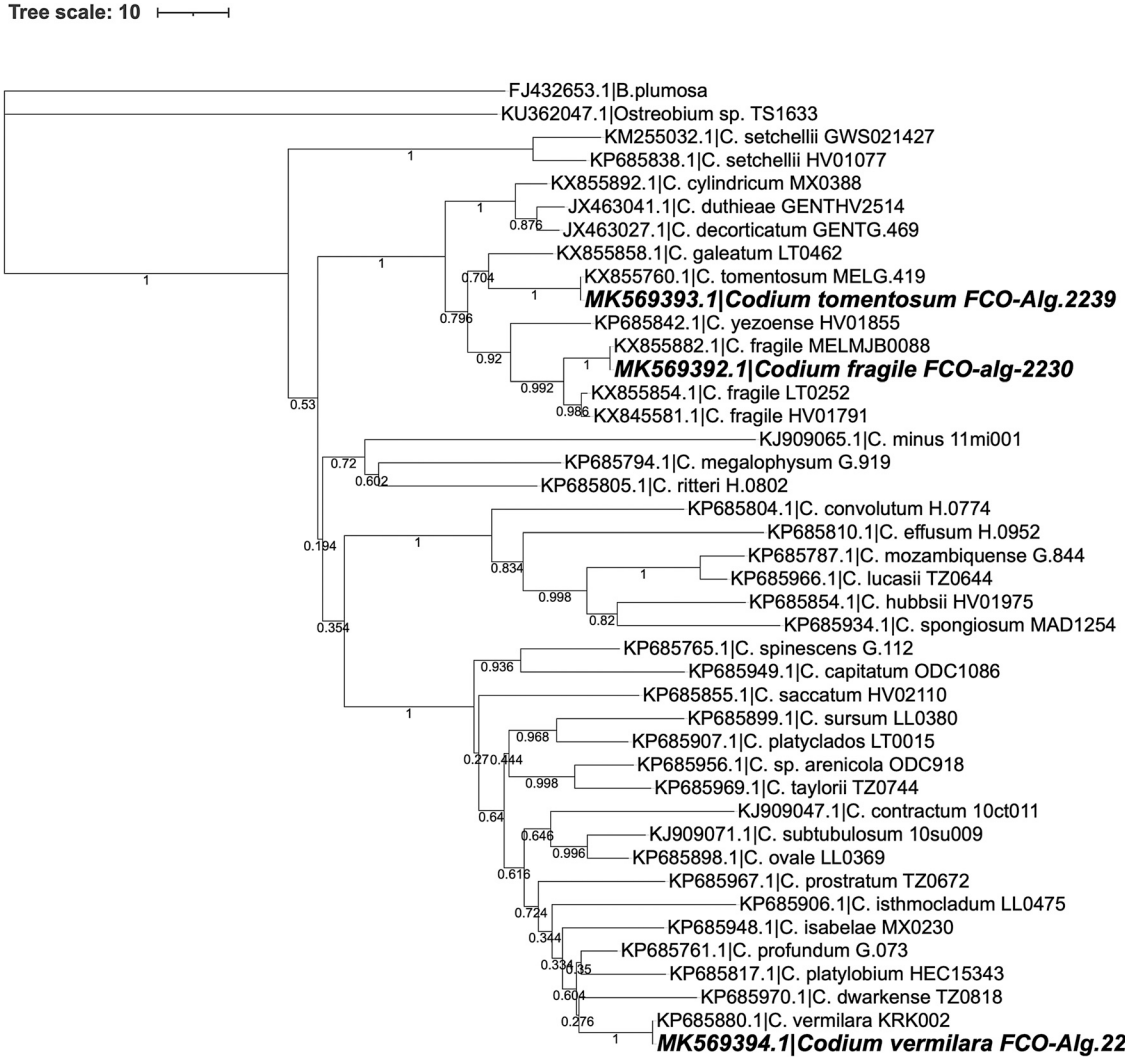

Figure 2: Neighbor-joining (NJ) tree showing the relationship among *Codium* spp. with tufA marker.83 sequences from this study and 58 from GenBank were used to assess species relationships (figshare https://doi.org/10.6084/m9.figshare.12176574). The samples from the central Cantabrian Sea are indicated in bold and italics.

bootstrap values for *tuf*A and *rbc*L (Figures 2 and 3). *rbc*L data for *C. tomentosum* were not available in the GenBank database. However, our *tuf*A analysis indicated that the haplotype MK569397 found in the sample FCO-Alg.2272 was clearly the species *C. tomentosum* (Table 1). Moreover, the invasive and native non-crustose *Codium* species under study revealed no differences in terms of genetic diversity within the area of study (a single haplotype in each case).

Fresh Korean samples and herbarium samples were successfully amplified with FCO*tuf*A (sequences in figshare, DOI: https://doi.org/10.6084/m9.figshare.12046407). *In silico* analyses showed that FCO*tuf*A may be useful for identifying *C. fragile*, *C. tomentosum*, *Codium yezoense*, *Codium galeatum*, *Codium cylindricum*, *Codium duthieae*, *C. decorticatum*, and *Codium contractum*. The new FCO*tuf*A marker differentiated the *C. fragile* samples and revealed one new haplotype in the Korean sample FCO-Alg. 2365 (Figure 4).

In this work, the presence of three non-crustose *Codium* spp. in the central Cantabrian Sea, the invasive variant of *C. fragile* and two native species (*C. tomentosum* and *C. vermilara*), were confirmed using the tufA and rbcL genes. Both genes supported morphological identification and revealed a lack of intraspecific genetic diversity within the area, as previously described for other geographic regions (i.e., Verbruggen and Costa 2015). The samples of *Codium* spp. collected in this study coincide with those of ecological studies of non-crustose *Codium* spp. in northern Spain (García et al. 2018), suggesting that there is a higher proportion of *C. fragile* in the summer months (July–September) and *C. tomentosum* in winter months and a lower abundance of *C. vermilara* in all localities.

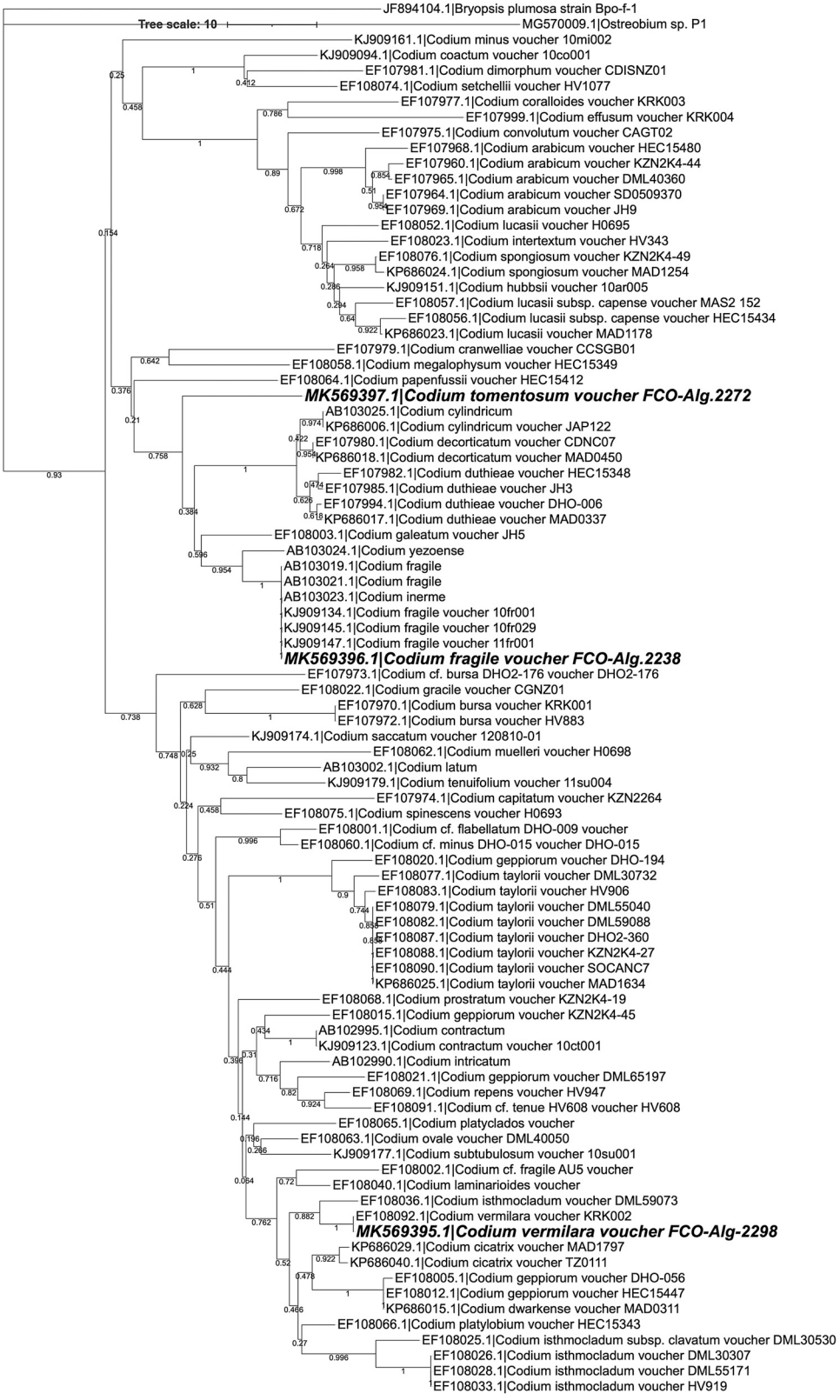

Figure 3: Neighbor-joining (NJ) tree showing the relationship among *Codium* spp. with *rbcL* marker.The *Codium* spp. genetic analyses included 132 sequences from this study and the 93 sequences downloaded from GenBank (figshare https://doi.org/10.6084/m9.figshare.12176574). The samples from the central Cantabrian Sea are indicated in bold and italics.

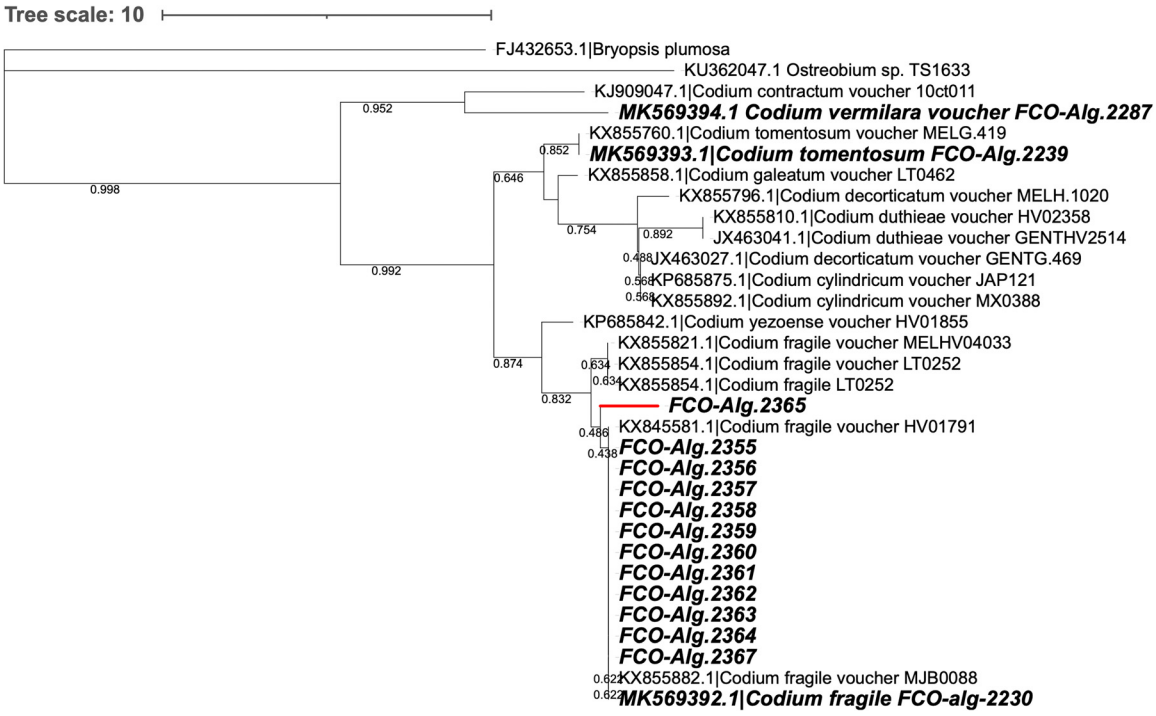

Figure 4: Neighbor-joining (NJ) tree showing the relationship among *Codium* spp. with *FCOtufA* marker.20 sequences from GenBank and 12 new sequences were obtained in this work (figshare https://doi.org/10.6084/m9.figshare.12991151). The new FCO*tuf*A marker differentiated *C. fragile* from the other species and the two previously described "native" haplotypes (KX855821.1|*C._fragile*, KX855854.1|*C._fragile*; Verbruggen et al. 2017) from the unique invasive haplotype detected in this work (KX855882.1|*C._fragile* = the Cantabrian Sea *C. fragile* haplotype MK569393), and from the new haplotype detected in the Korean sample, FCO-Alg. 2365 (red node). Samples processed in this work are indicated in bold and italics.

The FCO*tuf*A marker facilitated the successful amplification of problematic samples and revealed one new haplotype from the native range of *C. fragile* in South Korea. The small size of the FCO*tuf*A marker (<200 bp) constrains its utility in phylogenetic studies, although it could indicate the existence of higher genetic diversity in South Korea. Nevertheless, we suggest that this method can be used for the rapid identification of invasive *C. fragile* species.

Finally, the species *C. decorticatum* and *C. fragile* subsp. *atlanticum* were not found in this study or in previous studies based on morphological traits and the environmental DNA method (García et al. 2018, Muha et al. 2019). The sampling conducted here was limited to the intertidal zone, and thus, future studies should include subtidal areas to fully understand the *Codium* spp. distribution in the area studied in this work.

Acknowledgments: This work was supported by the EU Project Aquainvad-ED (H2020-MSCA-ITN-2014-ETN-642197). We are very grateful to Prof. Christine Maggs for providing herbarium samples and Dr. Hyul Je Lee for sending fresh samples from South Korea.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work has been funded by the University of Oviedo, the Port Authority of Gijón, and the Asturian and Spanish governments (FUO095-2016, MINECO-17-CGL2016-79209-R, and FC-GRUPIN-IDI/2018/000201).

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402.

Cires, R.E. and Moliner, C.C. (2010). Checklist of benthic algae from the Asturias coast (North of Spain). Bol. Ciencias Nat. R.I.D.E.A. 51: 135–212.

Famà, P., Wysor, B., Kooistra, W., and Zuccarello, G.C. (2002). Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tufA* gene. J. Phycol. 38: 1040–1050.

García, A., Olabarria, C., Arrontes, J., Losada Óscar, A., and Viejo, R. (2018). Spatio-temporal dynamics of *Codium* populations along the rocky shores of N and NW Spain. Mar. Environ. Res. 140, https://doi.org/10.1016/j.marenvres.2018.07.008.
Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.
Maggs, C.A. and Kelly, J. (2007). Codium. In: Brodie, J., Maggs, C.A., and John, D.M. (Eds.), *The green seaweeds of Britain and Ireland*. Dunmurry: British Phycological Society, pp. 189–201.
Montes, M., Rico, J.M., García-Vázquez, E., and Borrell, Y.J. (2016). Morphological and molecular methods reveal the Asian alga *Grateloupia imbricata* (Halymeniaceae) occurs on Cantabrian Sea shores (Bay of Biscay). Phycologia 55: 365–370.
Muha, T.P., Skukan, R., Borrell Pichs, Y., Rico, J., García de Leaniz, C., García-Vázquez, E., and Consuegra, S. (2019). Contrasting seasonal and spatial distribution of native and invasive Codium seaweed revealed by targeting species-specific eDNA. Ecol. Evol. 9: 1–13.
Pedroche, F. (2001). Estudios filogenéticos del género *Codium* (Chlorophyta) en el Pacífico Mexicano. Use de ADNr mitocondrial. An. Esc. Nac. Cienc. Biol. Mex. 47: 109–123.
Provan, J., Booth, D., Todd, N.P., Beatty, G.E., and Maggs, C.A. (2008). Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. Divers. Distrib. 14: 343–345.
Saunders, G.W. and Kucera, H. (2010). An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. Cryptogam. Algol. 31: 487–528.
Silva, P.C. (1955). The dichotomous species of *Codium* in Britain. J. Mar. Biol. Assoc. U. K. 34: 565–577.
Steinhagen, S., Karez, R., and Weinberger, F. (2018). Surveying seaweeds from the Ulvales and Fucales in the world's most frequently used artificial waterway, the Kiel Canal. Bot. Mar. 62: 51–61.
Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35: W71–W74.
Verbruggen, H. and Costa, J.F. (2015). Molecular survey of *Codium* species diversity in Southern Madagascar. Cryptogam. Algol. 36: 171–187.
Verbruggen, H., Leliaert, F., Maggs, C.A., Shimada, S., Schils, T., Provan, J., and Littler, M.M. (2007). Species boundaries and phylogenetic relationships within the green algal genus *Codium* (Bryopsidales) based on plastid DNA sequences. Mol. Phylogenet. Evol. 44: 240–254.
Verbruggen, H., Brookes, M.J., and Costa, J.F. (2017). DNA barcodes and morphometric data indicate that *Codium fragile* may consist of two species (Bryopsidales, Chlorophyta) may consist of two species. Phycologia 56: 54–62.

Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/bot-2020-0060).

Bionotes

Roberta Skukan
Department of Biology of Organisms and Systems (BOS), University of Oviedo, C/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain
Department of Functional Biology, Genetics, University of Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Spain
Neoalgae Micro Seaweeds Products, Polígono de Lloreda, C/Carmen Leal Mata 191, 33211 Gíjon, Asturias, Spain

Roberta Skukan obtained a PhD degree (University of Oviedo, Spain) studying the ecology and genetics of *Codium fragile* and raising awareness about the consequences of invasive seaweeds (citizen science). Currently she is a research technician in Akay Lab (University of East Anglia, UK) where she performs laboratory research and support in the maintenance and genotyping of the model organism *C. elegans*.

José M. Rico
Department of Biology of Organisms and Systems (BOS), University of Oviedo, C/ Catedrático Rodrigo Uría s/n, 33071 Oviedo, Spain

José M. Rico is a full professor of ecology at the Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo. Research interests include macroalgal ecology and physiology, systematics of red algae, especially of the order Gelidiales, and anthropogenic changes in marine ecosystems. Currently, he is dean of the Faculty of Biology.

Yaisel J. Borrell
Department of Functional Biology, Genetics, University of Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Spain
borrellyaisel@uniovi.es
https://orcid.org/0000-0003-2840-4384

Yaisel J. Borrell is a professor of genetics at University of Oviedo, Spain since 2018 (associated professor since 2011). He is part of the staff of the Marine Observatory of Asturias (OMA) and Biotechnological Institute of Asturias (IUBA), coordinator in the Master Program for Marine Conservation (unioviedo.es/mcm/) and Erasmus mundus International Master of Science in Marine Biological Resources (imbrsea.eu) at UNIOVI. His research lines included conservation genetics, breeding, aquaculture, and biological invasions.