

## The Mediterranean endemic new genus *Felicinia* (Halymeniales, Rhodophyta) recognized by a morphological and phylogenetic integrative approach

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**Abstract** – Recently, DNA phylogenies resulted in the transfer of some species of the halymeniacean genus *Aeodes*, namely *A. orbitosa* and *A. ulvoidea*, to the genus *Pachymenia* leaving the former supposedly as a monotypic genus based on the type species, *A. nitidissima*. Nevertheless, that work overlooked the presence of *A. marginata*, a red foliaceous alga endemic to the Mediterranean Sea with a convoluted nomenclatural history.

In the present study, we provided a thorough description of *A. marginata* based on both anatomical and molecular data and inferred its phylogenetic relationships among halymeniacean genera using both chloroplastic (*rbcL*) and nuclear genes (LSU). Based on our data, *A. marginata* allied with *Halymenia*, *Cryptonemia*, and associated genera rather than with the generitype *A. nitidissima*, and presented a unique set of characters deserving the status of genus within the Halymeniaceae. Therefore, we proposed the new genus *Felicinia* based on *Felicinia marginata* comb. nov.

A description of the multiaxial vegetative of *F. marginata* construct was provided, highlighting a unique characteristic among red algae: the presence, in the medulla, of peculiar oblique multicellular filaments connecting periclinal filaments. These filaments are produced secondarily as branches from axial filaments and are possibly homologous to the anticlinal filaments observed in the medulla of *Halymenia*.

***Aeodes marginata* / auxiliary ampulla / COI-5P region / DNA barcoding / *Felicinia* gen. nov. / *Felicinia marginata* comb. nov. / Halymeniales / LSU rDNA / Mediterranean sea / multiaxial vegetative construct / Rhodophyta / *rbcL* gene**

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## INTRODUCTION

*Aeodes marginata* (Roussel) F. Schmitz is a red foliaceous alga (Halymeniales, Rhodophyta) found in the sublittoral zone, from a few meters down to 70 m deep, endemic to the Mediterranean Sea (Manghisi *et al.*, 2010a).

It was initially described as *Halymenia marginata* by Roussel in Montagne (1838) and then transferred by J. Agardh (1842) to the genus *Nemastoma* J. Agardh and subsequently (1851) to the genus *Schizymenia* J. Agardh. Differently, Endlicher (1843) considered it as congeneric to *Iridaea* Bory de Saint-Vincent, while Kuntze (1891) placed it into *Platymenia* J. Agardh. Meanwhile, in 1876, J. Agardh described the new genus *Aeodes* with a single species, *A. nitidissima*, from New Zealand. Schmitz (1894) widened the genus *Aeodes* by transferring *Schizymenia marginata*, together with *Iridaea orbitosa* Suhr from South Africa and by adding a new species also from South Africa, *A. ulvoidea*. *Aeodes marginata* was also erroneously identified as *Schizymenia minor* (J. Agardh) J. Agardh (1851) by Zanardini (1866). Later, Rodriguez (1889), Falkenberg (1879), Berthold (1884) and Hauck (1885) credited Zanardini's identification. An exhaustive account of the plethora of nomenclature changes and synonymies to this species was provided by Schmitz (1894).

More recently, phylogenetic relationships inferred from molecular data revealed that species assigned to the genera *Aeodes* and *Pachymenia* J. Agardh were resolved within a strongly supported lineage, despite the polyphyly of each genus, leading some authors to suggest that the two genera should be merged (Fredericq *et al.*, 1996; Wang *et al.*, 2001; Hommersand & Fredericq, 2003; Faye *et al.*, 2004; Kawaguchi *et al.*, 2004). A different conclusion was proposed by Russell and coworkers (2009), who transferred the species *A. orbitosa* and *A. ulvoidea* to *Pachymenia*, leaving supposedly *Aeodes* as a monotypic genus left over with the type species, *A. nitidissima*, ignoring nonetheless the presence of *A. marginata*.

It is noteworthy that all known species of the genera *Aeodes* and *Pachymenia* are presently distributed in the southern hemisphere (Hommersand & Fredericq, 2003; Hommersand *et al.*, 2009) with the exception of *A. marginata*, which is endemic to the Mediterranean Sea (Manghisi *et al.*, 2010a).

The aim of the present study was to provide a thorough description of *A. marginata* based on the observation of vegetative and reproductive features as well as to infer the phylogenetic relationships of this Mediterranean endemic among halymeniacean genera using chloroplastic (*rbcL*) mitochondrial (COI-5P) and nuclear (LSU) genes.

## MATERIALS AND METHODS

### *Morphological observations*

Samples observed in this study are listed in Table 1. The collected material was pressed as herbarium specimens and fragments of the plants were preserved in 4% formalin for the morphological studies or dried in silica gel for DNA extraction. Voucher specimens (Table 1) were housed in the Herbarium of the University of Barcelona, Spain (BCN-Phyc), in the Muséum National d'Histoire Naturelle of Paris, France (PC), or in the Phycological Lab Herbarium of the University of Messina, Italy (PhL) (abbreviations follow the Global

Table 1. List of specimens examined in the present paper

Current name	Collection site	Collection date	Collector(s)	Voucher information	Notes	COI-5P (GB accession number)	LSU (GB accession number)	rbcL (GB accession number)
<i>Acrodiscus vidovichii</i> (Meneghini) Zarnardini	Vis Island, Croatia	20 Jun 2007	J.M.Utge Buil, L. Le Gall	PC0152091		-	KJ594947	KJ594952
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Algeria	Feb 1837		PC0474570 (AR5446 in Herb. Roussel)	Lectotype	-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	L'Imbutu, Scandola, Corse, France	24 Aug 1992	C. Rodriguez- Prieto	HGI-A 2746		-	KJ594949	KJ594954
<i>Aeodes marginata</i> (Roussel) F. Schmitz	La Gabimière, Port-Cros, France	10 Jun 2009	J.M.Utge Buil, L. Le Gall	PC0162467		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Capo dell'Armi, Reggio Calabria, Calabria, Italy	13 Apr 2011	J.M.Utge Buil, L. Le Gall	PC0142662		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Capo dell'Armi, Reggio Calabria, Calabria, Italy	13 Apr 2011	J.M.Utge Buil, L. Le Gall	PC0142660		KJ594945	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	S. Maria La Scala, Acireale, Sicily, Italy	21 Apr 2011	J.M.Utge Buil, L. Le Gall	PC0142661		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Nisida, Naples, Campania, Italy	23 Jul 1981	-	MS 35112-0204F		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Ischia, Naples, Campania, Italy	01 Aug 1981	-	MS 35112-0237F		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Cannizzaro, Aci Castello, Catania, Sicily, Italy	1983	-	MS35112	Gametophyte	-	-	-

Table 1. List of specimens examined in the present paper (*continued*)

<i>Current name</i>	<i>Collection site</i>	<i>Collection date</i>	<i>Collector(s)</i>	<i>Voucher information</i>	<i>Notes</i>	<i>COI-5P (GB accession number)</i>	<i>LSU (GB accession number)</i>	<i>rbcL (GB accession number)</i>
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Cala Figuera (Norte), Mallorca, Balearic Islands, Spain	05 Jun 2004	A. Manghisi, J. Rull, N. Salvador	BCN-Phyc 5915		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Porto Colom, Mallorca, Balearic Islands, Spain	04 Jun 2004	Fishermen	BCN-Phyc 5914	Gametophyte	-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Addaia, Menorca, Balearic Islands, Spain	21 Jun 2003	A. Manghisi, L. Lavelli, J. Rull, N. Salvador	BCN-Phyc 5910	Gametophyte	-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Cala Piques, Menorca, Balearic Islands, Spain	24 Jun 2003	A. Manghisi, L. Lavelli, J. Rull, N. Salvador	BCN-Phyc 5913		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Cap Caballeria, Menorca, Balearic Islands, Spain	23 Jun 2003	A. Manghisi, L. Lavelli, J. Rull, N. Salvador	BCN-Phyc 5912		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	S'Olla, Cap Caballeria, Menorca, Balearic Islands, Spain	21 Jun 2003	A. Manghisi, L. Lavelli, J. Rull, N. Salvador	BCN-Phyc 5911		-	-	-
<i>Aeodes marginata</i> (Roussel) F. Schmitz	Illa de l'Aire, Menorca, Balearic Islands, Spain	20 Jun 2003	A. Manghisi, L. Lavelli, J. Rull, N. Salvador	BCN-Phyc 5909		-	-	-
<i>Aeodes nitidissima</i> J. Agardh	Taroona (end of Flinders Esp.), south of Hobart, Tasmania, Australia	29 November 2002	G. W. Saunders	GWS001525 <sup>1</sup>		-	-	-

Table 1. List of specimens examined in the present paper (continued)

Current name	Collection site	Collection date	Collector(s)	Voucher information	Notes	COI-5P (GB accession number)	LSU (GB accession number)	rbcL (GB accession number)
<i>Corynomorpha prismatica</i> (J. Agardh) J. Agardh	Rocher de l'Albatros, Beloha, Androy, Madagascar	18 May 2010	E.H. Coppejan	PC0171835		–	KJ594950	KJ594955
<i>Cryptomenia lomation</i> (Bertoloni) J. Agardh	La Gabinière, Port-Cros, French Riviera, France	06 Aug 2009	F. Rousseau, J.M. Utge Buil	PC0162380		–	KJ594948	KJ594953
<i>Democorynus horridus</i> (Kützing) Gargiulo, M. Morabito et Manghisi	Stazzo, Catania, Sicily, Italy	11 Apr 2003	G. Genovese, M. Morabito, G.M. Gargiulo	PhL010		–	KJ511244	JX070627
<i>Grateloupia flicina</i> (J.V. Lamouroux) C. Agardh	Livorno, Tuscany, Italy	01 Jul 2002	G. Sartoni	PhL430		–	KJ511243	JX070629
<i>Grateloupia proteus</i> Kützing	Stazzo, Catania, Sicily, Italy	23 Jul 2004	G. Genovese, M. Morabito, G.M. Gargiulo	PhL060		–	KJ511245	JX070626
<i>Halymenia floresii</i> (Clemente) C. Agardh	S. Maria La Scala, Catania, Sicily, Italy	14 Jun 2005	A. Manghisi	PhL-MING026		–	KJ594951	KJ594956
<i>Pachymeniopsis</i> sp.	Villa S. Giovanni, Reggio Calabria, Calabria, Italy	17 Mar 2004	M. Morabito	PhL064		–	KJ511246	AY651060

1. LSU sequence unpublished, courtesy of dr. Gary W. Saunders, University of New Brunswick, Canada.

Registry of Biorepositories, [grbio.org](http://grbio.org)). Additional material was examined in the herbaria of the main Spanish universities and institutions involved in the project “*Flora phycologica iberica*”, and it is listed in Manghisi *et al.* (2010a).

Anatomical observations were performed on sections realized by hand with a razorblade, stained with a 1% aniline blue solution. Microphotography were obtained using a microscope Nikon Optiphot-2 equipped with a Nikon Coolpix 4500 digital camera (Nikon Corporation, Spain) or using a Diaplan Leica equipped with a Leica DFC 500 camera (Leica Microsystems, Italy).

### DNA sequencing and analysis

DNA was isolated from a fragment of tissue recovered from a herbarium sheet of a specimen of *A. marginata* collected in Corsica (France, see Table 1 for details) and from silica dried specimens of other species of Halymeniales. Sequence data generated for COI-5P, *rbcL* and LSU genes were submitted to BOLD (<http://www.barcodinglife.org>) and to GenBank. Accession numbers, together with collection information, are given in Table 1.

DNA isolation was performed as outlined in Manghisi *et al.* (2010b). The barcode region COI-5P was PCR amplified as detailed in Saunders and McDevitt (2012), the nuclear LSU rRNA gene was PCR amplified as detailed in Harper & Saunders (2001) and the plastid *rbcL* gene was amplified with various primer combinations from both Freshwater & Rueness (1994) and Wang *et al.* (2000). Sequences were generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems [ABI], Foster City, CA, USA) and analyzed using an ABI Prism 3130XL genetic analyzer at the CEMAR, University of New Brunswick, Canada, or an ABI Prism 3730XL at the Genoscope ([www.genoscope.fr](http://www.genoscope.fr), Evry, France). Forward and reverse sequence reads were assembled into contigs with the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and edited. Multiple sequence alignments were constructed in SeaView v. 4.3.3 (Gouy *et al.*, 2010) including both data from GenBank and sequences generated for the present study.

An initial alignment, including 603 *rbcL* sequences and 1258 nucleotide positions, was subjected to neighbor-joining (NJ) distance analysis under a general time-reversible model (GTR, Lanave *et al.*, 1984) in PAUP\* (Swofford, 2002) to identify species groups. The resulting tree was used to prepare a second alignment for subsequent phylogenetic analyses with 39 sequences representative of most genera of the order Halymeniales, including the generitypes, by the exclusion of duplicate or similar sequences (poor quality sequences, *i.e.*, those missing more than 30% of data, were also removed), and 1258 nucleotide positions.

Similarly, an initial alignment was built with 122 LSU sequences and 2921 nucleotide position, from which, after NJ analyses, a fourth alignment was built with 30 LSU sequences representative of most genera of Halymeniales, including the generitypes, and 2662 nucleotide positions, excluding those ambiguously aligned.

A fifth alignment, consisted of the concatenation of LSU and *rbcL* sequences for the same species, and when possible for the same specimen, and included 19 sequences and 3821 nucleotide positions.

Further alignments were also built adding sequences of species of Rhodymeniales as outgroup taxa.

All phylogenetic analyses were performed in MrBayes 3.1.2 (MPI versions for Unix clusters; Ronquist & Huelsenbeck 2003, Altekar *et al.* 2004), RAXML (on line version, <http://embnet.vital-it.ch/raxml-bb/>; Stamatakis *et al.*

2008), and PhyML 3.0 (on line version, <http://atgc.lirmm.fr/phyml>; Guindon & Gascuel, 2003).

Maximum Likelihood analyses were performed with bootstrap resampling to estimate robustness of the internal nodes (Felsenstein, 1985), based on 1000 replicates in PhyML, with a GTR+G+I substitution model (with all parameters estimated during the search), starting from ten random BIONJ trees (Gascuel, 1997) with subtree pruning and regrafting (SPR) as branch-swapping algorithm. Furthermore, Maximum Likelihood (ML) analyses were also performed with bootstrap resampling based on 100 replicates in RAxML, with a GTR+G+I substitution model partitioned by codon positions in *rbcL* analyses and by LSU and codon positions of *rbcL* in concatenate analyses.

For bayesian inference both the *rbcL* and the concatenate data sets were also partitioned as above. The covarion-like model (Huelsenbeck, 2002) was combined with the GTR+G model of sequence evolution unlinking parameters among partitions (shape, statefreq, revmat, switchrates) and setting the prior for the site specific rates as "variable". Each analysis consisted of two parallel runs, each run using four chains, one cold and three incrementally heated (temp = 0.10). A single run consisted of 5 million generations that were sampled every 1000th tree. After completion of the two runs, likelihood values were plotted against the number of generations to evaluate when MCMC chains reached stability, in order to set an appropriate burn-in value for each analysis. Only trees saved during the stationary phase were used to reconstruct a majority rule consensus tree and calculate the distribution of posterior probability.

In all phylogenetic analyses, unrooted trees were constructed, the root was subsequently designated based on previous knowledge.

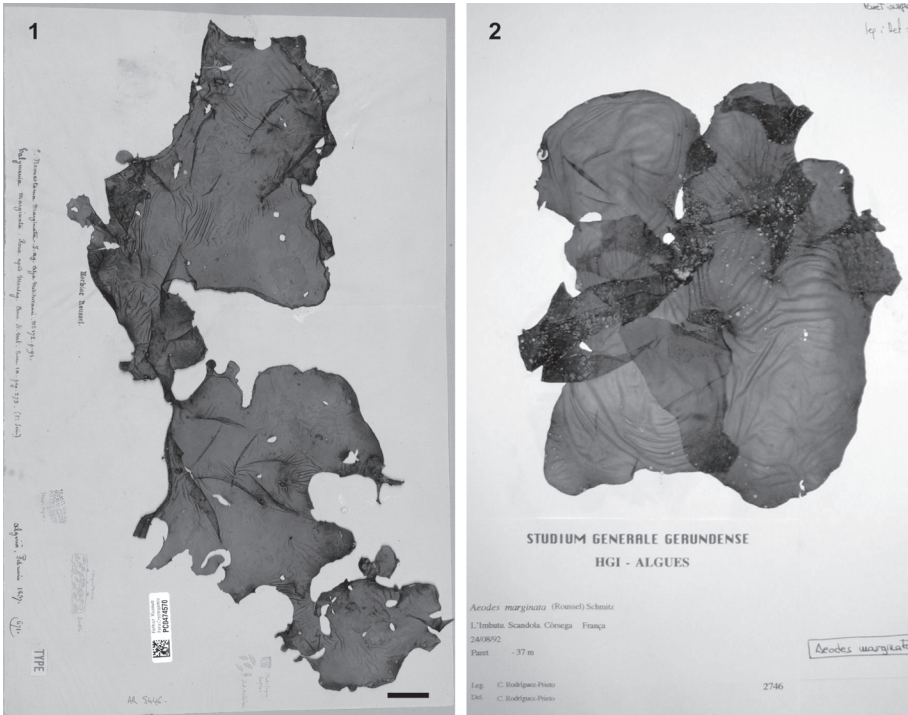
Alternative topologies were tested by means of the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) (SH test) performed in PAUP\* to test for significant difference between the best tree found in the raxML analysis of the combined dataset and a topology where either *Aedes marginata* would join members of the genera *Pachymenia* and *Aedes*.

## RESULTS

### Morphological observations

Seventeenth samples from most of the distribution area were observed (Table 1) and characters were shared and stable among all samples from different localities.

Fronde blade was flat, rounded to elliptical and lobed (Figs 1-2). The blade could be deeply incised at the adult stage with some perforations. Average size was around 15 cm high, but some specimens of 30 cm in height and width were found. The thallus was fixed to the substrate by a small discoid holdfast of about 5 mm in diameter that continues in a stipe lessening to about 2 mm at a height of 5 mm. The blade grew prostrate to the substrate or rarely upright. The edge, undulate and lightly enlarged, was deeply notched by a furrow (Fig. 3), irregular, toothed, finely serrated, as well as the margin of perforations. Consistence was membranous-coriaceous when young, coriaceous-fleshy when old. Colour was purple red in fresh specimens, purple-bloody in dried ones.

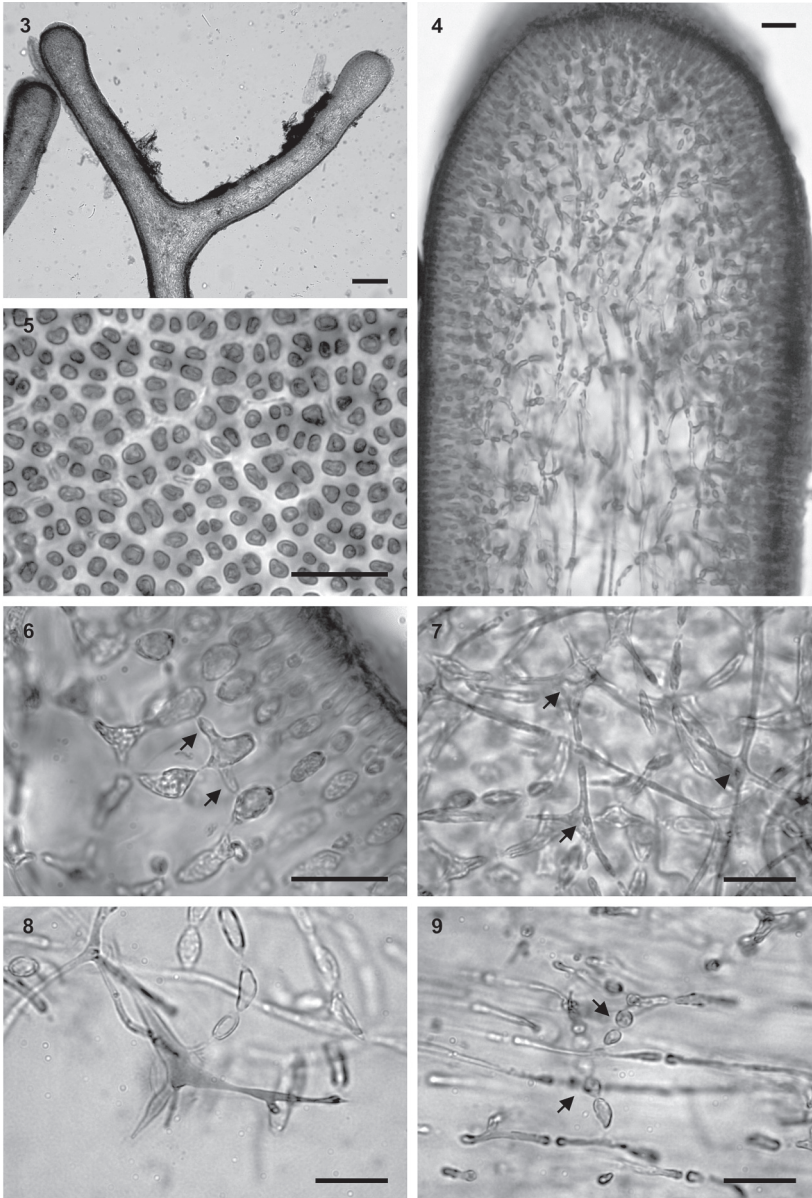


Figs 1-2. Herbarium specimens of *Felinicia marginata* gen. et comb. nov. **1.** The type specimen of *Halymenia marginata* Roussel (PC 0474570); scale bar: 2 cm. **2.** The specimen from Corse (France) used for DNA extraction (HGI-A 2746); ruler in cm.

### Anatomical observations of vegetative features

In longitudinal section, a multiaxial structure with a pseudo-parenchymatous cortex and a filamentous medulla was observed (Fig 4). In surface view, cells were approximately isodiametric or slightly elongated, about 4  $\mu\text{m}$  in diameter, and appear single or in pair (Fig. 5). The cortex was formed by pseudo-dichotomous anticlinal filaments composed of 5-7 ovoid (or rarely spherical) cells which size decreased from innermost cortical cells (20  $\mu\text{m}$  in diameter) to outermost cortical cells (4  $\mu\text{m}$  in diameter) (Fig. 6). The latter were united exclusively by primary pit connections and were more closely arranged than the former (Fig. 6). In the transition zone between cortex and medulla, lateral projections between cells of adjacent cortical filaments were common, forming secondary pit connections that linked either two cortical filaments (Fig. 6, arrows) or a cortical filament with a medullary filament. Consequently, innermost cortical cells became stellate, with 3-4 short arms of about 15  $\mu\text{m}$  and an elongated cell body (Fig. 7, arrows). The medulla was formed by abundant filaments and by very rare, darkly stained, refractive stellate cells (Figs 8-9). Two types of medullary filaments were present (Fig. 9): a) primary medullary filaments, *i.e.* axial longitudinal filaments made by longitudinally elongate and narrow cells (2-3  $\mu\text{m}$  in diameter), and b) oblique filaments, produced secondarily as branches from primary axial filaments, made of 2-4 short and rounded cells, 4-5  $\mu\text{m}$  in diameter (Fig. 9, arrows),





Figs 3-9. Vegetative anatomy of *Felicinia marginata* gen. et comb. nov. **3.** Longitudinal section of the margin of the blade, showing the typical furrow. **4.** Longitudinal section of the marginal part of the blade showing the multi-axial organization with a pseudo-parenchymatous cortex and a filamentous medulla. **5.** Superficial view with cortical cells either single or in pairs. **6.** Section of the cortex showing pseudo-dichotomous cortical filaments and secondary pit connections between inner cortical cells (arrows). **7.** Inside-out view of the cortex and part of the medulla, showing inner cortical stellate cells with short arms (arrows) and medullary stellate cells with longer arms (arrow head). **8.** Darkly stained refractive medullary stellate cell. **9.** Longitudinally and obliquely oriented filaments in the medulla (arrows). Scale bars: Fig. 3 = 200  $\mu\text{m}$ , Figs 4-9 = 20  $\mu\text{m}$ .

connecting the longitudinal filaments. Primary medullary filaments also branched to produce either cortical filaments or secondary pit-connections with adjacent axial filaments. Medullary stellate cells might be produced as a consequence of the connections of longitudinal filament cells by secondary pit connections. They had 3-4 arms, up to 60  $\mu\text{m}$  long (Figs 7, arrowhead, 8), and could also be connected to the oblique secondary filaments (Fig. 8).

Observed characters were shared and stable among all observed samples from different localities (Table 1).

### ***Anatomical observations of reproductive features***

The carpogonial branch and the auxiliary cell branch were in distinct *ampullae* borne by a cortical supporting cell. The carpogonial branch was 3-celled (Figs 10-11). The carpogonium was falcate, almost enveloping the prominent hypogynous cell. The latter and the first cell of the carpogonial branch each bore a lateral branch. The auxiliary cell was the terminal cell of the 2-celled auxiliary branch (Figs 12-13). It was elongated, large, and prominent. Both the first cell and the auxiliary cell had a lateral branch. After the fertilization, a connecting filament reached the auxiliary ampulla and fused with the auxiliary cell, which cut off a gonimoblast initial (Fig. 14). In some occurrences, a connecting filament also leaved the diploidized auxiliary cell. The diploidized auxiliary cell did not fuse with adjacent cells. Gonimoblast grew outwardly forming a spherical mass of tightly clustered carposporangia (Fig. 15). The thin pericarp was formed by the proliferation of ampullary filaments, which produced elongate filaments growing towards the thallus surface. The participation of some adjacent cortical filaments was unclear. Pericarp was not ostiolated.

Cystocarps were partially sunken in the thallus, slightly protruding on thallus surface, about 220-280  $\mu\text{m}$  wide and 180  $\mu\text{m}$  high (Fig. 15). Cystocarps were spread all over the blade on both faces. After the release of carpospores, the blade was perforated by many holes. The carpospores were elliptical-round, 7-10  $\mu\text{m}$  wide.

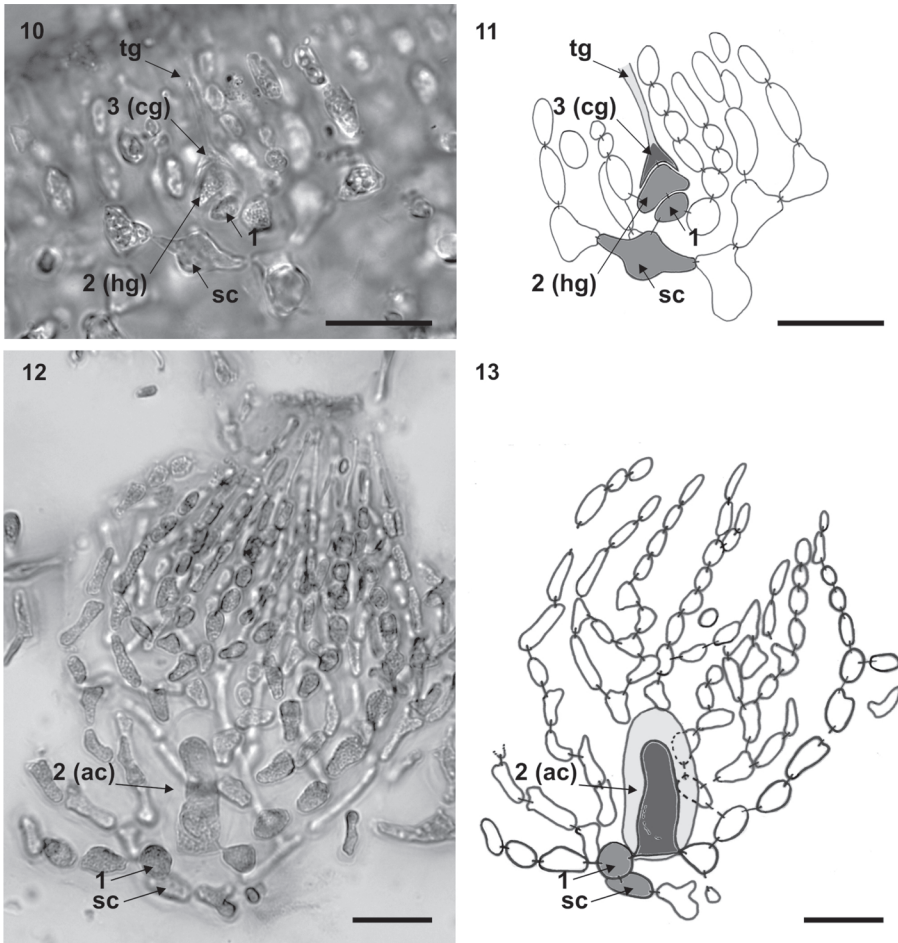
Characters were shared and stable among all observed samples from different localities (Table 1). Tetrasporophyte and male gametophyte were not observed.

### ***Molecular analyses***

The DNA barcode region generated for *A. marginata* is available in BOLD and Genbank (BOLD process ID: FELI003-14; GB #: KJ594945).

Trees inferred by different analytical strategies (e.g. moving taxa in and out from the alignments, extending and reducing outgroup taxa, either partitioning analysis parameters by codon positions and genes or not) gave similar results. We presented trees inferred from alignments restricted to Halymeniales, choosing sequences of *Tsengia* as outgroup.

Phylogenetic analyses inferred from both *rbcL* and LSU genes (Figs 16-17) resolved three strongly supported supergeneric lineages within the Halymeniales: a) one regrouped *A. nitidissima* and species of *Pachymenia*, b) a second comprised *Polyopes* J. Agardh species and *Glaphyrosiphon intestinalis* (Harvey) Leister & W.A. Nelson, c) a third included *Grateloupia* C. Agardh, *Yonagunia* Kawaguchi & Masuda, *Pachymeniopsis* Y. Yamada ex S. Kawabata, *Prionitis* J. Agardh, *Phyllymenia* J. Agardh and *Dermocorynus* P.L. Crouan &

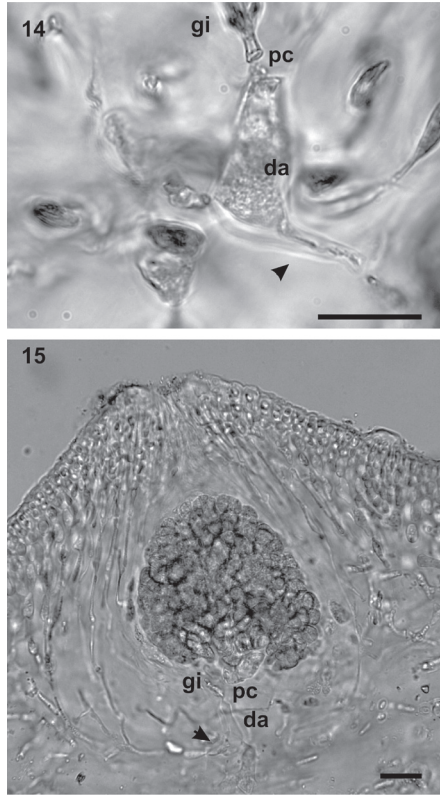


Figs 10-13. Reproductive features of *Felicinia marginata* gen. et comb. nov. **10-11.** Carpogonial ampulla with 3-celled carpogonial branch. **12-13.** Auxiliary cell ampulla with 2-celled auxiliary cell branch. Abbreviations: ac: auxiliary cell; cg: carpogonium; hg: hypogynous cell; sc: supporting cell; tg: trichogyne. Scale bars: 20  $\mu$ m.

H.M. Crouan species, all the above mentioned genera represented in the analyses by their respective generitypes. The relationships among the remaining halymeniacean taxa included in our analyses were poorly or not resolved, the genera *Cryptonemia* J. Agardh and *Thamnoclonium* Kützing were non monophyletic in *rbcL* analyses, and the genus *Halymenia* C. Agardh was non monophyletic in both *rbcL* and LSU trees.

*Aeodes marginata* failed to join the generitype *A. nitidissima* in all analyses (Figs 16-18), regardless of the method or gene considered. The monophyly of *A. marginata* either with *A. nitidissima* or with *Pachymenia* species were all significantly rejected by the SH tests.

The exact alliance of *A. marginata* was uncertain, varying according to the analyses.



Figs 14-15. Reproductive anatomy of *Felicinia marginata* gen. et comb. nov. **14.** Diploidized auxiliary cell with a lateral protuberance where the connecting filament fused (arrowhead) and a gonimoblast initial cut towards thallus surface. **15.** Mature cystocarp with pericarp; at the base diploidized auxiliary cell with a lateral protuberance where the connecting filament fused (arrowhead) and a gonimoblast initial cut towards thallus surface. Abbreviations: da: diploidized auxiliary cell; gi: gonimoblast initial; pc: pit connection. Scale bars: 20  $\mu$ m.

In *rbcL* analyses, (Fig. 16) *A. marginata* was included in a lineage encompassing *A. vidovichii* (Meneghini) Zanardini and species of the genus *Corynomorpha* J. Agardh without support (0.53 posterior probability [PP] in Bayesian analysis, no bootstrap support [BS] in ML analyses).

This assemblage grouped with various species of *Halymenia*, *Cryptonemia*, *Carpopeltis* F. Schmitz, *Codiophyllum* J.E.Gray, *Spongophloea* Huisman, De Clerck, Prud'homme van Reine & Borowitzka, and *Thamnoclonium*, all including respective generitypes, and *Epiphloea bullosa* (Harvey) De Toni, and *Gelinaria ulvoidea* Sonder, the latter's type species of the respective genera. This alliance gained variable support (0.78 PP; <50% BS).

In LSU trees (Fig. 17), *A. marginata* grouped (1 PP; 69% BS in PhyML; 74% BS in RAxML) with various species of *Halymenia*, *Cryptonemia*, both including the respective generitypes, *E. bullosa*, and *G. ulvoidea*. It should be noted that the genera *Carpopeltis*, *Codiophyllum*, *Spongophloea* and *Thamnoclonium*, were not included in LSU analyses.

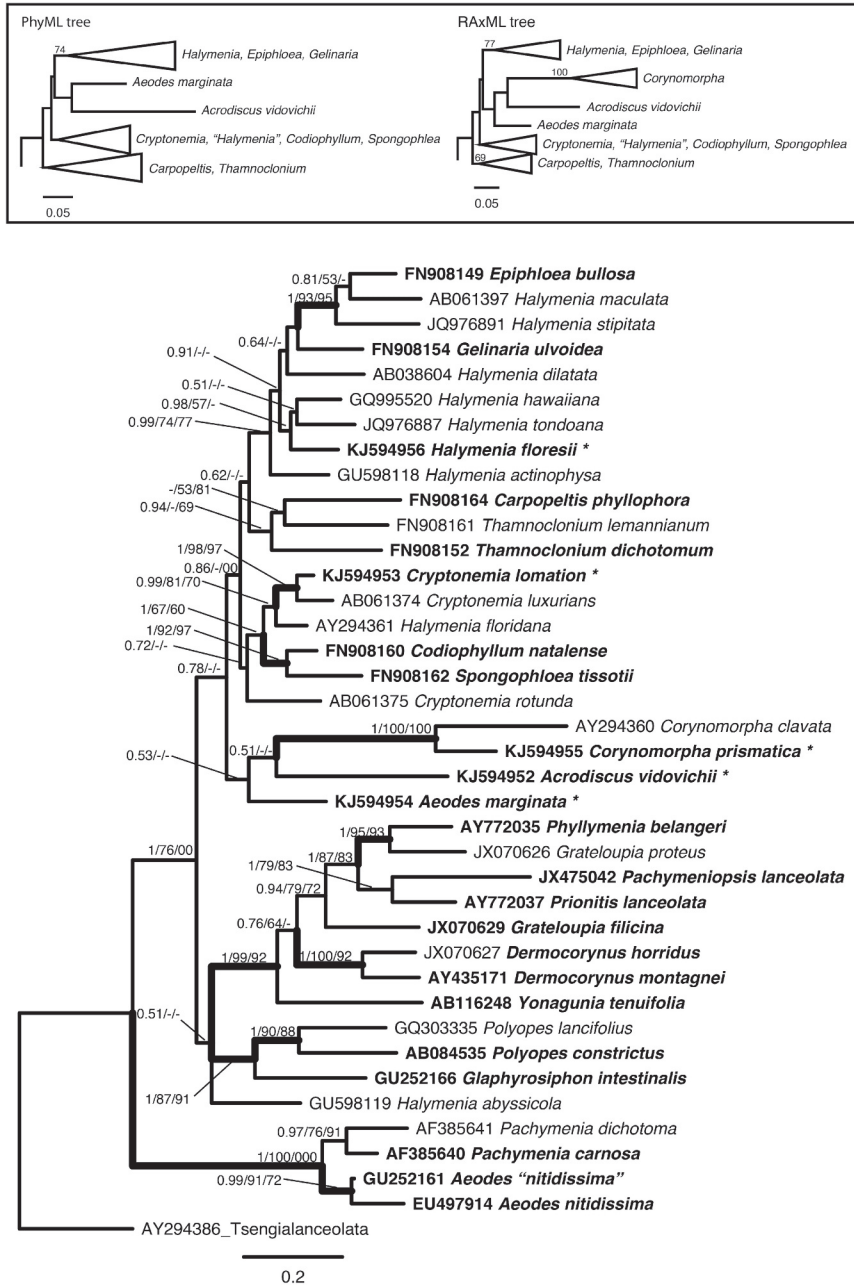


Fig. 16. Bayesian phylogram inferred from the *rbcL* data. Supports at nodes indicate Bayesian posterior probabilities and bootstrap values inferred from ML analyses (PhyML/RAxML). Sequences generated in the present study are indicated with an asterisk, generic type species are indicated in bold, supported nodes (>0.85/85/85) within ingroup are indicated by thick internal branch. Alternative topologies recorded in PhyML and RAxML trees are depicted in the top box.

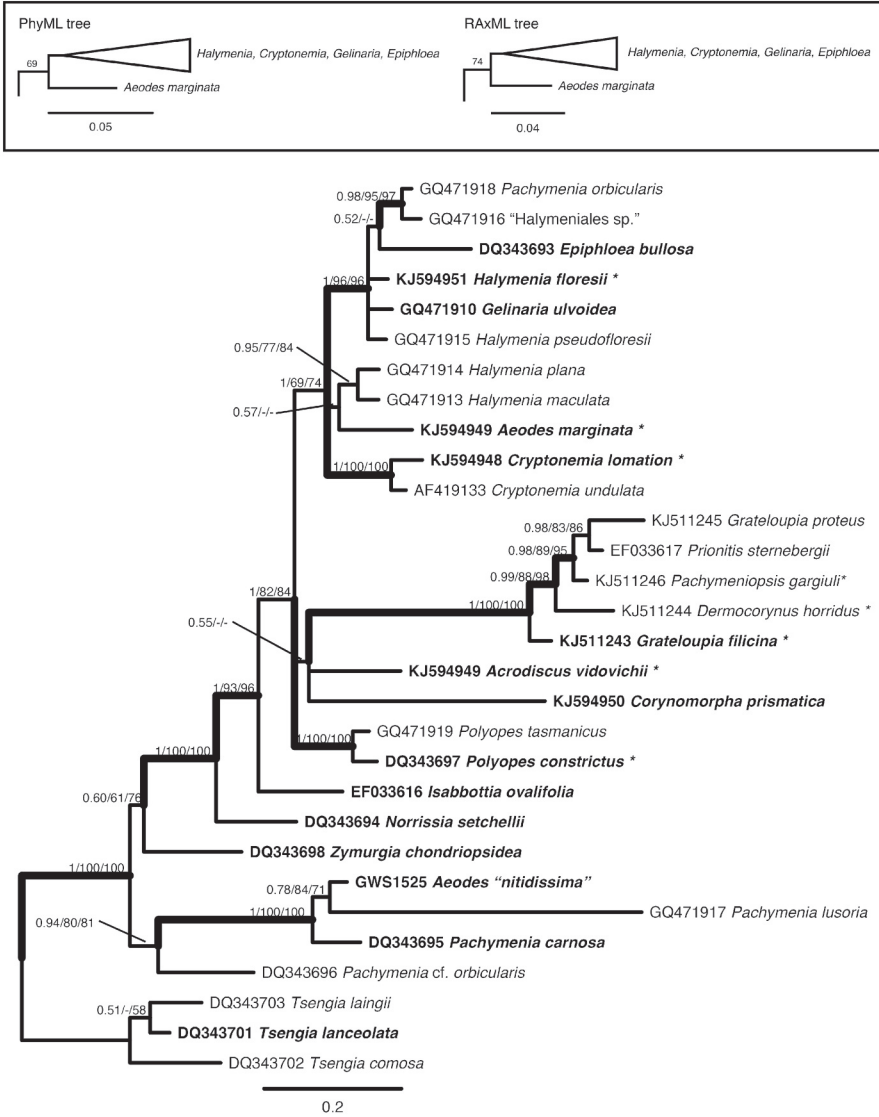


Fig. 17. Bayesian phylogram inferred from the LSU data. Supports at nodes indicate Bayesian posterior probabilities and bootstrap values inferred from ML analyses (PhyML/RAxML). Sequences generated in the present study are indicated with an asterisk, generitype species are indicated in bold, supported nodes (>0.85/85/85) within ingroup are indicated by thick internal branch. Alternative topologies recorded in PhyML and RAxML trees are depicted in the top box.

In concatenate LSU-*rbcl* analyses (Fig. 18), *A. marginata* was included in a lineage with *H. floresii* (Clemente) C. Agardh, *E. bullosa*, *G. ulvoidea*, *C. lomation* (Bertoloni) J. Agardh (0.97 PP; 60% BS in PhyML) with the inclusion of *A. vidovichii* only in the RAxML tree (<50% BS).

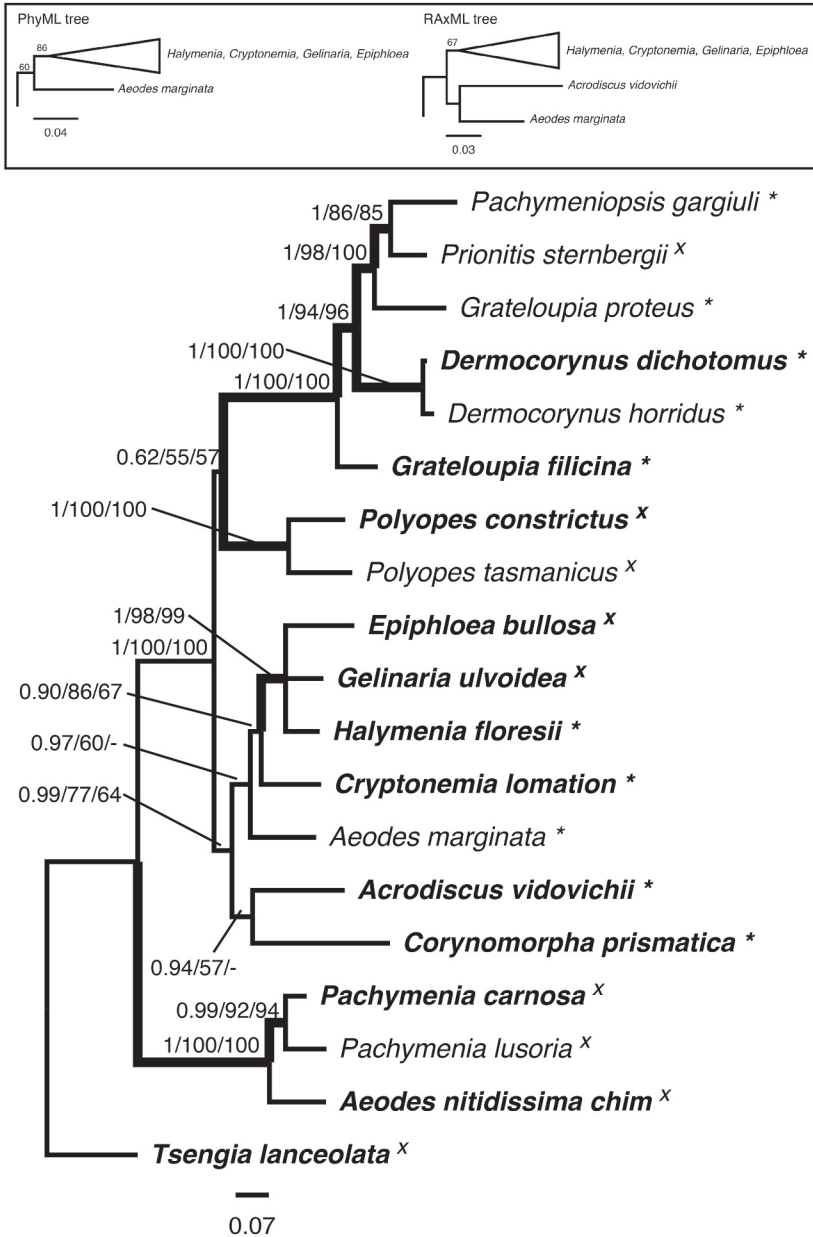


Fig. 18. Bayesian phylogram inferred from the combined LSU-*rbcL* data. Supports at nodes indicate Bayesian posterior probabilities and bootstrap values inferred from ML analyses (PhyML/RAxML). Sequences generated in the present study are indicated with an asterisk, genotype species are indicated in bold, concatenate sequences obtained from different samples for LSU and *rbcL* are indicated with an X, supported nodes (>0.85/85/85) within ingroup are indicated by thick internal branch. Alternative topologies recorded in PhyML and RAxML trees are depicted in the top box.

## DISCUSSION

Phylogenetic analyses inferred from *rbcL* gene led Wang *et al.* (2001) to hypothesize a fusion of the genera *Aeodes* J. Agardh and *Pachymenia* J. Agardh, although the authors refrained from formally lumping the two taxa because the sequences from the respective generitypes were missing. Hommersand & Fredericq (2003) also shared this view. Conversely, Russell *et al.* (2009), in light of a phylogeny inferred from Halymeniaceae from New Zealand, proposed to transfer South African species of *Aeodes* to the genus *Pachymenia* and considered *Aeodes* as a monotypic genus based on the generitype species, *A. nitidissima* J. Agardh, ignoring the presence of *A. marginata* (Roussel) F. Schmitz. All other species of the genus *Aeodes* had already been transferred to other genera (Table 2), *A. marginata* being the only species awaiting for a reassessment.

In the present study, some genera within the Halymeniaceae were not resolved as monophyletic lineages in all analyses, although with differences in tree topologies, which may depend both on taxa sampling and gene phylogenetic signal (Figs 16-18).

The genus *Pachymenia* was monophyletic only in trees inferred from *rbcL* data where the two species included (*P. carnosa* and *P. dichotoma*) were resolved as sister taxa. In trees inferred from LSU data, the four *Pachymenia* species included in the analysis were resolved in distinct lineages.

In our phylogenetic analyses, *A. marginata* never clustered with *A. nitidissima* regardless of the method or gene considered.

None of the analyses conclusively resolved the position of *A. marginata*, whose alliance changed depending on the reconstruction method. Nevertheless, this species was never closely related to either *Aeodes nitidissima* or *Pachymenia* (alliances significantly rejected using SH tests), however it rather joined a large assemblage including various species of *Halymenia* C. Agardh, *Cryptonemia* J. Agardh, *Carpopeltis* F. Schmitz, *Codiophyllum* J.E. Gray, *Spongophloea* Huisman, De Clerck, Prud'homme van Reine & Borowitzka, *Thamnoclonium* Kützing, *Epiphloea* J. Agardh, and *Gelinaria* Sonder.

Table 2. Species previously assigned to the genus *Aeodes*

<i>Species</i>	<i>Current name</i>	<i>References</i>
<i>A. ecuadoreana</i> W.R. Taylor	<i>Schizymenia ecuadoreana</i> (W.R. Taylor) Abbott	Taylor 1945, Abbott 1967b
<i>A. gardneri</i> Kylin	<i>Halymenia gardneri</i> (Kylin) Parkison	Kylin 1925, Parkinson 1980
<i>A. lanceolata</i> Okamura	<i>Pachymeniopsis laceolata</i> (Okamura) Kawabata = <i>Grateloupia lanceolata</i> (Okamura) S. Kawaguchi	Okamura 1934, Kawabata 1954, Kawaguchi 1997
<i>A. orbitosa</i> (Suhr) F. Schmitz = <i>Iridaea orbitosa</i> Suhr	<i>Pachymenia orbitosa</i> (Suhr) L.K. Russell	Suhr 1840, Schmitz 1894, Russell <i>et al.</i> 2009
<i>A. ulvoidea</i> F. Schmitz	<i>Pachymenia ulvoidea</i> (F. Schmitz) L.K. Russell	Schmitz 1894, Russell <i>et al.</i> 2009



It is noteworthy to mention that published *rbcL* sequences revealed an overlooked diversity within *A. nitidissima*, even from the type area, with two cryptic species (sp.1: EU497914, from type locality; sp.2: GU252161, AY583300) all collected in New Zealand, along the north-eastern coasts of North Island. A detailed work is needed to clarify the taxonomy of this species complex, an objective which is beyond the scope of the present paper, *A. marginata* being manifestly only remotely related with them.

The generic delineation of *Halymenia*, *Thamnoclonium* and *Cryptonemia* have to be refined, similarly to the process just initiated for the complex including *Grateloupia* C. Agardh, *Pachymeniopsis* Y. Yamada ex S. Kawabata, *Yonagunia* Kawaguchi & Masuda and related genera (Gargiulo *et al.*, 2013).

We analyzed different genes and different analytical strategies (e.g. moving taxa in and out from the alignments, extending and reducing outgroup taxa, either partitioning analysis parameters by codon position and genes or not, data not shown) in order to improve the resolution of Halymeniacean genera with presently available DNA data. However, all phylogenies failed to satisfactorily resolve relationships among genera. This lack of resolution might be due to the fact that most genera are underrepresented in DNA analyses, as most species (i.e. in the huge genus *Halymenia*) are not sequenced. An extensive reassessment of the boundaries of all genera within Halymeniaceae is strongly needed but is beyond the scope of this study and requires a comprehensive taxon sampling as well as a thorough revision of morpho-anatomical data as well as reproductive features in both type and freshly collected specimens. A comprehensive and fully resolved phylogeny of Halymeniaceae is far to come: our approach was to proceed step by step, trying and characterize particular genera especially within the Mediterranean Sea.

Therefore, the present study focused only on the revision of the taxonomic status of *A. marginata*.

Records of *Aeodes* and *Pachymenia* are restricted to the southern hemisphere, from southern Africa to Australia and New Zealand (Hommersand & Fredericq, 2003; Hommersand *et al.*, 2009), with the only exception of *A. marginata*, which is endemic to the Mediterranean Sea (Manghisi *et al.*, 2010a). *Aeodes marginata* was included in the genus *Aeodes* by Schmitz (1894), who nonetheless noted that the anatomic and reproductive characters of this taxon were close to some species of *Grateloupia* and *Halymenia*, while it differed in the habit, which resulted closer to *Iridaea*. He decided to accommodate the species into *Aeodes*, because J. Agardh (1876) had defined the latter genus as having “*Frons habitu et substantia Iridæae, structura et fructu fere Grateloupiæ*”.

According to Chiang (1970), “a feature peculiar to *Cryptonemia*, *Thamnoclonium*, *Codiophyllum* and *Halymenia* is the presence, among the ordinary filaments, of usually enlarged and branched filaments with a refractive material. This kind of medulla, to our knowledge, has not been reported in any other group of red algae. These filaments stain deeply with aniline blue (...)”. Interestingly, all the generitypes of the above mentioned halymeniacean genera were resolved in the same lineage which was always related to *A. marginata* (Figs 16-18). It is noteworthy to mention that *Aeodes marginata* presents in its medulla refractive stellate cells that stains darkly with aniline blue (Fig. 8). In light of our phylogenies, these refractive stellate cells could be interpreted as homologous to the refractive material described by Chiang.

The association shown in *rbcL* trees of *A. marginata* with *A. vidovichii* or *Corynomorpha* spp., which is not confirmed by neither LSU nor concatenate

analyses, might be just due to an artefact of the analyses such as long branch attraction.

Various authors (Balakrishnan, 1961; Abbott, 1967a; Chiang, 1970; Codomier, 1974; Kraft, 1977; Maggs & Guiry, 1982; Scott *et al.*, 1982; Gargiulo *et al.*, 1986; Guimaraes & Fujii, 1998; De Smedt *et al.*, 2001) pointed out how the main diagnostic character of the genus *Halymenia* is the presence of anticlinal filaments crossing the medulla from cortex to cortex. Others character, such as the slippery texture and the lateral stellate cells, are shared with other genera (also belonging to other families). Most authors (Abbott, 1967a; Chapman & Parkinson, 1974; Guiry, 1974; Irvine & Farnham, 1983; Desikachary *et al.*, 1990; Guimaraes & Fujii, 1998) emphasized the importance of the secondary thickening of the stipe, the presence of a midrib, and the medulla of predominantly periclinally directed filaments as main diagnostic characters of *Cryptonemia*. *Aeodes marginata* lacks the main diagnostic characters of both *Halymenia* and *Cryptonemia*.

*A. marginata* differs from *H. floresii* (Clemente) C. Agardh, the type species of *Halymenia*, because the latter has a thin cortex of rounded to stellate cells and a lax medulla of anticlinally oriented filaments, while the former has a thicker cortex with cells in anticlinal rows, ovoid to stellate inner cortical cells and a medulla formed by very rare stellate cells and abundant filaments of two types: axial longitudinal filaments made by elongate and narrow cells, and oblique secondary filaments made of small rounded cells connecting the longitudinal ones (Figs 4-9).

*Aeodes marginata* also differs from *C. lomation* (Bertoloni) J. Agardh, because the latter has a thin (2-3 layers) and compact cortex of rounded cells and a compact filamentous medulla of longitudinally elongated cells. The anatomical structure of *Codiophyllum*, *Thamnoclonium* and *Carpopeltis* is similar to that of a *Cryptonemia*.

According to the present observations, a characteristic unique to *A. marginata* is the presence in the medulla of peculiar oblique multicellular filaments connecting periclinal filaments. They are produced secondarily as branches from axial filaments, made of 2-4 short and rounded cells, 4-5 µm in diameter (Fig. 9, arrows). Possibly those filaments are homologous to the anticlinal filament in the medulla of *Halymenia*.

At the best of our knowledge, this is the first paper describing the anatomical construct of a Halymeniacean species at this level of detail. In order to achieve a comprehensive taxonomical revision of the genera included in the family, a similar description would be necessary for other taxa.

Referring to Chiang's (1970) ampullary types, Codomier (1972) did not find any difference between the auxiliary ampullae of *A. marginata* and the *Halymenia*-type, pointing that it should not be attributed to the *Aeodes* type. Furthermore, he affirmed that the germination of the spores of *A. marginata* was of the *Halymenia* type. However, the auxiliary ampulla of *A. marginata* drawn by Codomier (1972, Fig 97A) seems to be not fully developed. The auxiliary ampullae that we observed were more developed, being bushier, with various orders of branching (4-5 or rarely more), with a more or less conical shape. Using Chiang's ampullary types, we could say that *A. marginata* has a *Cryptonemia* type auxiliary ampulla.

Anyway, the auxiliary-cell ampullae of *Cryptonemia* and *Halymenia* have not been critically investigated from a developmental standpoint, and their complete structure and the exact position of the auxiliary cell in the ampulla remains to be determined (Hommersand *et al.* 2010). It is noteworthy that the auxiliary cell in *A. marginata* is the terminal cell of the auxiliary branch, as already

reported in *Isabbottia ovalifolia* (Kylin) Balakrishnan (Balakrishnan 1980), *Zymurgia chondriopsidea* (J. Agardh) J.A. Lewis & G.T. Kraft, "*G. filicina*" from Australia (presumably *G. subpectinata* Holmes) (Lewis & Kraft, 1992), and other Mediterranean species of "*Grateloupia*" *sensu lato* (Gargiulo *et al.*, 2013).

Considering the carpogonial ampulla branching order, as suggested by Kawaguchi *et al.* (2004), *A. marginata* has a quite simple carpogonial ampulla with a second order branching, which differs substantially from what has been reported for *Cryptonemia*. The auxiliary cell, after diploidization, does not fuse with adjacent cells.

Tetrasporangia were not observed in the present work, but are reported in literature (Agardh 1851; Zanardini 1866; Hauck 1855; Preda 1908).

Vegetative and reproductive observations, as well as molecular phylogenies inferred in the present study, provide compelling support to hypothesize the affinity of *A. marginata* with *Halymenia*, *Cryptonemia*, and associated genera rather than with *A. nitidissima*. Nevertheless, *A. marginata* presents its own unique set of characters suggesting that it deserves the status of genus within the Halymeniaceae. Hereafter, we propose the new genus *Felicinia* to accommodate this species.

### ***Felicinia* Manghisi, Le Gall, Ribera, Gargiulo & M. Morabito, gen. nov. Figs 1-15**

*Description*: Thallus decumbent, flat, rounded to elliptical and lobed, fixed to the substrate by a small discoid holdfast, with a very short stipe. The blade deeply incised in the adult stage with some perforations. The edge, undulate and lightly enlarged, is deeply notched by a furrow. Edge of the blade and perforations are irregular, toothed, finely serrated, and 'double'. Multiaxial structure. Pseudo-dichotomous cortical filaments. Secondary pit connections in the inner cortex. Rare refractive stellate cells in the medulla. Medulla with long slender periclinal primary filaments connected by short stout oblique secondary filaments, made of 2-4 short and rounded cells. 3-celled carpogonial branch in an ampulla. Auxiliary cell terminal of a 2-celled branch in a distinct ampulla. Gonimoblasts arising outwardly from the diploidized auxiliary cell and forming a spherical mass of tightly clustered carposporangia. Protruding cystocarps scattered all over the blade on both faces of the thallus. Pericarp not ostiolate and formed from elongated ampullary filaments. Male gametangia unknown. Tetrasporophytes isomorphic with tetrasporangia oblong, cruciately divided, immersed in outer cortex, scattered.

*Etymology*: named after Prof. Gianni P. Felicini, prominent Italian phycologist and mentor of the first author.

*Type species*: *Felicinia marginata* (Roussel) Manghisi, Le Gall, Ribera, Gargiulo & M. Morabito *comb. nov.*

### ***Felicinia marginata* (Roussel) Manghisi, Le Gall, Ribera, Gargiulo & M. Morabito *comb. nov.***

Basionym: *Halymenia marginata* Roussel in Montagne 1838, *Annales des Sciences Naturelles, Botanique*, série 2, tome 10: p. 273. Type Locality: Algiers, Algeria. Lectotype: PC0474570 (AR5446 in Herb. Roussel in PC, Fig. 1), designated here.

Homotypic synonyms: *Nemastoma marginatum* (Roussel) J. Agardh 1842: 91; *Iridaea marginata* (Roussel) Endlicher 1843: 38; *Schizymenia marginata* (Roussel) J. Agardh, (1851: 171); 171; *Platymenia marginata* (Roussel) Kuntze 1891: 910; *Aeodes marginata* (Roussel) F. Schmitz 1894: 630.

Heterotypic synonyms: *Schizymania minor sensu* Zanardini 1866: 423 *nom. illeg.*, not *Schizymania minor* (J. Agardh) J Agardh (1851: 172) (Schmitz 1894).

Iconography: Boudouresque *et al.* 1977, p 58, fig. 1; Codomier 1972, fig. 97; Preda 1908, p. 79, pl. XXXI; Rull Lluch *et al.* 1991, p 154, fig. 3 (as *Aeodes marginata*); Montagne 1846, pl. 10 fig. 3 (as *Iridaea marginata*); Ardissonne 1868(1874), pl. VII (as *Schizymania marginata*); Hauck 1885, p 119, fig 43; Zanardini 1866, pl. XIII (LXII) (as *Schizymania minor*).

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