Molecular barcoding confirms the presence of exotic Asian seaweeds (*Pachymeniopsis gargiuli* and *Grateloupia turuturu*) in the Cantabrian Sea, Bay of Biscay

Marcos Montes¹,², Jose M. Rico², Eva García-Vazquez¹ and Yaisel J. Borrell Pichs¹

¹ Biología Funcional, University of Oviedo, Oviedo, Asturias, Spain
² Biología de Organismos y Sistemas (BOS), University of Oviedo, Oviedo, Asturias, Spain

**ABSTRACT**

**Background.** The introduction of exotic species can have serious consequences for marine ecosystems. On the shores of the Cantabrian Sea (North of Spain) there are no routine examinations of seaweeds that combine molecular and morphological methods for early detection of exotic species making it difficult to assess in the early stages their establishment and expansion processes as a result of anthropogenic activities (e.g., shipping and/or aquaculture).

**Methods.** In this work we used both morphological identification and molecular barcoding (COI-5P and *rbcL* genes) of red algae collected in Asturias, Bay of Biscay (Gijón and Candás harbours) and from the University of Oviedo’s herbarium samples.

**Results.** The results confirmed the presence of exotic Asian seaweeds *Pachymeniopsis gargiuli* and *Grateloupia turuturu* Yamada on Cantabrian Sea shores. Several individuals of these species were fertile and developing cystocarps when collected, underlining the risk of possible expansion or continued establishment. This study constitutes the first report of the Asian *P. gargiuli* in this area of the Bay of Biscay.

**Conclusions.** Here the presence of the exotic species of the Halymeniales *P. gargiuli* is confirmed. We hypothesize that this species may have been established some time ago as a cryptic introduction with *G. turuturu* in Galician shores. The detection of these species on the shores of the Cantabrian Sea is relevant since introductions of *Pachymeniopsis* species could have been overlooked on other European coasts, probably mixed with *G. turuturu* and *P. lanceolata*. Our results confirm one new alien seaweed species that has been detected using molecular methods (COI-5P region and *rbcL* genes barcoding) on North Atlantic shores: the Asian native *P. gargiuli*. This demonstrates that routine screening for early detection of exotic algae in the Cantabrian Sea can be used for risk assessment. Genetic barcoding should be done using both *rbcL* gene and COI-5P regions since, although COI-databases are still poorer in sequences and this inhibits successful outcomes in *Grateloupia*-related species identifications, it is nonetheless a useful marker for species-level identifications in seaweeds.

**Subjects** Biodiversity, Ecology, Genetics, Marine Biology, Molecular Biology

**Keywords** Exotics, *rbcL*, COI, Seaweeds, Bay of Biscay, Rhodophyta, Introduced species, *Grateloupia*, Halymeniaceae, DNA barcoding

How to cite this article Montes et al. (2017), Molecular barcoding confirms the presence of exotic Asian seaweeds (*Pachymeniopsis gargiuli* and *Grateloupia turuturu*) in the Cantabrian Sea, Bay of Biscay. PeerJ 5:e3116; DOI 10.7717/peerj.3116
INTRODUCTION

The problem of invasion is considered one of the main threats to global biodiversity (Nunes et al., 2014). Seaweed species introductions are a significant component of Non-Indigenous Marine Species (NIMS) introductions. When these become invasive they can rapidly spread and monopolize space, alter food webs, and modify both ecosystem structure and function (Thresher, 2000; Katsanevakis et al., 2014). Shipping has been reported as the most important pathway for the introduction of NIMS and in recent years more than a thousand marine alien species have been reported in European seas, even though efforts to monitor and report alien species vary among European countries (Katsanevakis et al., 2013).

Grateloupia C. Agardh is the largest, least characterized and most taxonomically fluctuating genus of the family Halymeniaceae, with many poorly characterized species that display a wide array of morphological traits ranging from pinnate to subdichotomous to foliose morphologies (Guiry & Guiry, 2015; Kim et al., 2014). Recent studies (Gargiulo, Morabito & Manghisi, 2013) have resulted in a continued taxonomical rearrangement of this genus, including reinstatement of the genera Pachymeniopsis Y. Yamada ex S. Kawabata (Kawaguchi, 1997), Prionitis J. Agardh (Wang et al., 2001), Dermocorynus H. Crouan et P. Crouan (Wilkes, Mcivor & Guiry, 2005) and Phyllymenia J. Agardh (De Clerck et al., 2005b).

Some Grateloupia species have also been shown to be highly invasive via marine transportation and/or aquaculture activities (e.g., Gavio & Fredericq, 2002; Nyberg & Wallentinus, 2005; Verlaque et al., 2005; Verlaque, Boudouresque & Mineur, 2007; Hewitt, Campbell & Schaffelke, 2007; Saunders & Withall, 2006; Mathieson et al., 2008; Miller, Hughy & Gabrielson, 2009; DePriest & Lopez-Bautista, 2012). Grateloupia is represented in the North European Atlantic by more than six species (Gavio & Fredericq, 2002; Wilkes, Mcivor & Guiry, 2005) of which only two, G. filicina (J.V. Lamouroux) C. Agardh, and G. turuturu Yamada, are considered introduced (De Clerck et al., 2005a; De Clerck et al., 2005b).

Morphological analysis alone can be ineffective for species identification and leave cryptic introductions undetected (e.g., Saunders, 2009). Molecular methods have been proven to be more effective, but morphological analysis is still the most frequent (and sometimes the only) method used for seaweed NIMS routine screenings in northern Spain (e.g., Bárbara & Cremades, 2004; Cires & Moliner, 2010). Recently four non-foliose Grateloupia-like samples from Gijón marina and University of Oviedo FCO Herbarium samples were identified as G. imbricata Holmes and the Mediterranean G. filicina (J.V. Lamouroux) C. Agardh, two Halymeniaceae exotic in that area (Montes et al., 2016). This demonstrated the potential for molecular methods to detect previously overlooked species introductions and in particular, other possible introduction events for Grateloupia and other Halymeniaceae similar to the ones found on Galician coasts (G. doryphora and G. turuturu), in the coasts of the Bay of Biscay.

Grateloupia turuturu, a species considered highly invasive on a global scale (Nyberg & Wallentinus, 2005 as G. doryphora [Montagne] M. Howe) is noted as being among the invasive Grateloupia species on the northern coast of Spain, in Galicia (Bárbara et al., 2005; Boletín Oficial Del Estado, España, 2013). Grateloupia turuturu was discovered in samples considered to be G. doryphora that were screened using molecular identification
tools (Bárbara & Cremades, 2004). Curiously, the most recent checklist for benthic algae in Asturias (Cires & Moliner, 2010) does not cite records of G. turuturu on Asturian coasts but it does mention the high probability of an undetected establishment. Since some confusion exists in Grateloupia species identification (e.g., Gavio & Fredericq, 2002; Kim et al., 2013; Montes et al., 2016), and since there are no routine NIMS screenings combining both molecular and anatomical methods on the coasts of the Cantabrian Sea, it is likely that a foliose Grateloupia species similar to the G. doryphora group of poorly identified species (e.g., G. turuturu) could be expanding via anthropogenic activities (i.e., shipping and aquaculture) as been demonstrated for many other marine species in this region (Arias et al., 2014a; Arias et al., 2014b; Habtemariam et al., 2015; Semeraro et al., 2016).

This work describes a routine screening of foliose, putatively identified Grateloupia specimens using morphological identification as well as COI-5P and rbcL genes as barcodes. Both genes were proven to be effective for species identification in red algae after blasting new sequences against BOLD (Barcode of Life Database) and GenBank databases (e.g., Saunders, 2005; Saunders & McDevit, 2012). The aim of this study was to evaluate the status of possible exotic Halymeniales introductions to the Cantabrian Sea (as outlined in Cires & Moliner, 2010).

**MATERIALS AND METHODS**

In this work, a barcoding routine screening of foliose Grateloupia seaweeds was carried out in the large commercial port of Gijón (González, 2012 and references therein) and Candás harbour, a nearby smaller fishing and recreational harbour. Seaweed specimens were collected from jetties in the sport wharf of the inner part of the harbour of Gijón (43°32′34″N–5°39′44″W) and from the main wharf of Candás (43°58′79″N–5°75′85″W) during a low tide (Fig. 1). Samples collected were foliose Halymeniales (24 samples) preliminarily identified as Grateloupia turuturu on the basis of external morphology: long foliose fronds and pseudo-dichotomously branched blades of a dark reddish brown colour and mucilaginous-but-firm texture. Samples were air-dried and stored at −4 °C in the FCO Herbarium of the University of Oviedo (http://www.unioviedo.es/bos/Herbario/FCO.htm) (Table 1).

Samples already deposited at FCO were also used as external controls to this study and for species confirmation using genetic tools. The samples with voucher codes FCO 1583 and FCO 1584 were collected in 2001 from La Coruña (43°48′05″N–6°47′16″W) (Fig. 1) and classified, through classical taxonomy, as G. doryphora. In addition, we included the samples with the voucher codes FCO 2076 and FCO 2077, collected in 2010 from Candás (43°58′79″N–5°75′85″W) with previous morphological classifications as G. turuturu. Old voucher samples were rehydrated during one day in seawater prior to morphological analysis.

Freezing microtome sections and staining were carried out on all samples following Rico & Guiry (1997) using a cryotome (Leica, Germany, Model CM1510-1, Fabr. No. 2303/07.2000, Cat. No. 043631515) and blue aniline for staining, to conduct morphological analyses. DNA was extracted using the GeneMATRIX Plant and Fungi DNA
Table 1  Species assignments of the Grateloupia related algae found in Gijón, Candás and University of Oviedo Herbarium historical samples (Asturias, Bay of Biscay), using genetic data and BLAST procedures.

| Location       | Collector/ Date | FCO Numbers | COI-5P                   | rbcL                      |
|----------------|-----------------|-------------|--------------------------|---------------------------|
|                |                 |             | GenBank Number           | Results of Assignments    | GenBank Number           | Results of Assignments |
|                |                 |             |                          | in BOLD System            |                          | in GeneBank database.  |
|                |                 |             |                          | Results of Assignments    |                          | Results of Assignments |
|                |                 |             |                          | in GeneBank database.     |                          | in GeneBank database.  |
| ASTURIAS       |                 |             | KP271163                 | Priodontis sp. 3jeju       | KP281326                 | Grateloupia sp.        |
| GIJÓN          | JM Rico M Montes| FCO 2134, (FCO 2141-FCO 2143) | ABMMC 11722-10 (99.7%)    | Grateloupia sp. KJ648553.1 (99.9%) |                 | ABMMC 1360-07 (100%)  |
|                 | 4/4/2014        |             |                          |                          |                          |                         |
|                 |                 |             | KP271166                 | Grateloupia turuturu       | KP281329                 |                         |
|                 |                 |             |                          | ABMMC 1360-07 (100%)      |                 |                         |
|                 |                 |             |                          | KP475725.1 (100%)         |                 |                         |
|                 |                 |             |                          |                          |                 |                         |
| CANDÁS         | JM Rico M Montes| FCO 2135, FCO 2136, (FCO 2138–FCO 2140) | FCO 2076                  | Grateloupia sp. AY651060.1 (100%) |                 |                         |
|                 | 4/21/2014       |             |                          |                          |                 |                         |
|                 | J Raboso.       | FCO 2076    |                          |                          |                 |                         |
|                 | 5/6/2010        |             |                          |                          |                 |                         |
|                 | J Raboso        | FCO 2077    |                          |                          |                 |                         |
|                 | 5/25/2010       |             |                          |                          |                 |                         |
| GALICIA        | JM Rico 9/18/2001| FCO 1584   | KP271166                 | Grateloupia turuturu       | KP281329                 |                         |
| LA CORUÑA      |                 |             |                          | ABMMC 1360-07 (100%)      |                 |                         |
|                 |                 |             |                          | KP475725.1 (100%)         |                 |                         |
|                 |                 |             |                          |                          |                 |                         |
|                 |                 |             |                          |                          |                 |                         |

Montes et al. (2017), PeerJ, DOI 10.7717/peerj.3116
purification Kit (EURx Cat. No. E3595, Roboklon GmbH, Berlin, Germany; GeneMATRIX purification Kit) using 20–70 mg of each sample for both FCO Herbarium and the fresh samples obtained at Candás and Gijón. Plant material was ground in a mortar using liquid nitrogen until pulverized material for DNA extraction was obtained. The extracted DNA was stored at −20 °C. PCRs were performed for both *rbcL* gene and COI-5P regions. Following Freshwater & Rueness (1994) and Gavio & Fredericq (2002), three different combinations of primers (F7-R753, F577-R1381 and F993-RrbcS) were used to obtain three overlapping fragments of the *rbcL* gene. The primer pair GazF1 (Saunders, 2005) and GazR4 (Saunders, 2008) were used for COI amplification. PCRs used these general conditions: 3 mM MgCl₂, 1x of PCR Buffer, 0.4 mM dNTPs, 0.3 µM from both primers and 1u of Taq Polymerase, all in a 20 µl volume (including 2 µl of DNA extracts). PCR amplification profiles were 95 °C for 5 min; 5 cycles of 95 °C for 30 s, 42 °C annealing for 1 min, 72 °C extension for 1 min; followed by 35 cycles of 95 °C for 30 s, 46.5 °C annealing for 1 min, 72 °C extension for 1 min followed by 72 °C final extension for 10 min for COI-5P; and 95 °C for 5 min; 40 cycles of 95 °C for 1 min, 42 °C annealing for 1 min, 72 °C extension for 1 min 30 sec; followed by 72 °C final extension for 10 min for *rbcL*. These profiles are similar to those from Saunders & Moore (2013). The PCR products were electrophoresed in a 2% agarose gel, containing SimplySafe™ (EURx Cat. No. E4600-01) and using Promega 100 bp DNA Ladder Molecular Weight Marker (Promega Corporation 2800 Woods Hollow Road Madison, WI 53711, USA) for band sizes inspections. Bands were cut from agarose gels and were purified using the standard protocol of the EURx agarose purification kit (EURx Cat. No. E3540-02, Pryzodnikow, Gdansk, Poland) and sent to MACROGEN (Amsterdam, Netherlands) for sequencing using the standard Sanger sequencing method (Sanger & Coulson, 1975).

The new sequences were manually checked and edited using the freeware BIOEDIT (Hall, 1999). Alignments were made using CLUSTALW (Thompson, Higgins & Gibson, 1994). The
different sequences found in this study were submitted to GenBank. After alignment and corrections, species identification was carried out using Blast to search BOLD and GenBank databases. Species identifications were accepted if they showed more than 98% similarity to the reference sequences available in both databases. Additional *Grateloupia* sequences obtained from GenBank were used in downstream phylogenetic analyses, and *Halymenia floresii* (Clemente) C. Agardh (KJ594956 and GQ862071) was used as an outgroup (Table S1). Cluster analysis was performed using the Neighbor-joining method in MEGA v6 software (*Tamura et al., 2013*), the Tamura-Nei DNA evolution model with invariable sites (TN93 + I) for COI-5P, and Tamura 3-Parameter with gamma distribution evolution model (T92 + G) for *rbcL*. Sequences were trimmed to 615 bp (located on the 216–831 bp region in the 3′ end) in order to include database derived sequences of varying lengths that were identified as the most likely DNA models (ModelTest software available inside MEGA v6). A total of 2,000 bootstrap steps were conducted for testing branch supports.

This study was approved by the Committee of Ethics of the Principado de Asturias, with the reference 100/06 for GRUPIN-2014-093.

**RESULTS**

**Morphology**

**Samples FCO 2077, FCO 2134, FCO 2140, FCO 2143**

The morphological characters of the University of Oviedo herbarium (FCO 2077) and freshly obtained samples (FCO 2134, FCO 2140, FCO 2143) were clearly those of the genus *Grateloupia* (Fig. 2) as they presented a habit typical of this genus, with specimens ranging from 4 to 30 cm long, with a firm texture, colors ranging from purplish-red to reddish brown and large fronds with lanceolate to linear, pseudo-dichotomously branched blades. The blades were membranaceous, lubricious and pseudo-dichotomously branched, branching from the discoid holdfast (Figs. 2A and 2B). Multiaxial section of internal structures showed a narrow cortical zone and a broad filamentous medullary zone; the cortical zone was composed of 6 layers of cells, 3 cylindrical-roundish cells of 5–10 µm long and 2 µm wide; medulla consisting of loose medullary anticlinally arranged filaments; inner cortex composed of 2–3 roundish cells of 5–10 µm diameter and extracellular mucilaginous material (Figs. 2A and 2B). The specimens found in both harbours during this study were fertile. Carpogonial branch 6-celled ampullar structure and post-fertilization events were in agreement with those in the type of the genus (Figs. 2D and 2E; *Gargiulo, Morabito & Manghisi, 2013*). Auxiliary cell ampullae consisted of an oval auxiliary cell, and 2–3 unbranched ampullary filaments, up to 10-cells long (Figs. 2D and 2E), similar to illustrations shown in *Wilkes, Morabito & Gargiulo* (2006). Tetrasporangia were detected in herbarium sample FCO 2077 and were isomorphic, arising from inner cortical cells, cruciately divided, embedded beneath the cortical surface and 25–30 µm in diameter (Fig. 2C). Cystocarps were similar to others reported both for *G. lanceolata* (Okamura) Kawaguchi (*Gargiulo, Morabito & Manghisi, 2013*) and to the FCO 2137 sample (*Montes et al., 2016*) with a diameter around 120 µm (Fig. 2F).
Different morphological characteristics were found for the University of Oviedo herbarium sample FCO 1583. Fronds were 15 cm long and thinner than all the other samples (Fig. 3A). The transverse section of the middle of the frond showed a narrow cortical zone and a broad filamentous medullary zone; the cortex was formed by a 5–6 cell layer, with a conspicuous transition to the medulla (Figs. 3C, 3D). The outer cortical cells were roundish or cylindrical and 8–4 \( \mu m \) in diameter; medulla consisted of loose anticlinal arranged filaments. Tetrasporangia were isomorphic, arising from inner cortical cells as well but with slightly different morphology and 20–25 \( \mu m \) long and 5–20 \( \mu m \) wide (Figs. 3B–3D).

### Genetics

Four sequences, two for each gene under analysis (\( rbcL \) and COI-5P), were obtained in this work. The BLAST and NJ tree analyses demonstrated that these sequences belong to two different species *Pachymeniopsis gargiuli* S.Y. Kim, A. Manghisi, M. Moribato & S.M. Boo (*Kim et al., 2014*) and *Grateloupia turuturu*.

**Pachymeniopsis gargiuli**

Genetic analysis of the COI-5P gene from the samples collected from Gijón and Candás (Table 1), as well as from herbarium samples FCO 2076, FCO 2077 and FCO 1584 revealed a unique COI haplotype (KP271163) of 530 bp for all of them. Analysis of the \( rbcL \) gene in these samples revealed also only one haplotype (KP281326) of 1,190 bp (Table 1).
Blast results for the COI KP271163 haplotype revealed unspecific genetic identification matching with an unidentified Halymeniales species found in Korea labelled as ‘Prionitis sp. 3jeju’ (99.7% similarity) in the BOLD database and with Grateloupia sp. voucher GT103 (KJ648553; 99.9% similarity) in GenBank; both vouchers came from samples collected in Asia.

Blast of the rbcL KP281326 haplotype against the GenBank database did not support precise genetic identification. It matched only Grateloupia sp. Gra017 (100% similarity), an unspecified type of Grateloupia found in the Straits of Messina, Italy (Wilkes, Morabito & Gargiulo, 2006).

Neighbour-joining trees were generated using all available COI-5P sequences from the two databases (Fig. 4; Table S1). The COI KP271163 haplotype was located within a very well supported branch (clade) with Prionitis sp. 3jeju and G. divaricata Okamura and an unidentified Grateloupia species labelled as G. sp. MSK 2013-14 (KJ648553, KF475723) from Korea. All these sequences formed a monophyletic clade together, apart from the highly related G. lanceolata (which has been synonymised with Pachymeniopsis lanceolata (K. Okamura) Y. Yamada ex S. Kawabata (Kim et al., 2014) (Fig. 4).
Figure 4 Neighbor joining consensus trees using partial sequences of COI gene. Neighbor joining consensus trees using partial sequences (530 bp) of COI gene and the DNA evolution model TN93 + I. Nodes including samples from this study appear in color.
The rbcL NJ tree (Fig. 5) revealed for the haplotype KP281326 a localization within a clade with KJ648574 (G. sp. MSK 2013) and with a species labelled as G. lanceolata gargiuli (GU168560) (Fig. 5). This clade has been recently identified as a gene pool grouping for the new species Pachymeniopsis gargiuli S.Y. Kim, A. Manghis, M. Moribato & S.M. Boo (Fig. 5). This clade is clearly an independent entity in regard to P. lanceolata sequences (Fig. 5).

**Grateloupia turuturu**

Genetic analysis of the Herbarium sample FCO 1583 produced sequences for each of the genes under study, KP271166 for COI-5P and KP281329 for rbcL (Table 1). Blast for both genes gave a precise and specific identification. The COI KP271166 sequence showed significant similarity with G. turuturu using both the BOLD database and GenBank (G. turuturu, KF475725) (Table 1). The KP281329 sequence was also identified (99.9% similarity) as G. turuturu in both databases (Table 1).

The COI-5P NJ tree revealed that the sequence KP271166 was part of a well-supported monophyletic clade with other G. turuturu sequences from Asian areas (Fig. 4; Table S1). The rbcL NJ tree showed the sequence KP281329 forming a monophyletic clade with G. turuturu samples from various places including the native Asian areas (KJ648568 and GU168561) (Fig. 5).

**DISCUSSION**

Morphological analysis was not sufficient for precise species identification in this species complex. Despite this, we were able to determine the genus of some of the samples collected; the vegetative structure was typical of Grateloupia-type genera, and the reproductive structures analysis was similar to that used to separate between Grateloupia and Pachymeniopsis (Gargiulo, Morabito & Manghisi, 2013). Unfortunately not all the samples showed reproductive structures. Molecular analysis was by far the most effective method of species level identification in this work and rbcL sequences supported much more precise identifications than COI sequences as previously reported for G. imbricata and G. filicina in Montes et al. (2016). This was an expected outcome given that the systematics and taxonomy of the Grateloupia spp. complex and related genera (e.g., Pachymeniopsis) have been proposed, established, discussed and rearranged using the rbcL gene (i.e., Wang et al., 2001; Wilkes, Mcivor & Guiry, 2005; De Clerck et al., 2005a; De Clerck et al., 2005b; Lin, Liang & Hommersand, 2008; Lee et al., 2009; Zhang et al., 2012; Gargiulo, Morabito & Manghisi, 2013; Yang & Kim, 2015). However, the COI gene has potential to become an equally effective marker for species identifications in red algae in the future as outlined in the past in other genera (Saunders, 2005; Freshwater et al., 2010; Saunders & Moore, 2013) supported by our (limited) success here. More data in COI genetic databases would help to overcome this obstacle. Moreover, in the COI-5P tree (Fig. 4) the KP271163 sequence was grouped in a complex clade including incomplete labelled species (e.g., Grateloupia sp. 2 MSK-2013 voucher HAL019) and one species with a correct taxonomic label, G. divaricata Okamura (Wang et al., 2001), a species that shows pinnated frond morphology (different from our samples), suggesting a species misidentification. This pointed to several errors in species identifications that appear in genetic databases. Fortunately, the rbcL tree (Fig. 5)
Figure 5 Neighbor joining consensus trees using partial sequences of rbcL gene. Neighbor joining consensus trees using partial sequences (615 bp, located on the 216 to 831 bp region in the 3’ end) of rbcL gene and the DNA evolution model T92 + G. Nodes including samples from this study appear in color.
clearly showed the correct identity since samples from this study showed 100% identity with sequences of *P. gargiuli* (KJ125446, KJ214447), a recently described species closely related to *P. lanceolata* as described by *Kim et al. (2014)*.

Herbarium samples FCO 1583 and FCO 1584 were collected in the same area, the same day, and shared similar morphology; this led to both being identified as *G. doryphora* in 2001. The herbarium sample FCO 1583 has been unambiguously identified here as *G. turuturu*. However, the FCO 1584 sample was identified as *P. gargiuli*. Both samples were thus registered with incorrect species names in the FCO herbarium. *G. turuturu* presented a similar habit (Fig. 3A) to *P. gargiuli* (Figs. 2A and 2B) and vegetative and reproductive morphology was not sufficient for species identification. This underscores the value of genetic analyses if the aim is precise red algae species identifications when cryptic species complexes are considered with reproductive morphological features that are difficult to find if for example, samples do not include fertile specimens. Moreover, our results support the idea that both of the species we identified have been present in the Cantabrian Sea at least since 2001, although only *G. turuturu* has for the time being been described on Galician coasts (*Bárbara et al., 2005*). This last observation suggests that the presence of *P. gargiuli* in Asturias could be the result of a more recent introduction event (at least, since 2010) in comparison with other exotic seaweeds.

*Cires & Moliner (2010)* suggested that *G. turuturu* could be present in Asturian shores as a consequence of its proximity to coasts where it has been detected (Galicia) and because this seaweed has been reported showing invasive spread in nearby areas such as Portugal and France (*Simon, Gall & Deslandes, 2001; Araújo et al., 2011*), which makes expansion to other areas more probable. Although samples initially labelled by us as *G. turuturu* were collected in Asturias (including voucher samples from 2010 collected in Candás), they were found to all be *P. gargiuli*. These misidentifications are not surprising since *P. gargiuli* was also initially misidentified as *G. doryphora* and *G. turuturu* until genetic analyses were carried out on samples from the Strait of Messina (AY651060) (*Wilkes, Morabito & Gargiulo, 2006*). This suggests that the introduction of *P. gargiuli* in the Cantabrian Sea shores could be a cryptic introduction, thanks to its morphological similarity to *G. turuturu* and also to the similarity of *G. turuturu* in habit to the Galician native *G. lanceola* J. Agardh (J. Agardh). The latter similarity has resulted in a tendency to overlook the extent of *G. turuturu* presence in Galicia in the first place (*Bárbara & Cremades, 2004*).

*P. gargiuli* is also considered cryptic for *P. lanceolata*, sharing many morphological characteristics as well as distribution area in Korea (*Kim et al., 2014*). Samples of this species were detected in Italy (*Wilkes, Morabito & Gargiulo, 2006*) and, in the Canary Islands, initially identified as *P. lanceolata* (EU024819) (*García-Jiménez et al., 2008*), and also in the Madeira Islands at least since 2006; *P. lanceolata* was described, but only via morphological analysis (*Ferreira et al., 2012*). The similarity between these seaweeds raises the clear possibility of the presence of *P. gargiuli* in Madeira. The red algae *G. imbricata* was also found in Canarias through genetic analysis and in Madeira through classical taxonomy (*García-Jiménez et al., 2008; Ferreira et al., 2012*), but it has to date not been described anywhere in North Atlantic European shores or marinas except in the Gijón area (*Montes et al., 2016*). These similarities in locations where it was detected as an introduced species...
may suggest that these algae shared the same or similar introduction vectors/routes. The most likely and accepted hypothesis regarding introduction vectors of *Pachymeniopsis* spp. and of *G. turuturu* is oyster commerce to the Thau lagoon from the Pacific (*Verlaque et al., 2005; Verlaque, Boudouresque & Mineur, 2007*). It is likely that shipping will play a pivotal role in the range expansion of these species to the continent through ballast water and hull fouling (*Hewitt, Campbell & Schaffelke, 2007*).

This is the first report of a new genus, *Pachymeniopsis*, on the European shores of the North Atlantic. *P. gargiuli* is a species native to Asia, which was probably introduced long ago (at least since 2001) as a cryptic introduction within *G. turuturu* to Galician shores. Several individuals of these species were fertile as they were developing cystocarps and tetraspores when collected, emphasizing the risk of expansion or continued establishment. The detection of these species on the coasts of the Cantabrian Sea also indicates that *Pachymeniopsis* introductions may have been overlooked along other European coasts, especially if intermingled with *G. turuturu* and *P. lanceolata*. Our results highlight the potential for exotic algal introductions being missed when morphological identification fails to differentiate between highly similar species, and thus the importance of routine molecular barcoding surveys. This study also highlights the existence of gaps in the COI-5P records for *Grateloupia* spp., which might necessitate using an alternative barcode in the form of *rbcL*. We confirm previous findings and reports on two previously overlooked exotic algal introductions to an area of Europe where these had not been detected by morphology alone, the Asian native *G. imbricata* and the Mediterranean native *G. filicina* (*De Clerck et al., 2005a; De Clerck et al., 2005b*), and we add a newly reported species (*Montes et al., 2016*), also utilizing DNA barcoding, using both COI-5P region and *rbcL* gene: the exotic *P. gargiuli*. This demonstrates the utility of routine screenings that combine both anatomical investigation and barcoding procedures for early detection of exotic algae in the Cantabrian Sea. For such studies it would be ideal to combine anatomical and DNA barcoding procedures. In our case morphological examination was able to determine accurate genus placement, but for species identification, genetic methods proved to be more effective than conventional morphological identification.

**ACKNOWLEDGEMENTS**

Thanks to Gary W. Saunders (Centre for Environmental and Molecular Algal Research, University of New Brunswick, Canada), who kindly provided us with many research reports and information about the global DNA barcoding project for algae. Thanks also to the staff of the Puerto Deportivo de Gijón for helping us during the study.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**

This work was funded by the University of Oviedo and Spanish governments through the projects UNOV-13-EMERG-05, and the National Project MINECO CGL2013-42415-R. This paper is a contribution from the Marine Observatory of Asturias (OMA) and the
Research Group GRUPIN14-093. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
University of Oviedo: UNOV-13-EMERG-05.
Spanish governments: MINECO CGL2013-42415-R.
Marine Observatory of Asturias (OMA).
Research Group GRUPIN14-093.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
• Marcos Montes performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
• Jose M. Rico and Yaisel J. Borrell Pichs conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
• Eva García-Vazquez conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Field Study Permissions
The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):
Field experiments have been approved by the Committee of Ethics of the Principality of Asturias, with the reference 100/16 for GRUPIN-2014-093.

DNA Deposition
The following information was supplied regarding the deposition of DNA sequences:
Two different haplotypes of 530 bp were obtained in this study for the COI-5P gene. A unique haplotype (KP271163) was obtained from Gijón and Candás new samples, as well as from the herbarium samples FCO 2076, FCO 2077 and FCO 1584. Another haplotype (KP271166) was obtained from the herbarium sample FCO 1583.
Two different haplotypes of 1,190 bp were obtained in this study after the analyses of the sequences of the rbcL gene in our samples (KP281326, KP281329).

Data Availability
The following information was supplied regarding data availability:
The FCO Herbarium of the University of Oviedo.
FCO 2134 – FCO 2138
FCO 2139 – FCO 2143
http://www.unioviedo.es/bos/Herbario/FCO.htm.
Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3116#supplemental-information.

REFERENCES

Araújo R, Violante J, Pereira R, Abreu H, Arenas F, Sousa-Pinto I. 2011. Distribution and population dynamics of the introduced seaweed *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) along the Portuguese coast. *Phycologia* 50:392–402 DOI 10.2216/10-65.1.

Arias A, Bañón R, Almón B, Anadón N, Borrell YJ, Cremades J, Esquete P, Fernández-Álvarez FA, García-Vázquez E, Parapar J, Pérez J, Rico J, Salas C, San Martín G, Torralba-Burrial A, Trigo J. 2014a. Non-indigenous marine species (NIS) in the Cantabrian sea and adjacent Atlantic (NW-N Iberian peninsula): a first approach for the Marine Strategy Framework Directive in northern Spain waters. In: Ríos P, Suárez LA, Cristobo J, eds. *XVIII simposio ibérico de estudios de biología marina. Libro de resúmenes*. Centro Oceanográfico de Gijón, 252.

Arias A, Borrell YJ, Bañón R, Miralles L, Almón B, Pérez J, Trigo J, García-Vázquez E, Anadón N. 2014b. Alien marine fauna from north/northwestern Iberian coast identified through classical taxonomy and DNA barcoding. In: Ríos P, Suárez LA, Cristobo J, eds. *XVIII simposio ibérico de estudios de biología marina. Libro de resúmenes*. Centro Oceanográfico de Gijón, 252.

Bárbara I, Cremades J. 2004. *Grateloupia lanceola* versus *Grateloupia turuturu* (Gigarti-nales, Rhodophyta) en la Península Ibérica. *Anales del Jardín Botánico de Madrid* 61:103–118.

Bárbara I, Cremades J, Calvo S, López Rodríguez MC, Dosil J. 2005. Checklist of the benthic marine and brackish Galician algae (NW Spain). *Anales del Jardín Botánico de Madrid* 62:69–100.

Boletín Oficial Del Estado, España. 2013. Real Decreto 630/2013, de 2 de agosto, por el que se regula el Catálogo Español de especies exóticas invasoras. BOE núm. 185, de 3 de agosto de 2013: 56764-56786. Referencia: BOE-A-2013-8565.

Cires RE, Moliner CC. 2010. Checklist of benthic algae from the Asturias coast (North of Spain). *Boletín de Ciencias Naturales R.I.D.E.A.* 51:135–212.

De Clerck O, Gavio B, Frederick S, Cocquyt E, Coppejans E. 2005a. Systematic reassessment of the red algal genus *Phyllymenia* (Halymeniaceae, Rhodophyta). *Phycologia* 40:169–178.

De Clerck O, Gavio B, Fredericq S, Bábara I, Coppejans E. 2005b. Systematics of *Grateloupia filicina* (Halymeniaceae, Rhodophyta), based on rbcL sequence analyses and morphological evidence, including the reinstatement of *G. minima* and the description of *G. capensis* sp. nov. *Journal of Phycology* 41:391–410 DOI 10.1111/j.1529-8817.2005.04189.x.
DePriest M, Lopez-Bautista J. 2012. Sequencing of the rbcL marker reveals the non-native red alga *Grateloupia taiwanensis* (Halymeniaceae, Rhodophyta) in Alabama. *Gulf of Mexico Sciences* 30(1–2):7–13.

Ferreira S, Kaufmann M, Neto AI, Izaguirre JP, Wirtz P, De Clerck O. 2012. New records of Macroalgae from Madeira Archipelago. “International symposium FloraMac2012”, P05, abstract book, p. 60. Colégio dos Jesuítas, Funchal, Madeira, 5–8 de Setembro.

Freshwater DW, Rueness J. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species based on rbcL nucleotide sequence analysis. *Phycologia* 33:187–94 DOI 10.2216/i0031-8884-33-3-187.1.

Freshwater DW, Tudor K, O’shaunnessy K, Wysor B. 2010. DNA barcoding in the red algal order Gelidiales: comparison of COI with rbcL and verification of the “barcoding gap”. *Cryptogamie Algologie* 31:435–449.

García-Jiménez P, I. Geraldino PJ, Ming Boo S, Robaina R. 2008. Red alga *Grateloupia imbricata* (Halymeniaceae), a species introduced into the Canary Islands. *Phycological Research* 56:166–171 DOI 10.1111/j.1440-1835.2008.00498.x.

Gargiulo GM, Morabito M, Manghisi A. 2013. A re-assessment of reproductive anatomy and postfertilization development in the systematics of *Grateloupia* (Halymeniaceae, Rhodophyta). *Cryptogamie Algologie* 34:3–35 DOI 10.7872/crya.v34.iss1.2013.3.

Gavio B, Fredericq S. 2002. *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. *European Journal of Phycology* 37:349–359 DOI 10.1017/S0967026202003839.

González F. 2012. Investment and port traffic: an analysis of the situation in Spain. *Bulletin Facilitation of Transport and Trade in Latin America and the Caribbean* 313(9):8 pp.

Guiry MD, Guiry GM. 2015. *AlgaeBase*. Galway: World-wide electronic publication, National University of Ireland.

Habtemariam BT, Arias A, García-Vázquez E, Borrell YJ. 2015. Impacts of the supplementation aquaculture on the genetic diversity in the grooved carpet shell *Ruditapes decussatus* from northern Spain: species misidentifications and evidences of gene introgressions. *Aquaculture Environment Interactions* 6:241–254 DOI 10.3354/aei00128.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41:95–98.

Hewitt CHL, Campbell ML, Schaffelke B. 2007. Introductions of seaweeds: accidental transfer pathways and mechanisms. *Botanica Marina* 50:326–337 DOI 10.1515/BOT.2007.038.

Katsanevakis S, Gatto F, Zenetos A, Cardoso AC. 2013. How many marine aliens in Europe? *Managing Biological Invasions* 4:37–42 DOI 10.3391/mbi.2013.4.1.05.

Katsanevakis S, Wallentinus I, Zenetos A, Leppäkoski E, Çinar ME, Oztürk B, Grabowski M, Golani D, Cardoso AC. 2014. Impacts of invasive alien marine
species on ecosystem services and biodiversity: a pan-European review. Aquatic Invasions 9:391–423 DOI 10.3391/ai.2014.9.4.01.

Kim SY, Han EG, Kim SM, Park JK, Boo SM. 2013. Grateloupia jejuensis (Halymeniales, Rhodophyta): a new species previously confused with G. elata and G. cornea in Korea. Algae 28:233–240 DOI 10.4490/algae.2013.28.3.233.

Kim SY, Manghisi A, Morabito M, Yang EC, Yoon HS, Miller KA, Boo SM. 2014. Genetic diversity and haplotype distribution of Pachymeniopsis gargiuli sp. nov. and P. lanceolata (Halymeniales, Rhodophyta) in Korea, with notes on their non-native distributions. Journal of Phycology 50:885–896 DOI 10.1111/jpy.12218.

Lee JI, Kim HG, Geraldino LPJ, Hwang IK, Boo SM. 2009. Molecular classification of the genus Grateloupia (Halymeniacae, Rhodophyta) in Korea. Algae 24:231–238 DOI 10.4490/ALGAE.2009.24.4.231.

Lin SM, Liang HY, Hommersand MH. 2008. Two types of auxiliary cell ampuallae in Grateloupia (Halymeniaceae, Rhodophyta), including G. taiwanensis sp. Nov and G. orientalis sp. Nov from Taiwan based on rbcL gene sequence analysis and cystocarp development. Journal of Phycology 44:196–214 DOI 10.1111/j.1529-8817.2007.00443.x.

Mathieson AC, Dawes CJ, Pederson J, Gladych RA, Carlton JT. 2008. The Asian red seaweed Grateloupia turuturu (Rhodophyta) invades the Gulf of Maine. Biological Invasions 10:985–988 DOI 10.1007/s10530-007-9176-z.

Miller KA, Hughey JR, Gabrielson PW. 2009. Research note: first report of the Japanese species Grateloupia lanceolata (Halymeniacae, Rhodophyta) from California, USA. Phycological Research 57:238–241 DOI 10.1111/j.1440-1835.2009.00542.x.

Montes M, Rico JM, García-Vázquez E, García-Vázquez, Borrel YJ. 2016. Morphological and molecular methods reveal the Asian alga Grateloupia imbricata (Halymeniacae) occurs on Cantabrian Sea shores (Bay of Biscay). Phycologia 55:365–370 DOI 10.2216/15-112.1.

Nunes AL, Katsanevakis S, Zenetos A, Cardoso AC. 2014. Gateways to alien invasions in the European seas. Aq Inv 9:133–144 DOI 10.3391/ai.2014.9.2.02.

Nyberg CD, Wallentinus I. 2005. Can species traits be used to predict marine macroalgal introductions? Biological Invasions 7:265–279 DOI 10.1007/s10530-004-0738-z.

Rico JM, Guiry MD. 1997. Life history and reproduction of Gelidium maggsiae sp. Nov. (Rhodophyta, Gelidiales) from Ireland. European Journal of Phycology 32:267–277 DOI 10.1080/09670269710001737189.

Sanger F, Coulson AR. 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. Journal of Molecular Biology 94:441–448 DOI 10.1016/0022-2836(75)90213-2.

Saunders GW. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 360:1879–1888 DOI 10.1098/rstb.2005.1719.
Saunders GW. 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. *Molecular Ecology Resources* 9(Suppl. 1):140–150 DOI 10.1111/j.1755-0998.2009.02639.x.

Saunders GW, McDevit DC. 2012. Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. *Methods in Molecular Biology* 858:207–222.

Saunders GW, Moore TE. 2013. Refinements for the amplification and sequencing of red algal DNA barcode and RedToL phylogenetic makers: a summary of current primers, profiles and strategies. *Algae* 28:31–43 DOI 10.4490/algae.2013.28.1.031.

Saunders GW, Withall RD. 2006. Collections of the invasive species *Grateloupia turuturu* (Halymeniales, Rhodophyta) from Tasmania, Australia. *Phycologia* 45:711–714 DOI 10.2216/06-10.1.

Semeraro A, Mohammed-Geba K, Arias A, Anadon N, García-Vázquez E, Borrell Yj. 2016. Genetic diversity and connectivity patterns of harvested and aquacultured molluscs in estuaries from Asturias (northern Spain). Implications for management strategies. *Aquaculture Research* 47:2937–2950 DOI 10.1111/are.12745.

Simon C, Gall E, AR, Deslandes E. 2001. Expansion of the red alga *Grateloupia doryphora* along the coasts of Brittany (France). *Hydrobiologia* 443:23–29 DOI 10.1023/A:1017587918604.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30:2725–2729 DOI 10.1093/molbev/mst197.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673–4680 DOI 10.1093/nar/22.22.4673.

Thresher RE. 2000. Key threats from marine bioinvasions: a review of current and future issues. In: Pederson J, ed. Jan 24–27, 1999, Marine bioinvasions, proceedings of the first national conference. Massachusetts Institute of Technology, Sea Grant College Program, Boston, 24–36.

Verlaque M, Boudouresque CF, Mineur F. 2007. Oyster transfers as a vector for marine species introductions: a realistic approach based on macrophytes. In: Briand F, Moschella P, eds. *Impact of mariculture on coastal ecosystems*. vol. 32. Lisboa: CIESM Workshop Monographs 39–47.

Verlaque M, Brannock PM, Komatsu T, Villalard-Bohnsack M, Marston M. 2005. The genus *Grateloupia* C. Agardh (Halymeniales, Rhodophyta) in the Thau Lagoon (France, Mediterranean): a case study of marine pluri-specific introductions. *Phycologia* 44:477–496 DOI 10.2216/0031-8884(2005)44[477:TGGCAH]2.0.CO;2.

Wang HW, Kawaguchi S, Horiguch T, Masuda M. 2001. A morphological and molecular assessment of the genus *Prionitis* S. Agardh (Halymeniales, Rhodophyta). *Phycological Research* 49:251–226 DOI 10.1111/j.1440-1835.2001.tb00255.x.

Wilkes RJ, Mcivor LM, Guiry MD. 2005. Using rbcL sequence data to reassess the taxonomic position of some *Grateloupia* and *Dermocorynus* species (Halymeniales,
Rhodophyta) from the north-eastern Atlantic. *European Journal of Phycology* 40:53–60 DOI 10.1080/09670260400024634.

Wilkes RJ, Morabito M, Gargiulo GM. 2006. Taxonomic considerations of a foliose *Grateloupia* species from the Straits of Messina. *Journal of Applied Phycology* 18:663–669 DOI 10.1007/s10811-006-9069-z.

Yang MY, Kim MS. 2015. Taxonomy of *Grateloupia* (Halymeniales, Rhodophyta) by DNA barcode marker analysis and a description of *Pachymeniopsis volvita* sp. Nov. *Journal of Applied Phycology* 27:1373–1384 DOI 10.1007/s10811-014-0432-1.

Zhang W, Wang HW, Sheng YW, Zhao D. 2012. *Grateloupia orientalis* (Rhodophyta), a new record of the Chinese mainland, on the basis of morphological observation and *rbcL* gene sequence analysis. *Marine Sciences* 36:109–116.