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Gelidium adriaticum sp. nov. and *Gelidium carolinianum sp. nov.* (Gelidiales, Rhodophyta) from the Mediterranean Sea

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ABSTRACT

Identification of small *Gelidium* species based on morphology is difficult; as a consequence, the name *Gelidium pusillum* has been used for many small gelidiacean taxa throughout the world. Molecular-assisted identifications, however, are demonstrating that *G. pusillum* has a more restricted distribution than previously recognised. We used detailed morphological analyses combined with *rbc*L and *cox*1 sequence analyses to identify and assess the phylogenetic relationships of small Mediterranean *Gelidium* species. These analyses revealed the presence of two new species, *G. adriaticum sp. nov.* and *G. carolinianum sp. nov. Gelidium adriaticum* is a closely related sister species to *G. pusillum*, which was not found amongst our Mediterranean specimens. *Gelidium carolinianum* has been previously collected outside the Mediterranean, but it was misidentified as *G. americanum*. Morphological observations and sequence data generated from *G. americanum* types and other historical specimens clarified the status and distribution of this species. Phylogenetic analyses resolved *G. carolinianum* as a species in the Mediterranean and warm-temperate northwest Atlantic; whereas, *G. americanum* occurs in the Caribbean and tropical western Atlantic, and is sister to *G. calidum* from Brazil and closely related to *G. crinale*.

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INTRODUCTION

The identification of species in the Gelidiales on the basis of morphological characters alone is difficult, especially without fertile individuals, which are frequently rare. In addition, intraspecific morphological variation can be misinterpreted as multiple species and, conversely, convergent evolution has resulted in cryptic species identical in appearance but genetically distinct. Molecular markers from the nuclear, plastid and mitochondrial genomes have shed light on taxonomic, distributional and phylogenetic questions in the Gelidiales (e.g. Bailey & Freshwater 1997; Boo *et al.* 2013, 2014; Boo, Cai & Boo 2016; Boo, Hughey *et al.* 2016; Boo, Le Gall *et al.* 2016; Boo, Nguyen *et al.* 2016; Freshwater & Bailey 1998; Freshwater & Rueness 1994; Freshwater *et al.* 1995, 2010; Tronchin *et al.* 2002). These markers, however, have been applied to very few Mediterranean species (Boo, Cai & Boo 2016; Boo, Le Gall *et al.* 2016; Bottalico *et al.* 2014a, 2014b; Tronchin *et al.* 2003).

Initial steps towards improving the understanding of algal diversity and taxonomy in Mediterranean, and in particular Italian, Gelidiales, have focused on the smallest species that very often characterise the midlittoral zone of rocky shores. New species in the Gelidiellaceae have been described on the basis of morphological and/or molecular data (Bottalico *et al.* 2014b, 2015; Perrone & Delle Foglie 2006). Gelidiaceaen specimens provisionally attributed to *Gelidium pusillum* (Stackhouse) Le Jolis were reviewed, because the presence of this species in the Mediterranean has been repeatedly questioned (Fredriksen *et al.* 1994; Freshwater *et al.* 1995; Guiry & Womersley 1993; Maggs & Guiry 1987; Millar & Freshwater 2005). The most recent molecular analyses suggest that *G. pusillum* is restricted to North Atlantic waters; records outside this area should be treated with caution, and the intraspecific classifications of the species should be abandoned (Kim & Boo 2012).

Gelidium pusillum is amongst the most difficult red algal species to identify, and several other species have passed, and currently pass, under this name. It has been reported around the world (Guiry & Guiry 2018), including the Mediterranean Sea, and especially along Italian coasts (Furnari *et al.* 2010). The Adriatic Sea is a Mediterranean region in which the benthic algal flora has been intensively studied. Historically, species with morphological characters similar to those of *G. pusillum* were reported; for example, *Gelidium clavatum* (J.V. Lamouroux) J.V.Lamouroux, often under earlier synonyms (Naccari 1828a, 1828b; Vatova 1940; Zanardini 1840). Hauck (1882) also described *G. pusillum* as a perennial gelidiacean alga occurring in the Adriatic Sea. *G. pusillum* continues to be reported from many localities, especially from the Venice Lagoon (Sfriso & Curiel 2007), but these reports have not been well documented.

Preliminary molecular analyses carried out on collections from Apulia, Italy, that fit the previous concept of *G. pusillum* in the Mediterranean, showed that none of the specimens were

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Supplemental data for this article can be accessed on the publisher's website.

G. pusillum but rather resolved them as two *Gelidium* species. The first was an undescribed species, and the second shared identical *rbc*L sequences with a North Carolina specimen identified as *Gelidium americanum* (W.R.Taylor) Santelices. Consequently, the latter was provisionally attributed to *G. americanum* and published as the first record of the species in the Mediterranean Sea (Bottalico *et al.* 2014a). Later, it was reported as *Gelidium* sp. from the Salento peninsula, Apulia, Italy (Bottalico *et al.* 2016), pending a more thorough exploration of the status of *G. americanum*.

Although reported widely in the Western Atlantic (Guiry & Guiry 2018; Kapraun 1980; Schneider & Searles 1991; Taylor 1960; Wynne 2017), *G. americanum* has not been studied in depth. The species was described by Taylor (1943) as *Pterocladia americana*. Taylor's holotype is a Jamaican specimen from the *Phycotheca boreali-americana* (Collins *et al.* 1900), *P.B.-A.* #783 labelled '*Gelidium coerulescens* Crouan', an unpublished name with the epithet '*caerulescens*' misspelled, but he also listed specimens from North Carolina, Bermuda, Barbados, Costa Rica, and Venezuela. After observing bilocular cystocarps in some specimens in the *P.B.-A.* #783 collection, Santelices (1976) transferred *Pterocladia americana* to *Gelidium*.

Observations of herbarium specimens identified as *G. americanum* and *P. americana* from several western Atlantic localities confirmed that the specimens from Italy and North Carolina resembled some of them but were clearly different from most, including the holotype of *P. americana*. Thus, it became apparent that the name *G. americanum* had been misapplied to the North Carolina specimen from which the sequences in GenBank had been generated and that this specimen represented an undescribed species.

We carried out detailed morphological/anatomical observations, combined with analyses of plastid *rbc*L and mitochondrial *cox*1 gene sequences, on field-collected material as well as historically significant herbarium specimens. Two new species were revealed by these analyses: *Gelidium adriaticum sp. nov.* (previously reported as *G. pusillum* from Apulia, Italy, and Slovenia) and *Gelidium carolinianum sp. nov.* (previously reported as *G. americanum* from North Carolina, USA, Bermuda, and Apulia, Italy).

MATERIAL AND METHODS

From 2010 to 2015, vegetative, tetrasporangial and cystocarpic thalli of small-sized gelidiacean algae were collected on the Adriatic and Ionian coasts of Apulia (southeastern Italy) during targeted surveys (Fig. 1). Some specimens were preserved in 4% formalin–seawater for morphological observations and others carefully cleaned and dried in silica gel desiccant for DNA extraction. Additional dried samples from Koper Bay, Slovenia, were sent by C. Battelli, and formalin-preserved samples from the Cheradi Islands, Taranto, Italy, were sent by E. Cecere. The Macroalgal Herbarium Portal, the Naturalis Biodiversity Centre

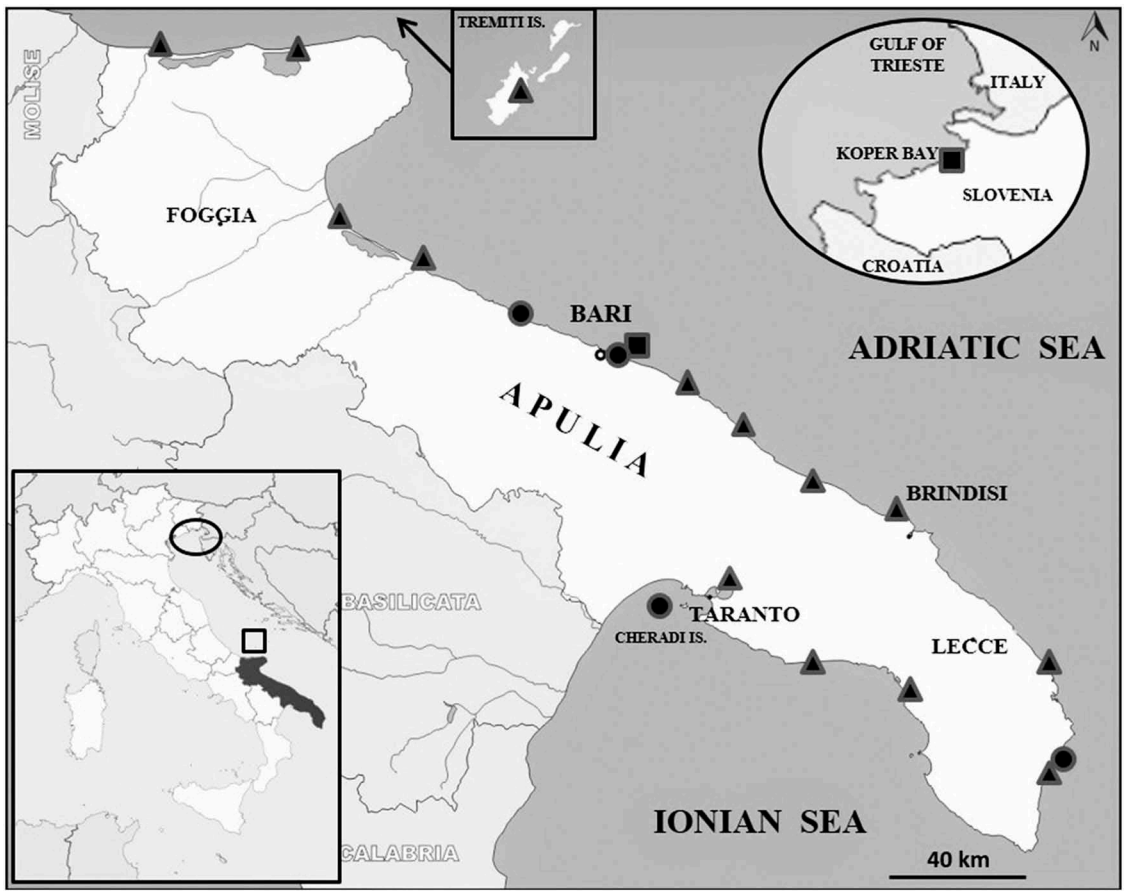


Fig. 1. Sampling sites (▲) along the Apulian coasts (south-eastern Italy, Mediterranean Sea). Localities where Gelidium adriaticum and G. carolinianum were collected are marked with (■) and (●), respectively.

Bioportal, the Muséum National d'Histoire Naturelle, Paris, France, and Web Ficoflora Venezuela were searched for specimens labelled '*G. coerulescens* Crouan', *Pterocladia americana*, and *G. americanum* to determine the distribution and potential misidentification of this species. Some of the specimens found in these searches, including multiple isotypes, were obtained on loan from WNC, NCU, UC and MICH for detailed observations and sequencing, and others were observed as online images (Table 1, Table S1). Herbarium abbreviations follow Thiers (2018).

Morphological and anatomical observations were carried out using a Leica MZ 7.5 stereomicroscope (Leica, Wetzlar, Germany) and an Olympus BX-40 light microscope (Olympus America Inc., Center Valley, Pennsylvania, USA). Thalli were sectioned freehand or with a DSK-1000 Vibratome (Dosaka, Kyoto, Japan) and stained with 1% aqueous aniline blue acidified with 1 N HCl. Observations under polarised light were made as described in Felicini & Perrone (1986). Photomicrographs were taken using a DP21 digital camera (Olympus) equipped with DP2-Twain software for measurements (Olympus). Newly collected voucher specimens were deposited in FI, BI and WNC.

DNA extraction, polymerase chain reaction amplification and sequencing were performed as described in Boo *et al.* (2013). The primers used for amplifying and sequencing were F7, F645, R753, and RrbcS-start for *rbc*L (Freshwater & Rueness 1994; Gavio & Fredericq 2002; Lin *et al.* 2001) and COXI43F and COXI1549R for *cox*1 (Geraldino *et al.* 2006). DNA from type and old herbarium specimens from UC, MICH, WNC was extracted and amplified following procedures described by Boo, Hughey *et al.* (2016). When large fragments of the analysed loci could not be amplified in these archival specimens, we were able to amplify and sequence 124 to 757 bp of *rbc*L and 230 bp of *cox*1 using primer pairs F145–R406, F577–R753, F753–R900, F993–R1144, and F1237–R1381 for *rbc*L (Boo *et al.* 2015; Freshwater & Rueness 1994; Iha *et al.* 2016; Kim *et al.* 2010) and C622F and C880R for *cox*1 (Yang *et al.* 2008). All *rbc*L and *cox*1 sequences generated in this study were deposited in GenBank (Table S1). Three outgroup species, representing three genera in the Gelidiaceae (Boo, Hughey *et al.* 2016), were included in the alignment.

Phylogenies of *cox*1 and *rbc*L datasets were inferred using maximum likelihood (ML) and Bayesian inference (BI). The ML analyses were reconstructed using the Pthreads version of RAxML v8.0.X (Stamatakis 2014) set as follows: a rapid bootstrap analysis and search for the best scoring ML tree in one single programme run with 1000 bootstrap replicates under the GTR + G + I substitution model. BI was performed for individual datasets with MrBayes v3.2.1 (Ronquist *et al.* 2012) using the Metropolis-coupled Markov chain Monte Carlo under the GTR + G + I model. For each matrix, two million generations of two independent runs were performed with four chains and sampling trees every 100 generations. The burn-in period was identified graphically by tracking the likelihoods at each generation to determine whether they reached a plateau. Twenty-five percent of the saved trees were removed, and the remaining trees were used to calculate Bayesian posterior probabilities (BPPs).

RESULTS

Gelidium adriaticum C.Perrone, A.Bottalico, G.H.Boo & S.M.Boo *sp. nov.*

Figs 2–13

DESCRIPTION: Thallus consisting of terete prostrate axes attached to the substratum by brush-like haptera and erect fronds proximally terete to compressed, distally flattened, spatulate to lanceolate, simple or sparingly and irregularly branched up to two orders; outermost cortical cells irregularly ovoid; medullary cells in the flattened parts of fronds aligned in a single median row and surrounded by internal rhizoidal filaments; tetrasporangial sori with a sterile margin; tetrasporangia cruciately divided and irregularly arranged; cystocarps bilocular, spherical, with one central, circular ostiole per chamber provided with a raised peristome; spermatangial sori not found; *rbc*L sequence = GenBank accession KU958475; *cox*1 sequence = GenBank accession KU958469.

HOLOTYPE: FI018628, collected 25 September 2012 by A. Bottalico.

TYPE LOCALITY: Torre a Mare, Bari, Italy (41°5.3′N, 16°59.85′E); upper intertidal zone, attached to rocks.

ISOTYPE: BI38468.

PARATYPES: BI38469, Torre a Mare, Italy; BI38470, Koper Bay, Slovenia (45°32.67′N, 13°43.183′E).

ETYMOLOGY: The epithet '*adriaticum*' refers to the Adriatic Sea where the species was collected.

Morphology

Gelidium adriaticum formed epilithic turfs at the upper intertidal level, in semishaded sites on exposed coasts. The purplish red, cartilaginous thalli consisted of long, terete and irregularly branched stolons, 60–130 μm in diameter, attached to the substratum by brush-like haptera, and flattened erect fronds, 4.5–8.0 mm tall, 180–550 μm wide, and 80–100 μm thick, initially spatulate becoming lanceolate and sometimes with long stipes, arising individually or in clumps. Branching of erect fronds was scarce and irregular, up to two orders, from the margins only (Figs 2, 3). Erect fronds showed dark longitudinal parallel striations when observed in surface view under magnification (Fig. 4) that corresponded to bundles of internal rhizoidal filaments when observed under polarised light (Fig. 5). The outermost cortical cells were ovoid in surface view, 5–6 × 4–-7 μm, and irregularly arranged (Fig. 6). Flattened fronds had two to three layers of small, pigmented cortical cells and hyaline medullary cells regularly aligned in the median plan and surrounded by internal rhizoidal filaments (Fig. 7). Terete stolons, in contrast, had irregularly arranged medullary cells and internal rhizoidal filaments (Fig. 8).

Tetrasporic plants were collected from September to January. Fertile branches often had one or two constrictions in the apical zone, probably a result of episodic growth during the development of sori (Fig. 9). Tetrasporangial sori formed in the apical parts of the main axis as well as in lateral or regenerated branchlets, and a well-defined, narrow sterile margin was always present. Mature tetrasporangia were subspherical, 34–37 μm in diameter, and cruciately divided. Tetrasporangia were initially arranged in regular V-shaped rows, but later no pattern was apparent, as secondary tetrasporangia were added randomly, resulting in patches of tetrasporangia of mixed ages (Fig. 10). Cystocarpic plants were collected only in January and were the smallest in the population

Table 1. Identification of historical herbarium specimens examined in this study.

Current label identification[a]	Herbarium number[b]	Collection data	New identification[c]
G. clavatum	PC0465633√	Adriatic Sea; G. Zanardini	*G. adriaticum* **m**
G. pusillum	PC0465632√	Cherchell, Algeria; 18 May 1930; J. Feldmann	*G. adriaticum* **m**
G. pusillum	NHA595257√	La Marsa, Tunisia; 04 Dec. 1973; Meñez & Cherif	*G. crinale* **m**
P. americana [*G. caerulescens*]	MICH654144*	Shark Shoal Breakwater, Beaufort, Carteret Co., North Carolina, USA; 19 Oct. 1940; H.L. Blomquist 11436	*G. carolinianum* **M**
G. americanum	NCU-A-0012735#	Radio Island, Carteret Co., North Carolina, USA; 23 Apr. 1994; M.H. Hommersand	*G. carolinianum* **M**
G. americanum	NCU-A-0012737#	Radio Island, Carteret Co., North Carolina, USA; 21 Aug. 1994; M.H. Hommersand	*G. carolinianum* **M**
P. americana [*G. caerulescens*]	MICH654094*	New River Inlet, North Carolina, USA; 07 Jul. 1949; L.G. Williams	*G. carolinianum* **M**
P. americana	UC1105413#	Shark Hole, Harrington Sound, Hamilton I., Bermuda; 22 Jun. 1956; W.R. Taylor	*G. carolinianum* **M**
P. americana	MICH654116*	Tuckers Bay, Harrington Sound, Hamilton Island, Bermuda; 26 Aug. 1949; A.J. Bernatowicz	*G. carolinianum* **M**
P. americana	MICH654115*	Port Royal Bay, Hunt Island, Bermuda; 11 May 1949; W.R. Taylor & A.J. Bernatowicz	*G. carolinianum* **M**
G. americanum	NCU-A-0012736#	Topsail Sound, Pender Co., North Carolina, USA; 02 Oct. 1993; M.H. Hommersand	*G. carolinianum* **m**
G. americanum [*G. pusillum*]	WNC8212#	Radio Island, Carteret Co., North Carolina, USA; 22 Jun. 1975; D.F. Kapraun	*G. carolinianum* **m**
G. americanum [*G. pusillum*]	WNC8213#	Radio Island, Carteret Co., North Carolina, USA; 22 Jun. 1975; D.F. Kapraun	*G. carolinianum* **m**
G. americanum	WNC15668#	Radio Island, Beaufort, North Carolina, USA; 14 Sep. 1981; C.W. Schneider	*G. carolinianum* **m**
P. americana [*G. pusillum*]	MICH654092*	Shark Shoals, Beaufort, North Carolina, USA; 04 Jul. 1949; L.G. Williams	*G. carolinianum* **m**
P. americana [*G. caerulescens*]	NY2224531√	Fort Macon, Beaufort, North Carolina, USA; 20 Aug. 1906; W.D. Hoyt	*G. carolinianum* **m**
G. pusillum	PC0467258√ (J.F. n° 74)	Herbier J. Feldmann, Carthage, Tunisia; 04 Jun. 1931	*G. carolinianum* **m**
P. americana [*G. corneum*]	MICH654085*	Port Aransas, Nueces, Texas, USA; 27 Mar. 1937	*G. crinale* **M**
P. americana	MICH654117 *	Shark Hole, Harrington Sound, Hamilton Island, Bermuda; 22 Apr. 1949; W.R. Taylor & A.J. Bernatowicz	*G. crinale* **M**
P. americana	UC1880503#	Guajataca, Puerto Rico; 01 Jan. 1962; M. Diaz-Piferrer	*G. crinale* **M**
P. americana [*G. crinale*]	MICH654145*	Shark Shoal Breakwater, Beaufort, Carteret Co., North Carolina, USA; 19 Oct. 1940; H.L. Blomquist 11432	*G. crinale* + *Gelidium* sp. **M**
'*G. coerulescens* Crouan'	UC1847858#	Port Antonio, Jamaica; Jul. 1900; C.E. Pease & E. Butler	*G. americanum* **M**
G. americanum [*P.?americana*]	MICH654147*	Saint Sauveur, Guadeloupe; 08 Oct. 1944; A. Questel	*G. americanum* **M**
P. americana ['*G. coerulescens* Crouan']	MICH1306116√ **Holotype**	Port Antonio, Jamaica; Jul. 1900; C.E. Pease & E. Butler	*G. americanum* **m**
'*G. coerulescens* Crouan'	WTU-A-014405√ 'Type'	Port Antonio, Jamaica; Jul. 1900; C.E. Pease & E. Butler	*G. americanum* **m**
G. americanum ['*G. coerulescens* Crouan'; *P. americana*]	MICH654084* **Isotype**	Port Antonio, Jamaica; Jul. 1900; C.E. Pease & E. Butler	*G. americanum* **m**

[a]Names in brackets are previous identifications given in chronological order.
[b]# indicates whole sheet; * indicates only a fragment provided on loan; √ indicates specimen observed online.
[c]**m** indicates identification based on morphological characters.
M indicates identification based on partial *rbc*L sequences (124–757 bp depending upon specimen).

(Fig. 11); they bore many single cystocarps in the subterminal zone of nonspecialised branches (Fig. 12). Cystocarps were bilocular, surrounded by a sterile margin, and spherical with one central circular ostiole per chamber provided with a prominent peristome (Fig. 13). Spermatangial sori have not been found (Table 2).

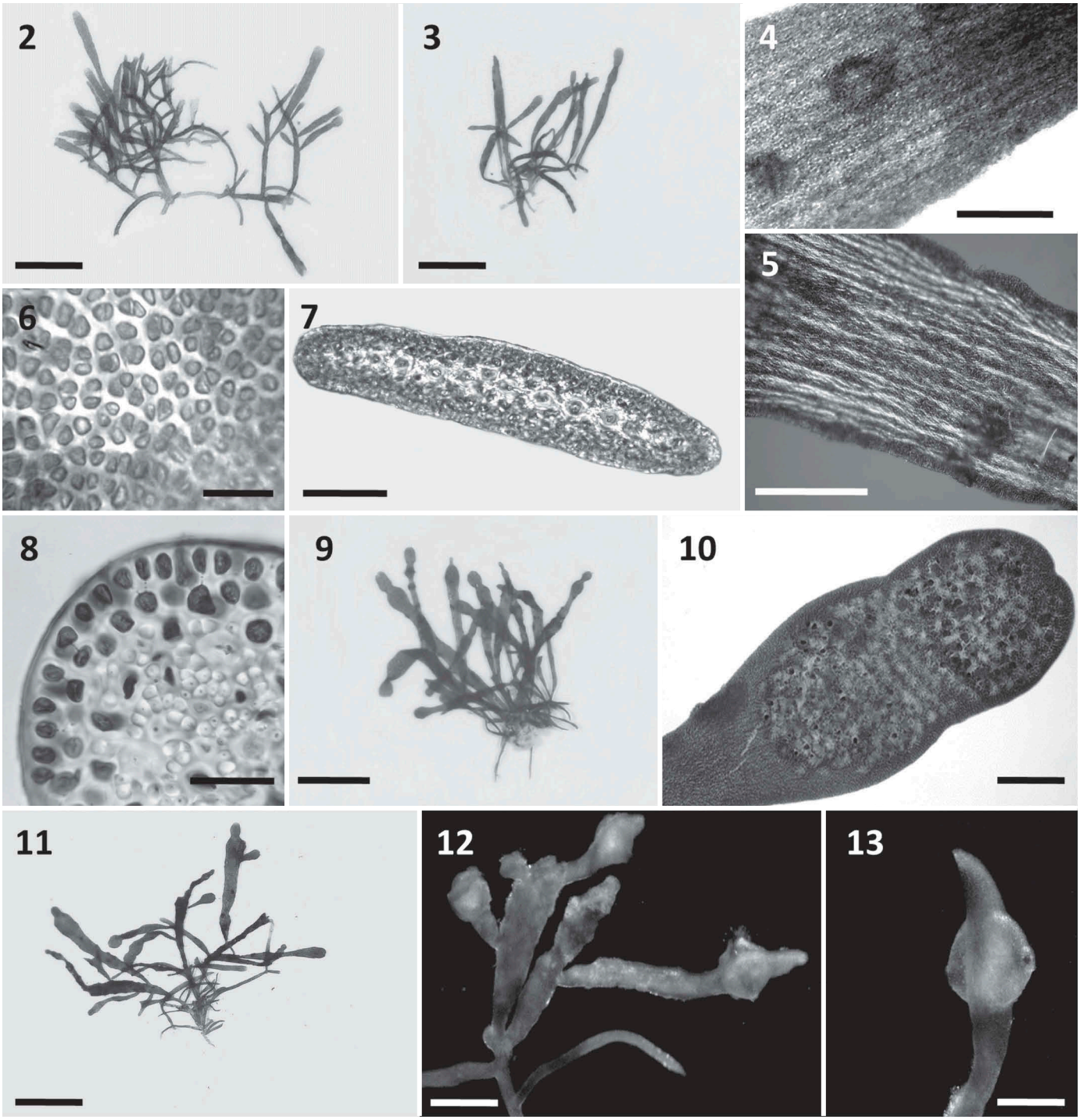
Remarks

Observations of numerous herbarium specimens labelled *G. pusillum* revealed that none of the Mediterranean ones represented this species. Specimens from the British and French coasts of the English Channel showed the characteristic features described and illustrated for this taxon, but many from other localities most likely represent *G. crinale* (Hare ex Turner) Gaillon, (e.g. NHA595257 from La Marsa, Tunisia), *G. pulchellum* (Turner) Kützing, or other gelidioids. Historical specimens from Mediterranean localities labelled *G. pusillum* were not sequenced, but they exhibited morphological characters that differed from those typical of *G. pusillum*. At least two of these, *G. pusillum* from Cherchell, Algeria, identified by J. Feldmann in 1930 (PC0465632), and *Gelidium clavatum* (J.V.Lamouroux) J.V. Lamouroux from the Adriatic Sea, identified by Zanardini (PC0465633, undated), could belong to *G. adriaticum* (Table 1).

Gelidium carolinianum C.Perrone, D.W.Freshwater, A. Bottalico, G.H.Boo & S.M.Boo *sp. nov.*

Figs 14–27

DESCRIPTION: Thallus cartilaginous and stiff, consisting of bushy erect fronds arising from an inconspicuous prostrate system attached to the substratum by brush-like haptera; uprights compressed to flattened; branching distichous, irregularly to regularly pinnate up to three orders; lateral branches constricted at their bases; outermost cortical



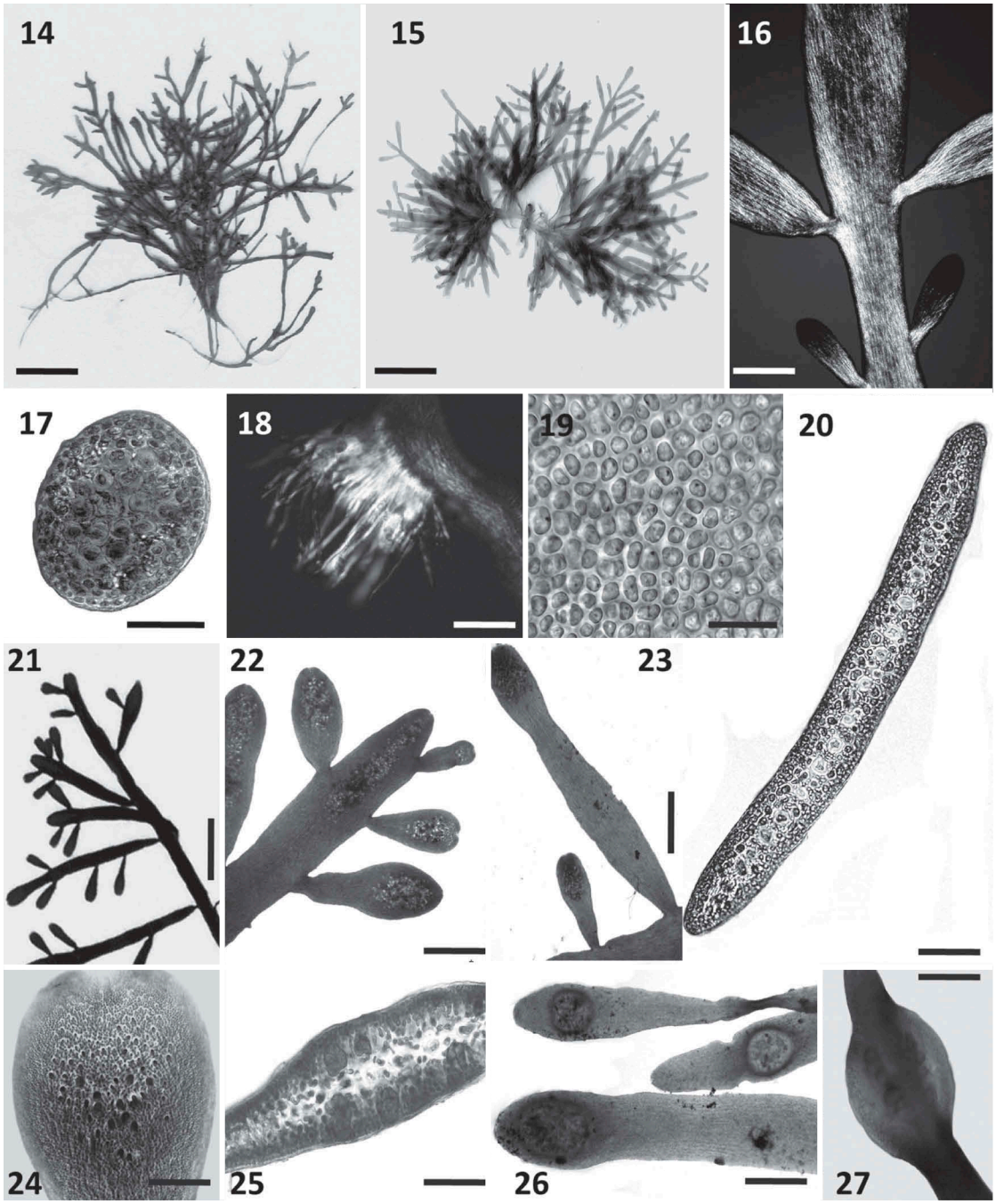
Figs 2–13. *Gelidium adriaticum sp. nov.*
Fig. 2. Detail of holotype specimen (lower right corner specimen on type sheet FI018628; Torre a Mare, Bari, Apulia, Italy). Scale bar = 3 mm.
Fig. 3. Isotype specimen (BI38468). Scale bar = 3 mm.
Fig. 4. Dark longitudinal striations observed in surface view (BI38469). Scale bar = 100 µm.
Fig. 5. Bundles of internal rhizoidal filaments longitudinally arranged (under polarised light; BI38469). Scale bar = 100 µm.
Fig. 6. Irregularly arranged outermost cortical cells in surface view (BI38470, Koper Bay, Slovenia). Scale bar = 25 µm.
Fig. 7. Transverse section through flattened distal portion of erect axis showing two to three layers of cortical cells and medullary cells regularly aligned in median plane, surrounded by internal rhizoidal filaments (BI38470). Scale bar = 100 µm.
Fig. 8. Transverse section through stolon showing irregularly arranged medullary cells (BI38468). Scale bar = 50 µm.
Fig. 9. Tetrasporic plant (BI38468, isotype). Scale bar = 1.5 mm.
Fig. 10. Tetrasporangial sorus surrounded by a well-defined sterile margin (BI38468). Scale bar = 200 µm.
Fig. 11. Cystocarpic plant (BI38470, isotype). Torre a Mare, Bari, Apulia, Italy. Scale bar = 1 mm.
Fig. 12. Single cystocarps in subterminal zone of nonspecialised branches (BI38468). Scale bar = 500 µm.
Fig. 13. Bilocular cystocarp with one central circular ostiole per chamber, provided with a prominent peristome (BI38468). Scale bar = 300 µm.

Table 2. Morphological character states of *Gelidium adriaticum sp. nov.*, *Gelidium carolinianum sp. nov.*, and similar species.

Features	*G. adriaticum*	*G. carolinianum*	*G. americanum*	*G. pusillum*	*G. crinale*
Type locality, geographic coordinates	Torre a Mare, Bari, Apulia, Italy, 41° 5.3′N, 16°59.85′E	Topsail Sound, Pender Co., North Carolina, USA, 34° 22.320′N, 77° 37.891′W	Port Antonio, Jamaica, 18° 10.57′N, 76°26.95′W	Sidmouth, Devon, England, 50°40.317′N, 3°14.183′W	Ilfracombe, Devonshire, England, 51°12.65′N, 4°7.53′W
Habitat	On rocks, upper intertidal	On rocks and shells, from lower intertidal to subtidal (to −18 m in Onslow Bay, North Carolina, USA)	Subtidal to −1 m in Guadeloupe	On rocks, upper intertidal	On rocks, upper to lower intertidal
Habit	Erect, turf-forming	Erect, bushy	Erect, bushy	Creeping, turf-forming	Erect, turf-forming
Thallus texture	Cartilaginous	Cartilaginous	Submembranous	Cartilaginous	Cartilaginous
Colour	Purplish red	Red to dark purple	Brownish red	Purplish to blackish	Dark red to blackish
Erect frond	Longitudinal striations in surface view	No longitudinal striations in surface view	No longitudinal striations in surface view	Longitudinal striations in surface view	No longitudinal striations in surface view
Shape	Flattened, spatulate to lanceolate, sometimes long stipitate	Compressed to flattened, linear; branches constricted at the base	Compressed to flattened, spatulate to lanceolate; branches slightly attenuated at the base	Flattened, obovate to spatulate	Terete to compressed, reproductive apices compressed to flattened, linear to narrowly lanceolate
Branching	Scarce and irregular, up to two orders	Irregular to pinnate up to three orders	Irregular in the lower part, alternate to subpinnate in the upper third, up to four orders	Scarce and irregular, mostly simple	Irregular to subdichotomous up to four orders
Height (cm)	0.4–0.8	0.8–2.5	0.5–6.0	0.2–1.0	1.0–3.0
Width (μm)	180–550	300–800	400–2000	500–2000	400–450
Thickness (μm)	80–100	60–80	75–150	–	60–160
Medullary cells	Single median row	Single median row	Alignment not evident	Alignment not evident	One or two rows
Internal rhizoidal filaments	Abundant, surrounding medullary cells	Abundant, surrounding medullary cells	Scarce and scattered in the medulla	In the medulla	Abundant in inner cortex and outer medulla
Tetrasporangial sori	Terminal on main axis and lateral branches; well-defined sterile margin	Terminal on main axis and lateral branches; ill-defined sterile margin	Terminal; no sterile margin	Terminal on main axis and lateral branches; no sterile margin	Terminal on main axis and lateral multilobate branchlets; no sterile margin
Tetrasporangia arrangement	Irregular	At first in regular rows, then irregular	At first in regular rows, then irregular	Relatively regular rows	Irregular
Cystocarps	Subapical, spherical, one central circular ostiole per chamber with a prominent peristome	Subapical, ovoid, one to two on the same branch; one or more simple openings per chamber	Unilocular[a] bilocular[b]	Observed only in cultured material: single, terminal; one or more simple openings per chamber	Globose, on fastigiate apices; one or two ostioles per chamber
Spermatangia	Not observed	Not observed	Not observed	Observed only in cultured material	Not observed
Geographic distribution (ascertained to date)	Apulia, Italy (southern Adriatic Sea); Koper Bay, Koper, Slovenia (northern Adriatic Sea)	North Carolina, USA, Bermuda (northwestern Atlantic); Apulia, Italy (Adriatic Sea and Ionian Sea)	Jamaica, Guadeloupe (Caribbean Sea); Brazil (southwestern Atlantic)	North Atlantic	Cosmopolitan
References	This study	Hoyt (1920), Kapraun (1980), Schneider & Searles (1991; as *G. americanum*); this study	Mazè & Schramm (1878; as *Gelidium caerulescens*); Taylor (1943; as *Pterocladia americana*); Santelices (1976; as *G. americanum*); this study	Stackhouse (1795); Feldmann & Hamel (1936); Maggs & Guiry (1987); Fredriksen *et al.* (1994); Rueness & Fredriksen (1998); Kim & Boo (2012)	Feldmann & Hamel (1936); Womersley & Guiry (1994); Millar & Freshwater (2005); Croce & Parodi (2013); Díaz-Tapia & Barbara (2014); Jamas *et al.* (2017); this study

[a]Taylor (1943; as *Pterocladia americana*).
[b]Santelices (1976; as *G. americanum*).

cells ovoid; inner cortex of two to three layers of pigmented cells; medullary cells regularly arranged in a single median row, surrounded by internal rhizoidal filaments; tetrasporangial sori in the apical part of both main axes and lateral branches, with ill-defined sterile margins; cruciately divided tetrasporangia irregularly arranged; cystocarps subterminal, bilocular, ovoid with one or more simple openings without peristomes; spermatangial sori not found; *rbc*L sequence = GenBank accession MG272418; *cox*1 sequence = GenBank accession MG820613; *psbA* sequence = GenBank accession MG820614; 28S rRNA gene sequence = GenBank accession MG818476.



Figs 14–27. *Gelidium carolinianum sp. nov.*

Fig. 14. Detail of holotype specimen (upper right corner specimen on type sheet WNC 33701, *Gelidium americanum*; Topsail Sound, Pender Co., North Carolina, USA). Scale bar = 2.5 mm.
Fig. 15. Specimen from Torre a Mare, Bari, Apulia, Italy (BI38471, paratype). Scale bar = 2.5 mm.
Fig. 16. Internal rhizoidal filaments longitudinally arranged and densely packed at the basal constrictions of upright branches (under polarised light; BI38471). Scale bar = 300 µm.
Fig. 17. Transverse section through stolon showing irregularly arranged medullary cells and very few internal rhizoidal filaments between cortex and medulla (BI38472). Scale bar = 100 µm.
Fig. 18. Brush-like hapteron (under polarised light; BI38472). Scale bar = 100 µm.
Fig. 19. Outer cortical cells in surface view (BI38471). Scale bar = 20 µm.
Fig. 20. Transverse section through flattened distal portion of an erect axis showing two to three layers of cortical cells and medullary cells regularly aligned in the median plane, surrounded by internal rhizoidal filaments (BI38471). Scale bar = 100 µm.
Fig. 21. Tetrasporangial branchlets (BI38471). Scale bar = 1.5 mm.
Fig. 22. First stages of the formation of the tetrasporangial sori. Scale bar = 300 µm.
Fig. 23. Tetrasporangial sori occurring in the apical part of lateral branches (NCU-A-0012736, Topsail Sound, Pender Co., North Carolina, USA). Scale bar = 300 µm.
Fig. 24. Young tetrasporangial sorus with the initial ordered tetrasporangium arrangement (BI38471). Scale bar = 150 µm.
Fig. 25. Transverse section through a tetrasporangial sorus with cruciately divided tetrasporangia (BI38471). Scale bar = 100 µm.
Fig. 26. Subterminal cystocarps with sterile margins (formalin-preserved specimen, Cheradi Islands, Italy). Scale bar = 200 µm.
Fig. 27. Bilocular ovoid cystocarp (NCweed-1060, Onslow Bay, North Carolina, USA). Scale bar = 150 µm.

HOLOTYPE: WNC33701; barcode WNC-A-0006691, collected 05 October 2014 by D.W. Freshwater & K.D. Turner, as *Gelidium americanum* (W.R.Taylor) Santelices.

TYPE LOCALITY: Topsail Sound, Pender County, North Carolina, USA (34° 22.320′N, 77°37.891′W); growing over mussels at the waterline on floating dock.

PARATYPES: WNC8212, WNC8213, WNC15668, NCU-A-0012735, NCU-A-0012736, NCU-A-0012737, MICH654092, MICH654094, MICH654144, (North Carolina, USA); BI38471, BI38472, BI38473, WNC34301 (Italy); UC1105413, UC948280, MICH654115, MICH654116 (Bermuda).

ETYMOLOGY: The epithet '*carolinianum*' refers to North Carolina, USA, where the oldest known collections were made.

Morphology

Gelidium carolinianum formed light red to purple-red hemispherical tufts on rocks and shells, in the lower intertidal, and has also been collected from subtidal hard bottom ledges. Plants were erect and bushy with upright fronds arising from short stolons. Fronds were linear, compressed to flat, 0.8–2.5 cm tall; lateral branches were inserted at acute angles and constricted at their bases. Branching was sometimes irregular, with laterals of various lengths and intervals, sometimes subpinnate to three orders (Figs 14, 15). The erect fronds contained abundant internal rhizoidal filaments longitudinally arranged and densely packed at the basal branch constrictions (Fig. 16). In contrast, internal rhizoidal filaments in stolons were scarce and scattered between the cortex and medulla (Fig. 17). Stolons were 130–-190 μm in diameter and attached to the substratum by brush-like haptera (Fig. 18). Outer cortical cells of both fronds and stolons were ovoid in surface view and approximately 5 × 7 μm diameter (Fig. 19). Flattened fronds were 300–800 μm wide and 60–80 μm thick and had three layers of cortical cells and thick-walled medullary cells, approximately 20 μm in diameter, that were aligned in a single row along the median plane and surrounded by internal rhizoidal filaments (Fig. 20).

Tetrasporic plants were more regularly pinnately branched than cystocarpic plants (Fig. 21). Tetrasporangial sori formed in the apical part of both main axes and lateral branches that frequently had retuse apices and were surrounded by an ill-defined sterile margin (Figs 22, 23). Tetrasporangia initially developed in regular rows but soon appeared irregularly arranged (Fig. 24). Mature tetrasporangia were subspherical, 27–35 μm in diameter, and cruciately divided (Fig. 25). Cystocarps were bilocular, ovoid, subterminal with sterile margins, one or two in a row on the same branch, and had one or more simple openings without organised cortical tissue as in true ostioles (Figs 26, 27). Spermatangial sori were not found (Table 2).

Remarks

Plants of *G. carolinianum* from different habitats exhibited varying morphologies. Mediterranean specimens were more robust and smaller than those from the western Atlantic; plants from Radio Island, near Beaufort, North Carolina, were more robust and branched than those from Topsail Sound. In Apulia, plants from S. Cesarea Terme, Lecce (in the Otranto Channel), growing on rocks exposed to strong wave motion, were more rigid and robust than those from the Cheradi Islands (Gulf of Taranto, Ionian Sea).

Among Mediterranean specimens labelled *G. pusillum*, those collected by J. Feldmann in 1931 from Carthage, Tunisia (PC0467258), shared many morphological characters with *G. carolinianum*. The 1906 Hoyt collection from Beaufort identified as *G. caerulescens* (NY2224531) also showed characters of *G. carolinianum*. Unfortunately, neither of these specimens could be sequenced. Specimens that we sequenced from North Carolina and Bermuda, labelled *P. americana* and *G. americanum*, were resolved in the *G. carolinianum* clade (see below) and exhibited morphological characters similar to those of the new species. Two 1940 Blomquist collections from Shark Shoal, Beaufort, North Carolina, USA, were annotated as *P. americana* by Taylor in 1942. Collection No. 11436, originally labelled *G. caerulescens* Kützing (MICH654144), corresponded in sequence and morphology to *G. carolinianum*. Collection No. 11432, originally labelled *G. crinale* (Turner) J.Agardh (MICH654145) was a mixture of *G. crinale* and *Gelidium* sp. (Table 1).

Gelidium americanum (W.R.Taylor) Santelices (1976)
Figs 28–32

BASIONYM: *Pterocladia americana* Taylor (1943): 154.

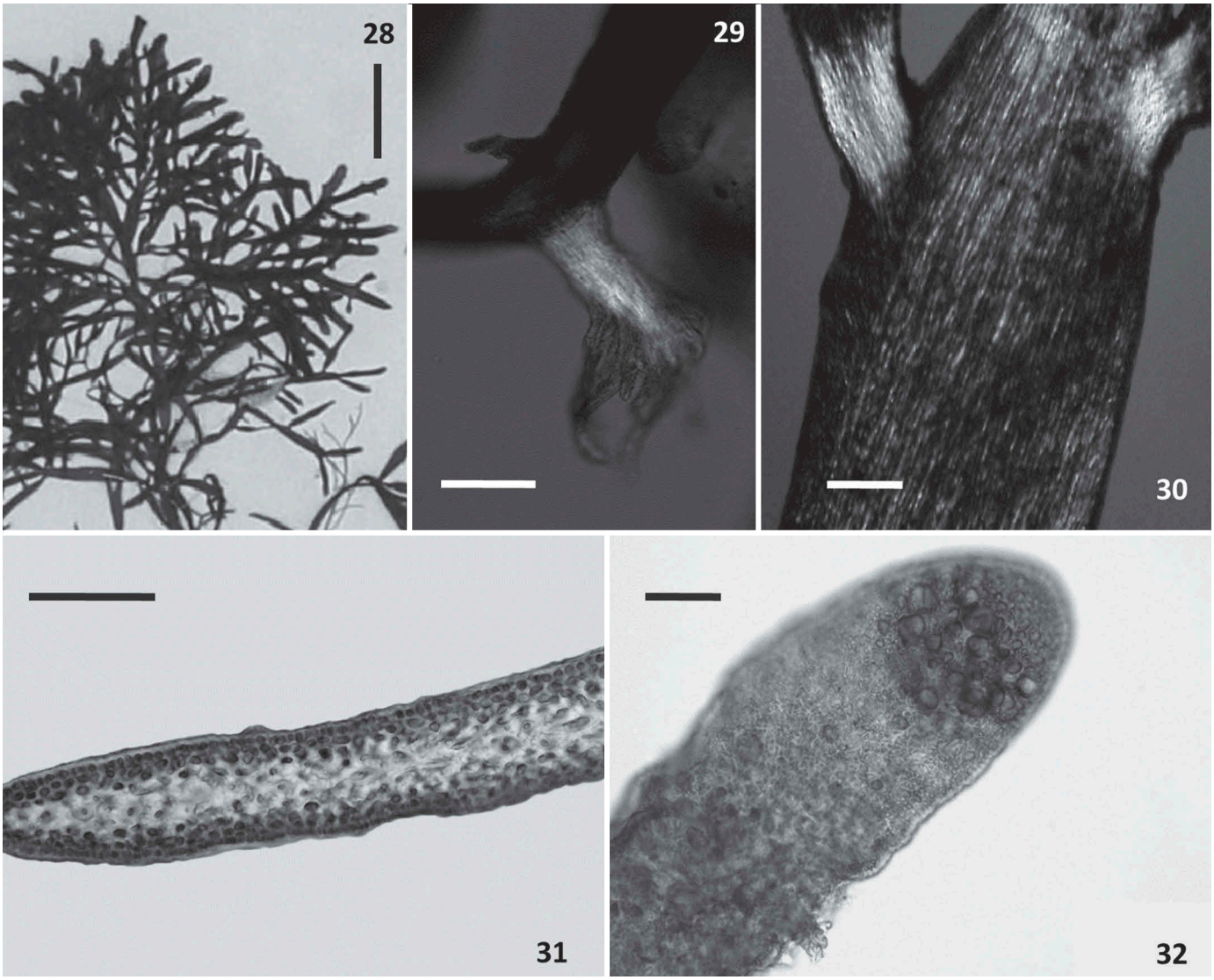
HOLOTYPE: MICH1306116, *P.B.-A.* #783, collected July 1900 by C.E. Pease and E. Butler.

TYPE LOCALITY: 18°10.57′N, 76°26.95′W; Port Antonio, Jamaica.

Remarks

Isotype specimens of '*Gelidium coerulescens* Crouan' (*P.B.-A.* #783) from Port Antonio, Jamaica, represented *P. americana*, as described by Taylor, very well and were morphologically distinct from *G. carolinianum* (Table 2; Figs 28–32). One specimen in the *P.B.-A.* #783 collection (WTU-A-014405) was marked 'Type' in pencil, indicating that this specimen could represent the intended holotype of '*Gelidium coerulescens* Crouan' selected by Collins but never published. The holotype of *P. americana* (MICH1306116) was unavailable, but its vegetative morphology was studied in online images. Vegetative characters were similar to those of many other *P.B.-A.* #783 specimens, including the putative Collins holotype (Table 1). Although reproductive structures on the *P. americana* holotype were undetectable in the online images, Taylor annotated on the holotype sheet '… tetrasporangia at first in V-s [V-shaped rows], oldest at the base, occupying the full width of the branchlet. … Cystocarps unilocular'. Taylor's comments suggest that it included both tetrasporangial and cystocarpic plants and that the structure of the cystocarps justified inclusion in *Pterocladia*.

The four herbarium specimens of *G. americanum* that we sequenced (Table S1; isotypes UC693255 and UC1847858 labelled '*Gelidium coerulescens* Crouan' from Jamaica; UC1105492 labelled *P. americana* from Jamaica; MICH654147 labelled *P.* ?*americana* from Guadeloupe) were also sectioned and stained for macro- and microscopic observations. Well-developed plants were 4–6 cm tall and branched to four orders. First-order branching was completely irregular at broad intervals



Figs 28–32. *Gelidium americanum*.
Fig. 28. Thallus morphology and branching (UC693255, isotype, '*Gelidium coerulescens* Crouan', Port Antonio, Jamaica, *P. B.-A.*#783). Scale bar = 1.5 cm.
Fig. 29. Stolon without internal rhizoidal filaments and with brush-like hapteron (under polarised light; UC693255). Scale bar = 100 µm.
Fig. 30. Apical region of flattened frond with few internal rhizoidal filaments (under polarised light; UC1105492, *Pterocladia americana*, Manchioneal, Jamaica). Scale bar = 150 µm.
Fig. 31. Transverse section of a flattened frond (UC1105492). Scale bar = 100 µm.
Fig. 32. Tetrasporangial sorus without sterile margin (UC1105492). Scale bar = 300 µm.

but branching became denser and alternate to subpinnate towards the apices. Branches were inserted at acute angles and often upcurved. The fronds were flattened, about 2 mm wide, spatulate to lanceolate and gradually attenuated towards their bases (Fig. 28). Erect fronds arose in close clusters from an entangled, stoloniferous prostrate system bearing brush-like haptera. Internal rhizoidal filaments were sparse or absent in the stolons and restricted to the haptera (Fig. 29). In the erect axes, especially in flattened distal portions, only a few internal rhizoidal filaments were present, mixed with irregularly arranged medullary cells (Figs 30, 31). Tetrasporangial sori, 700–1200 µm long and 250–400 µm wide, without sterile margins, were found in apical parts of ultimate branchlets (Fig. 32). Cystocarps and spermatangial sori were not found (Table 2).

Molecular analyses

Nineteen *rbc*L sequences generated in this study were aligned and analysed with 48 previously published *Gelidium* species sequences from GenBank (Fig. 33, Table S1). *G. adriaticum sp. nov*. from Italy was resolved as sister to *G. pusillum* from the United Kingdom (100% ML, 1.0 BPP), with a pairwise divergence of 0.6% (8 bp; Fig. 33). *G. carolinianum sp. nov*. from Italy, North Carolina, USA (as *G. americanum* and *G. caerulescens*), and Bermuda (as *P. americana*) were resolved as a monophyletic clade (100% ML, 1.0 BPP), clearly separated from other *Gelidium* species (Fig. 33). The intraspecific divergences of *G. carolinianum* were up to 0.4% (5 bp). Six short *rbc*L segments (124 bp) were obtained from

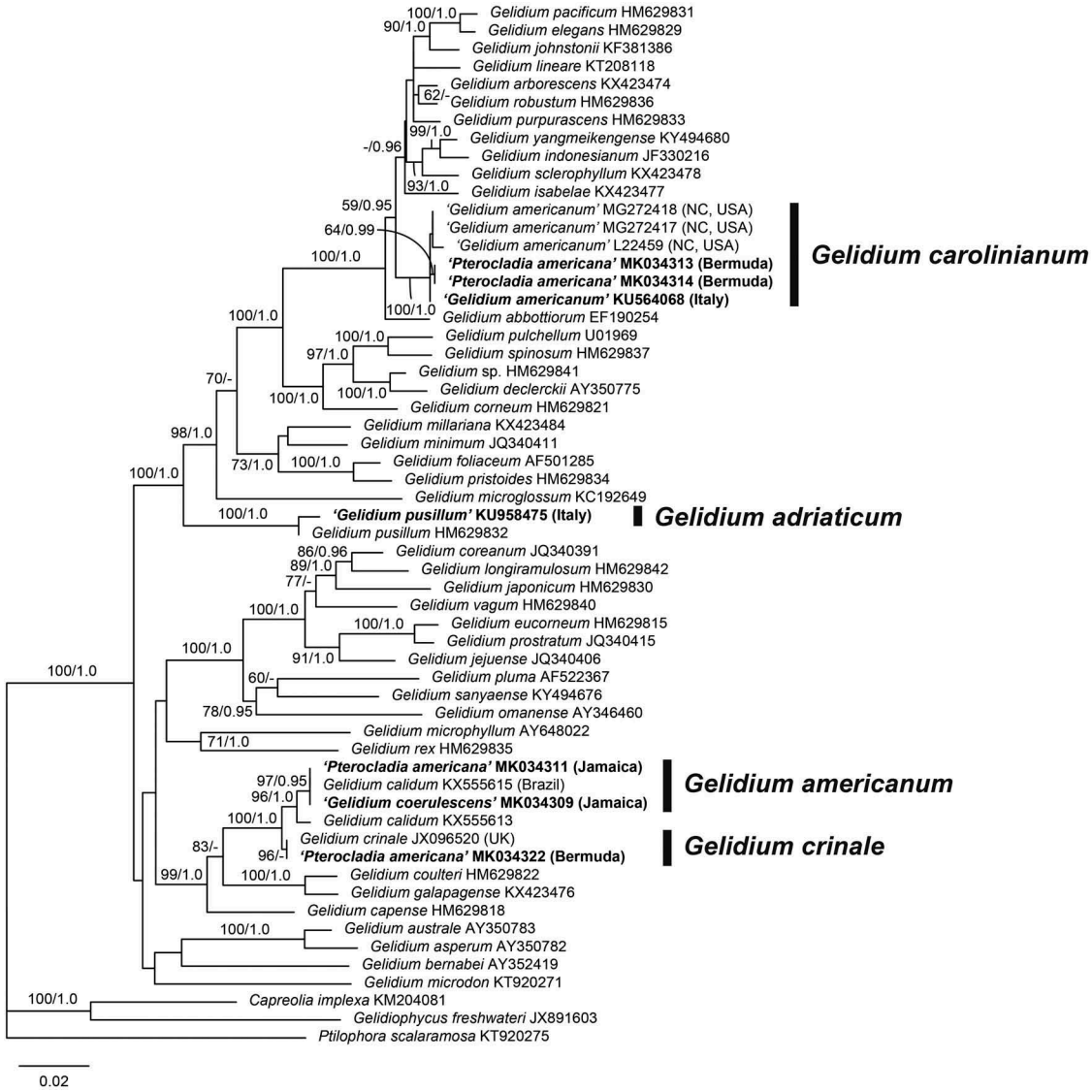


Fig. 33. Maximum-likelihood tree inferred from *rbc*L sequences of *Gelidium*. ML bootstrap values (≥ 50%) and Bayesian posterior probabilities (≥ 0.90) are shown at branches. Dashes indicate values < 50 or < 0.90. Bold letters indicate newly generated sequences in this study.

herbarium specimen fragments labelled *G. americanum, G. caerulescens*, and *Pterocladia americana* from Bermuda and North Carolina, USA. Sequences for all *G. carolinianum* specimens differed by only 0 or 1 bp over this segment of the gene (Table S2).

Sequences from two Jamaican specimens of *G. americanum*, one an isotype, were identical to that of a specimen labelled *Gelidium calidum* Jamas, Iha & M.T.Fujii from Brazil (KX555615) and these formed a strongly supported clade (96% ML, 1.0 BPP) with a second *G. calidum* specimen (KX555613). This *G. americanum/G. calidum* clade was resolved as sister to *G. crinale* (100% ML, 1.0 BPP). The *G. americanum rbc*L sequences differed by 0.7%–0.8% from *G. calidum* (KX555613) and by 0.8%–1.0% from *G. crinale*. Short *rbc*L segments (124–245 bp) from another isotype of '*Gelidium coerulescens* Crouan' (UC1847858) and a specimen of *P. americana* (MICH654147) from Guadeloupe were identical to the other *G. americanum* sequences (Table 1) and that of one '*G. calidum*' specimen (KX555615). Short *rbc*L sequences (124–782 bp) from five specimens, labelled *P. americana* from Bermuda, Puerto Rico, North Carolina, USA, and Texas, USA, were identical to those of *G. crinale* from the United Kingdom (Table 1, Table S2).

Nine *cox*1 sequences were generated in this study and aligned with 46 sequences of *Gelidium* species from GenBank (Fig. 34, Table S1). The pairwise divergence of *cox*1 within *G. carolinianum* from three different locations in Italy was 0.0%–0.5% (0–6 bp). All three specimens of *G. adriaticum* from Italy and Slovenia were identical in *cox*1 sequences and differed by 3.9% from *G. pusillum*. Two Brazilian specimens identified as *G. calidum* were identical to a Jamaican specimen of *G. americanum* (UC1105492). These sequences differed by 3.4%–3.6% from those of other *G. calidum* specimens, including the holotype, and by 5.3%–6.5% from *cox*1 sequences of *G. crinale* available in GenBank (Fig. S1).

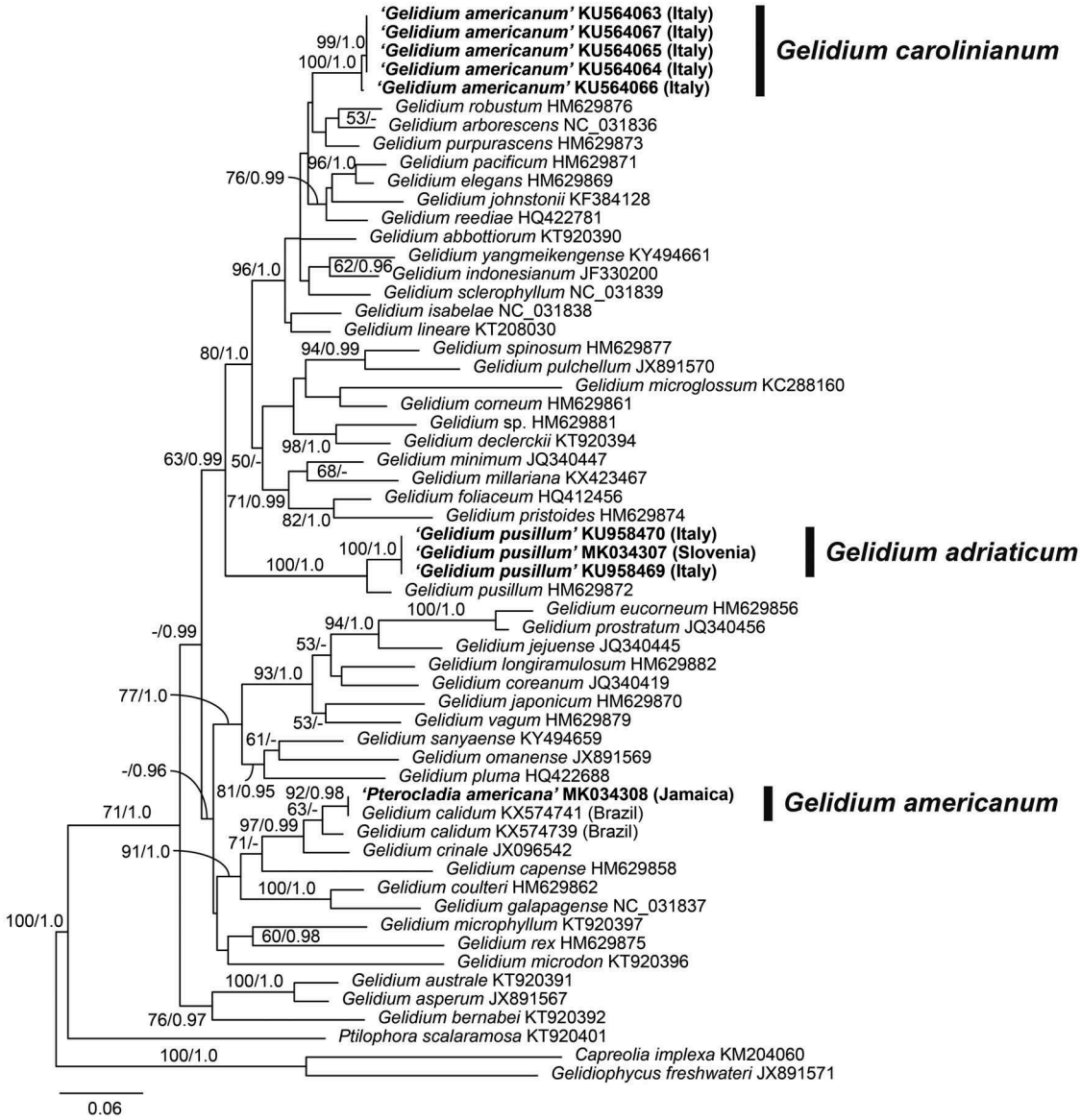


Fig. 34. Maximum-likelihood tree inferred from *cox*1 sequences of *Gelidium*. ML bootstrap values (≥ 50%) and Bayesian posterior probabilities (≥ 0.90) are shown at branches. Dashes indicate values < 50 or < 0.90. Bold letters indicate newly generated sequences in this study.

The *cox*1 phylogeny (Fig. 34) had a topology similar to that of *rbc*L. *G. carolinianum* was distinct from other species in the genus (100% ML, 1.0 BPP). *G. adriaticum* formed a fully supported clade with '*G. pusillum*' from Italy and Slovenia. *G. americanum* was identical to '*G. calidum*' (KX574741) and formed a strongly supported clade with *G. calidum* (KX574739) and *G. crinale* (97% ML, 0.99 BPP).

DISCUSSION

The last inventory of the Apulian macroalgae reported seven species and two varieties belonging to *Gelidium: G. crinale* (Turner) Gaillon, *G. minusculum* (Weber-van Bosse) R.E. Norris, *G. pectinatum* (Montagne) Montagne (as *G. bipectinatum* G.Furnari), *G. pulchellum* (Turner) Kützing, *G. pusillum, G. spathulatum* (Kützing) Bornet, *G. spinosum* (S. G.Gmelin) P.C.Silva var. *spinosum*, and *G. spinosum* (S.G. Gmelin) P.C.Silva var. *hystrix* (J.Agardh) G.Furnari (Cormaci *et al.* 2001; Furnari *et al.* 1999, 2010). These taxa have been well described in the literature (Croce & Parodi 2013; Díaz-Tapia & Bárbara 2014; Echegaray & Seoane-Camba 1982; Feldmann & Hamel 1936; Fredriksen *et al.* 1994; Gómez Garreta *et al.* 1982; Hatta & Prud'homme van Reine 1991; Kützing 1868; Millar & Freshwater 2005; Norris 1992; Weber van Bosse 1921), and all were collected except *G. minusculum, G. pulchellum* and *G. spinosum* var. *hystrix* during our surveys along the Italian Apulian coast, but molecular confirmation of their presence in the Mediterranean Sea is still needed.

Based on molecular and morpho-anatomical analyses of specimens resembling *G. pusillum*, as well as an in-depth

review of historical and recent herbarium specimens and literature, we described two new species, *Gelidium adriaticum* and *Gelidium carolinianum*, distantly related to *G. pusillum* and *G. crinale*, the only species of *Gelidium* with which they could be locally confused. Comparative data for *G. adriaticum, G. carolinianum*, and congeners with which they have been and still can be confused are summarised in Table 2. Young plants of *G. adriaticum* and *G. carolinianum* were morphologically nearly indistinguishable and shared the same thallus structure, with medullary cells aligned in a single median row and surrounded by internal rhizoidal filaments. Mature plants, in contrast, were distinct. Thalli of *G. adriaticum* were always smaller, with initially spatulate fronds that become lanceolate with long stipes and with branching absent or scarce to irregular up to two orders. In *G. carolinianum*, fronds were compressed to flattened, linear, basally constricted, and subpinnately branched to three orders.

Tetrasporangial sori had well-defined and ill-defined margins in *G. adriaticum* and *G. carolinianum*, respectively. This difference may be due to different developmental pathways. Well-defined margins could result from a developmentally programmed endpoint; whereas, ill-defined margins may represent transitional stages in which the formation of sporangia may extend up to the edges of the frond; the final appearance could be that of sori without margins. Ill-defined margins could also result from a cortex containing internal rhizoidal filaments, which produce margins of variable shape and thickness, depending on the developmental stage.

Cystocarpic plants of *G. adriaticum* had spherical cystocarps with one central ostiole with a raised peristome per chamber; whereas, *G. carolinianum* had ovoid cystocarps with simple openings that were variable in number, size and position. These differences, like the margins of tetrasporangial sori, are also a product of developmental differences. True ostioles begin to form early in pericarp development from predisposed initials that can also form peristomes. They are characterised by organised cortical tissue around the opening through the pericarp, as observed in *G. adriaticum*. In contrast, cystocarps of some species release their spores through simple openings resulting from breaks in the pericarp. These simple openings were the type observed in *G. carolinianum*.

Young plants of both *G. adriaticum* and *G. carolinianum* were almost indistinguishable from *G. pusillum*, as it has been described and illustrated. *G. adriaticum* had dark longitudinal striations in erect thalli when observed in surface view, as was described for *G. pusillum* (Fredriksen *et al.* 1994, fig. 8. However, the medullary structure of *G. pusillum* is different; a median cell row is not evident as in our new species (Maggs & Guiry 1987; Rico & Guiry 1997). Well-developed and reproductive thalli, in contrast, were easily distinguishable from those of *G. pusillum*. Mature plants of *G. adriaticum* were smaller than those of *G. pusillum* and *G. carolinianum* with fewer branch orders. Tetrasporangial sori in *G. pusillum* have been described as lacking well-defined sterile margins (Fredriksen *et al.* 1994), which were present in *G. adriaticum*. The two new species, especially *G. carolinianum*, could be confused with *G. crinale* when the apices of the latter become enlarged when fertile. *G. crinale* was often found together with one or the other new species during our Apulian coast surveys.

Specimens from North Carolina, Bermuda and Italy previously identified as *G. americanum* represent a distinct species, described here as *G. carolinianum*, which is distantly related to *G. americanum* (Figs 33, 34). Although the two species have historically been confused in the northwest Atlantic, there are morphological differences (Tables 1, 2). Young, short plants of *G. americanum* had axes that were wider than those of *G. carolinianum*, and many erect fronds arose close together from the stolons, giving *G. americanum* a caespitose appearance. Well-developed thalli of *G. americanum* were usually twice or more larger than *G. carolinianum* and branched to four orders. *G. americanum* fronds were spatulate to lanceolate, gradually attenuated towards their bases. In contrast, fronds of *G. carolinianum* were linear and abruptly constricted at the base. The single median row of medullary cells characteristic of *G. carolinianum* was not so evident in *G. americanum*, and although internal rhizoidal filaments were observed only in the medullary zone of both species, they were much fewer in *G. americanum*. This may explain why *G. americanum* has a less cartilaginous texture, a characteristic that is obvious by the way branches were arranged on pressed herbarium specimens.

Previous DNA sequence data for *G. carolinianum* were deposited in GenBank as *G. americanum* (Bailey & Freshwater 1997; Freshwater & Bailey 1998; Freshwater & Rueness 1994; Olson *et al.* 2004), and this misidentification led to initial reports of *G. americanum* in the Mediterranean (Bottalico *et al.* 2014a). *G. carolinianum* has also been confused with *G. crinale* and *G. pusillum* in the warm–temperate western Atlantic and this, along with the merger and subsequent separation of *G. crinale* and *G. pusillum* (Dixon & Irvine 1977; Fredriksen *et al.* 1994; Kim & Boo 2012), has made the identification of *Gelidium* species in the region inconsistent (e.g. Freshwater & Rueness 1994; Kapraun & Bailey 1989; Wiseman & Schneider 1976). All of these species share morphological characters, and only careful observations and molecular analyses, and sometimes only the latter, can distinguish them.

Multiple specimens of '*Gelidium coerulescens* Crouan' (*P.B.-A.* #783 collection) were distributed to numerous herbaria but very few retain the original identification (Table 1). Most of these isotypes have been annotated as *G. americanum* or its basionym *P. americana*, but some have been attributed to *Pterocladiella caerulescens* (Kützing) Santelices & Hommersand or its basionym *G. caerulescens* Kützing, perpetuating the original confusion between the two species.

The provisional name '*Gelidium coerulescens* Crouan' was used by F.S. Collins on labels of a gelidioid alga collected in both 1891 and 1900 at Port Antonio, Jamaica, by C.E. Pease and E. Butler and identified with the aid of E. Bornet. Jamaican samples were considered as the same species previously collected by Mazé and Schramm from Guadeloupe and identified by the brothers Crouan as *Gelidium caerulescens* Kützing (Mazé & Schramm 1878). Thus, Collins, uncertain whether it was the plant (Collins 1901), used the provisional name '*Gelidium coerulescens* Crouan', which should be interpreted as *G. caerulescens* Kützing *sensu* Crouan *frat*. However, Collins did not further pursue this species, despite designating a putative holotype.

Hoyt (1920) reported two gelidiacean algae, *Gelidium crinale* and *Gelidium caerulescens* Kützing, from the area around Beaufort, North Carolina, USA. The notes of Hoyt on this latter species closed with the following sentence: 'This species was identified by Mr. Collins on the basis of a Guadeloupe specimen determined by Crouan and it may perhaps be questioned whether it is really the species described by Kuetzing' (p. 475). Hoyt was clearly referring to the alga that Collins labelled '*Gelidium coerulescens* Crouan'; however, he identified the alga from Beaufort as *Gelidium caerulescens* Kützing, while at the same time perpetuating the doubt that it was indeed this Pacific taxon. Børgesen (1916) also highlighted the differences between the Atlantic and the Pacific taxa and treated '*Gelidium coerulescens* Crouan' as a synonym of *Gelidium corneum* (Hudson) J.V.Lamouroux from the Danish West Indies (Virgin Islands). To compare the Caribbean plants with *Gelidium caerulescens*, he sent one of his specimens to Weber-van Bosse, who had access to Kützing's type specimen. Weber-van Bosse confirmed that the West Indian plants were different from Kützing's species and stated that they belonged to *Gelidium corneum*. Blomquist, 20 years after Hoyt's North Carolina studies, also identified his collection (No. 11436 from Shark Shoal, Beaufort, North Carolina, USA) as *Gelidium caerulescens* Kützing (Table 1) without taking into account the opinions of Collins, Børgesen and Weber-van Bosse. Taylor (1943) also noted differences among specimens, but having described *Pterocladia americana* on the basis of Jamaican samples, he annotated numerous herbarium specimens as his new species, including those from North Carolina, previously labelled as *G. corneum, G. crinale, G. pusillum*, '*Gelidium coerulescens* Crouan' and *Gelidium caerulescens* Kützing.

The conflicting observations of unilocular versus bilocular cystocarps by Taylor (1943) and Santelices (1976) raise the possibility that the Collins collection is not homogeneous. Santelices (1976) re-examined the holotype of *P. americana* and found that it was only tetrasporangial. He questioned the observation of unilocular cystocarps by Taylor and observed bilocular cystocarps on multiple *P.B.-A.* #783 specimens, prompting his proposal of the new combination *G. americanum*. *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand, *P. bartlettii* (W.R.Taylor) Santelices, *P. sanctarum* (Feldmann & Hamel) Santelices, and *P. beachiae* Freshwater are found in the Caribbean (Freshwater *et al.* 1995; Santelices 2007; Thomas & Freshwater 2001). *P. beachiae* is part of a closely related species complex that includes *P. caerulescens*, and distinguishing these two species morphologically is unreliable (Freshwater *et al.* 2010; Iha *et al.* 2017; Tronchin & Freshwater 2007). Similarly, *G. americanum* and *P. beachiae* could be confused, and perhaps some thalli of the latter are present in the *P.B.-A.* #783 collection. However, analyses of *rbc*L data from both *G. americanum* isotypes sequenced in this study, as well as other specimens originally identified as *P. americana*, clearly demonstrate that they represent a *Gelidium* species (Fig. 33, Table 1, Table S2).

Gelidium americanum was also reported in Brazil (Creed *et al.* 2010; Joly 1965; Ugadim 1985, 1987), but its presence was not confirmed by recent molecular surveys of Brazilian Gelidiales (Iha *et al.* 2015; Jamas *et al.* 2017) because of the incorrect identification of the specimen from which all prior GenBank sequences attributed to *G. americanum* were generated (Bailey & Freshwater 1997; Freshwater & Bailey 1998; Freshwater & Rueness 1994; Olson *et al.* 2004). Our analyses of *rbc*L and *cox*1 sequence data indeed revealed that two paratype specimens identified as *Gelidium calidum*, a species recently described from Brazil by Jamas *et al.* (2017), are actually specimens of *G. americanum* (Figs 33, 34). This is especially apparent in *cox*1 data, in which these two '*G. calidum*' sequences are identical to a short sequence generated from one of the *G. americanum* specimens but 3.4%–3.6% different from the sequences of genuine specimens of *G. calidum*, including the holotype (Fig. S1). *G. americanum* and *G. calidum* are resolved as sister species closely related to *G. crinale* in both *rbc*L and *cox*1 phylogenetic analyses (Figs 33, 34).

In conclusion, the results of our research (1) led to the description of two new species of *Gelidium* from the Mediterranean Sea, *G. adriaticum* and *G. carolinianum*, the latter previously misidentified in the Western Atlantic as *G. americanum*; (2) have supported studies that suggested that *G. pusillum* is not present in the Mediterranean, showing that it is absent at least in the Adriatic Sea; and (3) have clarified both the phylogenetic relationships and the geographical distribution of *G. americanum* and its misidentification in the Western Atlantic with other species of *Gelidium*, including *G. pusillum, G. crinale, G. calidum, G. caerulescens* (currently *Pterocladiella caerulescens*) and *G. carolinianum sp. nov.*

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