





Systematic revision of the foliose Halymeniaceae (Halymeniales, Rhodophyta) from Europe, with the description of *Halymenia ballesterosii* sp. nov. from the Mediterranean Sea and *Nesoia hommersandii* from the Canary Islands

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Systematic revision of the foliose Halymeniaceae (Halymeniales, Rhodophyta) from Europe, with the description of *Halymenia ballesterosii* sp. nov. from the Mediterranean Sea and *Nesoiia hommersandii* from the Canary Islands

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ABSTRACT

In the past, large, foliose and unbranched *Halymenia*-like plants from Europe were often identified as '*Halymenia latifolia*', however recent taxonomic studies have shown that '*H. latifolia*' was a catch-all name for at least three cryptic species: *Neofolia rosea*, *Nesoiia mediterranea*, and the genuine *H. latifolia*, which has been transferred to the recently established genus *Nesoiia*. In this study, we further refine the taxonomy of European Halymeniaceae by examining new collections of foliose taxa from the Mediterranean Sea and Macaronesia. Analyses of *rbcL* gene sequences showed that unbranched foliose Halymeniaceae from Europe can be separated into five species, including three previously described species in *Neofolia* (*N. rosea*) and *Nesoiia* (*N. latifolia* and *N. mediterranea*) and two new species belonging to *Halymenia* (*H. ballesterosii* sp. nov.) and *Nesoiia* (*N. hommersandii* sp. nov.). *Halymenia ballesterosii* is the only known unbranched foliose species in the genus from Europe and can be separated from morphologically similar species in *Neofolia* and *Nesoiia* by protruding surface cells, two-celled carpogonial branches, a basal nutritive cell cluster in the carpogonial branch ampullae, and secondary connecting filaments issued from basal cells of ampullar filaments. *Nesoiia hommersandii* differs from *N. latifolia* by the lack of swollen arms in medullary stellate cells and cystocarps that slightly protrude above the thallus surface. We expect that the species diversity of foliose Halymeniaceae may increase when more foliose plants are collected and examined from different locations in Europe.


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Introduction

The Halymeniales (Rhodophyta) was one of the first orders segregated from the Gigartinales (Saunders & Kraft, 1996) following the merger of that order with the Cryptonemiales by Kraft & Robins (1985), characterized by its non-procarpic female reproductive structures, in which the carpogonial branches and auxiliary cells are protected by ampullar filaments (known as ampullae). Currently, the Halymeniales includes ~37 genera: one in the Tsengiaceae and 36 in the Halymeniaceae. Among them, the genera *Grateloupia* C.Agardh and *Halymenia* C.Agardh are by far the most species-rich, including, respectively, 97 and 80 described species (Guiry & Guiry, 2019). Previous studies have highlighted the polyphyletic nature of *Halymenia* (Hernández-Kantun *et al.*, 2009; D'Archino *et al.*, 2014; Lee & Kim, 2019). Recently, our research group undertook a series of systematic studies on European Halymeniales, resulting in the description of a new genus, *Neofolia* S.-M.

Lin, Rodríguez-Prieto, De Clerck & Huisman, and recognition of several cryptic species (Rodríguez-Prieto *et al.*, 2018, 2019) by characterizing the pre- and post-fertilization development of female reproductive structures in the generitype of *Halymenia*, *H. floresii* (Clemente) C.Agardh. A year later, Lee & Kim (2019) erected a new genus, *Nesoiia* H.W.Lee & M.S.Kim, based on a foliose species, *N. delicatula* H.W.Lee & M.S.Kim from Jeju Island, South Korea, and transferred *Halymenia latifolia* to *Nesoiia*, as *N. latifolia* (P.Crouan & H.Crouan in Lloyd ex Kützing) H.W.Lee & M.S.Kim. In the same year, we characterized the *Halymenia*-like species, *Nesoiia latifolia*, and described a new species, *N. mediterranea* Rodríguez-Prieto, S.-M.Lin, De Clerck & Huisman, a species morphologically similar to *N. latifolia*. Our studies detailing the ontogeny of female reproductive structures and post-fertilization development in the Halymeniaceae provide some useful clues for resolving the polyphyletic genus *Halymenia*.

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In this study, we continue our systematic studies on European Halymeniaceae based on our recent new collections of specimens, which were difficult to assign to any named species based on vegetative structures alone. Therefore, we focused on these *Nesoia latifolia*-like specimens, examined their vegetative and reproductive structures and sequenced related species from the North-eastern Atlantic Ocean, Macaronesia and the Mediterranean Sea in order to infer their phylogenetic relationships. These investigations led to the recognition of two new species, one in each of the genera *Halymenia* and *Nesoia*.

Materials and methods

Collections were made by SCUBA or trawling on circalittoral detritic bottoms. Algal samples were preserved in 5% formalin-seawater for the morphological study or pressed as herbarium vouchers. Materials used in the molecular studies were desiccated in silica gel or preserved in 95% ethanol. Specimens were sectioned by hand or with a freezing microtome and were stained with 1% aniline blue acidified with 1% HCl or were treated with Wittmann's aceto-iron-haematoxylin-chloral hydrate (Wittmann, 1965) modified by Rodríguez-Prieto & Hommersand (2009), and mounted in 50% Hoyer's mounting medium. Habit images were taken with a Canon EOS 350D (Canon, Tokyo, Japan) and photomicrographs were made with an AxioCam MRc attached to an Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany). Voucher specimens were deposited in the Herbarium of the University of Girona, Spain (HGI) and in the herbarium of the University of La Laguna (TFC). Herbarium abbreviations follow Thiers (2019).

DNA samples were prepared using a DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. DNA sequencing procedures of the *rbcL* gene are as described in De Clerck *et al.* (2005a, 2005b) and Lin *et al.* (2008). Newly generated sequence data were combined with a selection of sequences available from GenBank and aligned with Sequencher (Gene Codes Corp., Ann Arbor, Michigan, USA). The taxon sampling aimed to present the diversity of Halymeniales at the genus-level as comprehensively as possible, while expanding sequence data on *Halymenia*, *Neofolia* and *Nesoia* (see Supplementary table 1). A Maximum likelihood (ML) species tree was generated using MEGA6 under the Tamura 3-parameter model (Tamura *et al.*, 2013). The robustness of the resulting phylogeny was tested using 500 replicates of a rapid bootstrap heuristic (Tamura *et al.*, 2013). In addition, a Bayesian tree was estimated using MrBayes 3.2. (Ronquist *et al.*, 2012), applying a GTR model. Two runs consisting of 4 chains each were run for 5 million generations. Stationarity and convergence of the runs were assessed visually using

Tracer v.1.6 (Rambaut *et al.*, 2014) and a majority rule consensus tree was calculated after removal of a burn-in fraction of 10%.

Results

Molecular analyses

A total of 57 *rbcL* sequences from representative taxa belonging to the genera *Halymenia*, *Nesoia* and *Neofolia* along with related genera in the family Halymeniaceae were selected for analysis, with *Sebdenia monnardiana* (Montagne) Berthold and *Tsengia lanceolata* (J. Agardh) Saunders & Kraft serving as outgroup (Fig. 1). The alignment included 1257 sites, omitting 106 and 103 base pairs from the 5' and 3' end of *rbcL* (1467 bp) gene due to missing information. The ML, Maximum parsimony and Bayesian trees were overall congruent, differing only in the relative placement of clades that received only low support. Foliose Halymeniacean taxa from Europe were clustered into three natural lineages: *Halymenia*, *Neofolia* and *Nesoia*. Our proposed new foliose species, *Halymenia ballesterosii* sp. nov. was resolved in a moderately well-supported clade containing the generitype of *Halymenia* (*H. floresii*) and most named *Halymenia* species worldwide, along with *Epiphloea bullosa* (Harvey) De Toni and *Gelinaria ulvoidea* Sonder, two monospecific genera endemic to Australia. The proposed species *Nesoia hommersandii* sp. nov. was resolved in a well-supported clade containing the generitype of *Nesoia* (*N. delicatula*) and all other currently known species of *Nesoia*. Our analyses showed that the foliose *Halymenia latifolia* from Europe contained five species belonging to three genera: *Halymenia* (*H. ballesterosii* sp. nov. herein), *Neofolia* (*N. rosea*) and *Nesoia* (*N. latifolia*, *N. mediterranea*, *N. hommersandii* sp. nov. herein).

Morphological observations

***Halymenia ballesterosii* Rodríguez-Prieto, S.-M. Lin, De Clerck & Huisman sp. nov.** (Figs 2–23)

DIAGNOSIS: Thalli erect, foliose, simple or rarely bifurcate, 3–13 cm long by 1.4–4.0 cm wide, rose-pink in colour, soft and gelatinous in texture; blades 90–345 µm thick in upper parts; cortex made up of 2–4 cell layers, outer cortical cells ovoid and protruding above thallus surface, 6.5–11.6 µm long in longitudinal view; medulla mostly hollow, composed of an open network of stellate cells with long arms and sparse filaments, anticlinal to thallus surface, up to 83–340 µm long and 5–17 µm in diameter. Gametophytes and tetrasporophytes isomorphic. Tetrasporangia cruciately divided, 14.8–21.0 µm long by 11.5–19.0

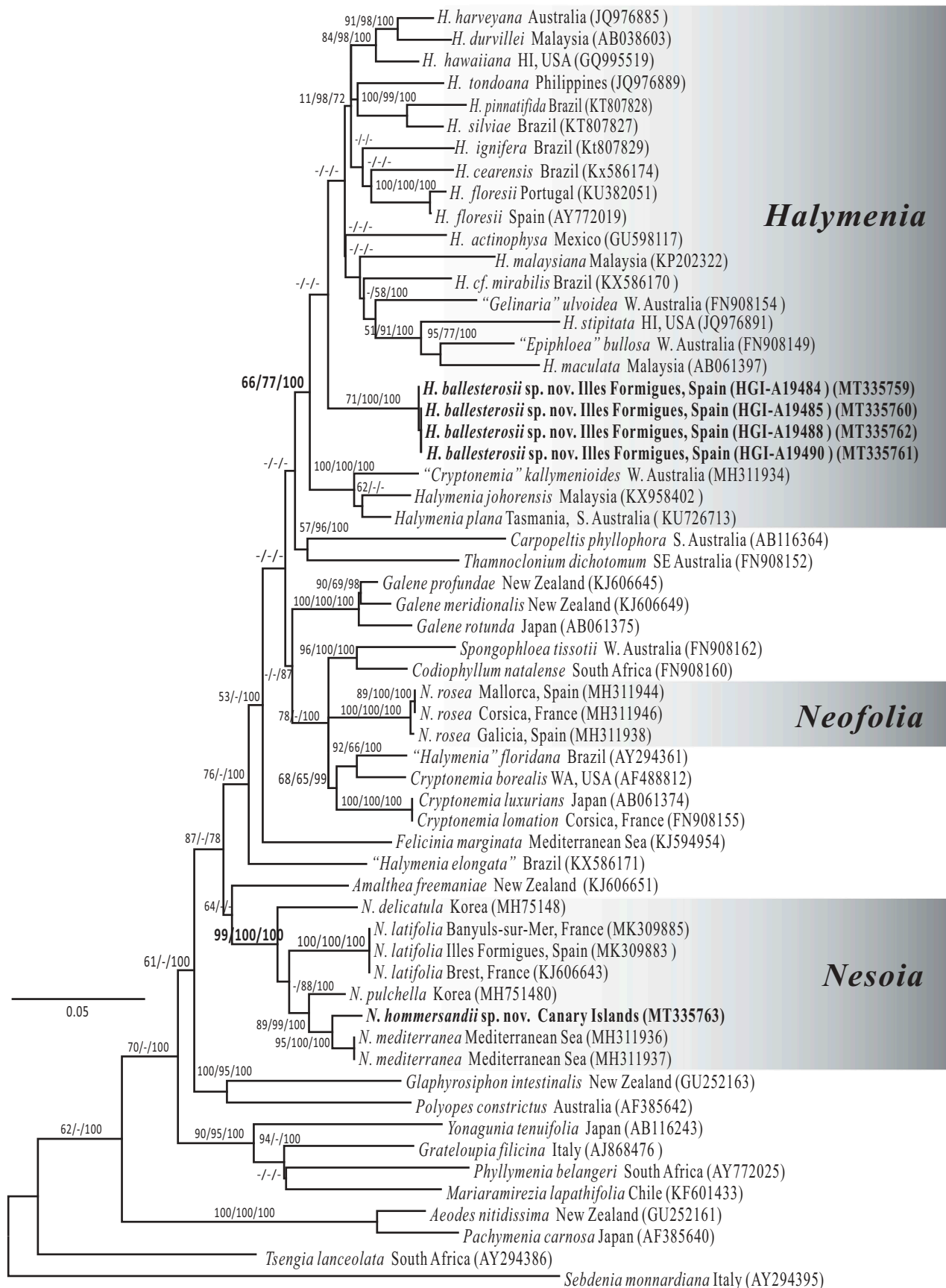
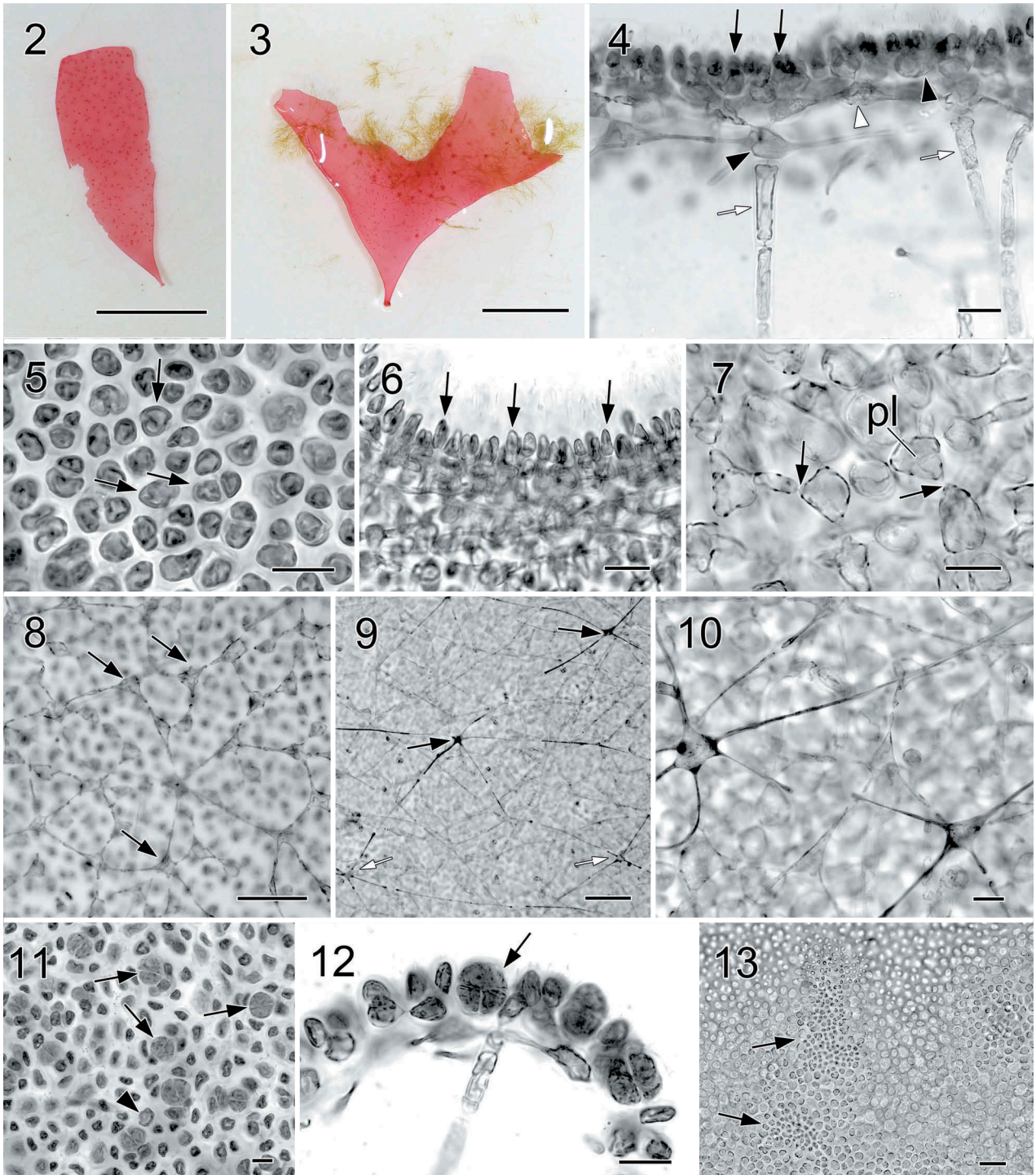


Fig. 1. Maximum likelihood phylogeny of the Halymeniales based on the *rbcL* gene using the Tamura 3-parameter + GAMMA model in MEGA6 ($-\ln = 11385.4511$). Support values resulting from the Maximum likelihood, Maximum parsimony and Bayesian analyses are indicated as branch labels (ML/MP/BI). Scale indicates substitutions per site.



Figs 2–13. *Halymenia ballesterosii* Rodríguez-Prieto, S.-M. Lin, De Clerck & Huisman sp. nov. Thallus morphology, vegetative structure, sporophyte and male reproductive structure. **Fig. 2.** Holotype, a monoecious specimen with cystocarps scattered over fertile thallus surface (HGI-A 19484). **Fig. 3.** Isotype, a female gametophyte consisting of a bifurcating and apically eroded blade (HGI-A 19490). **Fig. 4.** Cross section of a young blade showing ovoid surface cells (black arrows), ovoid subcortical pit-connected cells (black arrowhead at right), third layer of stellate cells with short arms (white arrowhead), inner cortical cell with long arms (black arrowhead at left) and anticlinally arranged medullary filament (white arrows) (HGI-A 19490). **Fig. 5.** Surface view of a blade showing rounded to irregularly polygonal cortical cells with reticulate plastid (arrows) (HGI-A 19484). **Fig. 6.** Close up of a cross section of the blade showing ovoid and protruding surface cells (arrows) (HGI-A 19499). **Fig. 7.** Subsurface view of thallus blade showing subcortical ovoid to slightly stellate cells with very short arms. Note that cells are secondarily pit connected (arrows) and bear a reticulate plastid (pl) (HGI-A 19490). **Fig. 8.** Subsurface view of thallus blade showing the inner subcortical cells layer, composed of stellate cells with quite long arms (arrows) (HGI-A 11077). **Fig. 9.** Subsurface view of thallus blade showing the network of medullary stellate cells with long arms (arrows). Note that the network is composed both by darkly staining (black arrows) and non-darkly staining medullary cells (white arrows) (HGI-A 6552). **Fig. 10.** Detail of the medulla and medullary stellate cells of Fig. 9 (HGI-A 6552). **Fig. 11.** Subsurface view showing a tetrasporangial initial (arrowhead) and many scattered tetrasporangial initials that are divided once (arrows) (HGI-A 19488). **Fig. 12.** Cross section through a tetrasporangial sorus showing cruciately divided tetrasporangium (arrow) (HGI-A 19498). **Fig. 13.** Surface view of spermatangial sori (arrows) (HGI-A 19477). Haematoxylin (Figs 4, 5); Aniline blue (Figs 6–12); Not stained (Fig. 13). Scale bars: Figs 2, 3 = 1 cm; Figs 4, 5, 7, 12, 13 = 20 μ m; Figs 6, 10, 11 = 10 μ m; Fig. 8 = 50 μ m; Fig. 9 = 100 μ m.

µm wide. Gametophytes monoecious. Spermatangial sori scattered over both thallus surfaces; spermatangia 11.2–17.7 µm long by 2.9–5.4 µm wide. Carpogonial branch ampullae composed of a two-celled carpogonial branch with a small network of nutritive cells; auxiliary cell ampullae abundant, composed of three orders of branched ampullar filaments; mature carposporophyte composed of two gonimolobes up to 60–195 µm in diameter; carposporangia 12.5–15.0 µm in diameter; mature cystocarps ostiolate, slightly swelling the thallus.

ETYMOLOGY: The species name honours Dr Enric Ballesteros, for his contribution to the knowledge of the biodiversity and ecology of marine macroalgae from the Mediterranean Sea.

TYPE COLLECTION: Holotype, monoecious gametophyte, collected from a detritic bottom off the Formigues Islands, Palamós, Spain, 08 October 2017 by C. Rodríguez-Prieto, at a depth of 37 m (Fig. 2), currently deposited in the algae section of the Herbarium of the University of Girona (HGI-A 19484). Isotypes in HGI-A # 19485 (female), 19488 (tetrasporic), 19490 (Fig. 3, female), 19498 (tetrasporic), and 19499 (male and female).

TYPE LOCALITY: Formigues Islands, Palamós, Spain (North-western Mediterranean).

DISTRIBUTION: This species is so far known only from the Western Mediterranean (Banyuls-sur-Mer, France and Catalonia and Balearic Islands, Spain).

HABITAT AND SEASONALITY: This is a rare species. The plant from Banyuls-sur-Mer was found in August at –12 m, those from Catalonia were found between August and October at depths of 30–37 m, and those from the Balearic Islands were found in May, September and October, at depths of 50–87 m. Plants grew on circalittoral detritic bottoms, often mixed with *Nesovia latifolia* and *N. mediterranea*.

SPECIMENS EXAMINED: Spain: (1) Palamós, Formigues Islands, –37 m, 08.x.2017, coll. C. Rodríguez-Prieto, HGI-A 19484 (male and female), HGI-A 19485 (female), HGI-A 19488 (tetrasporic), HGI-A 19490 (female), HGI-A 19498 (tetrasporic), HGI-A 19499 (male & female); Platja d'Aro, La Roja, –30 m, 17. ix.2017, coll. C. Rodríguez-Prieto, HGI-A 19477 (male and female). (2) Balearic Islands, Menorca Channel, –65 m, 07.ix.2011, coll. S. Joher, HGI-A 11077 (tetrasporic and female); Balearic Islands, Eivissa, –50 m, 16. ix.2004, coll. E. Ballesteros, HGI-A 6552 (tetrasporic, male and female). France: Banyuls-sur-Mer, –12 m, 10. viii.1997, coll. C. Rodríguez-Prieto, HGI-A 4246.

Habit and vegetative structure: Thalli erect, foliose, simple (Fig. 2) or seldom bifurcate (Fig. 3), usually minute, up to 3.0 cm long and 1.4 cm wide, occasionally up to 13 cm long and 4 cm wide when growing in nutrient-rich waters. Thalli arise from a small discoid holdfast and a well-defined stipe is absent (Figs 2, 3). Blades are rose-pink, with a thin, slightly darker margin, and are

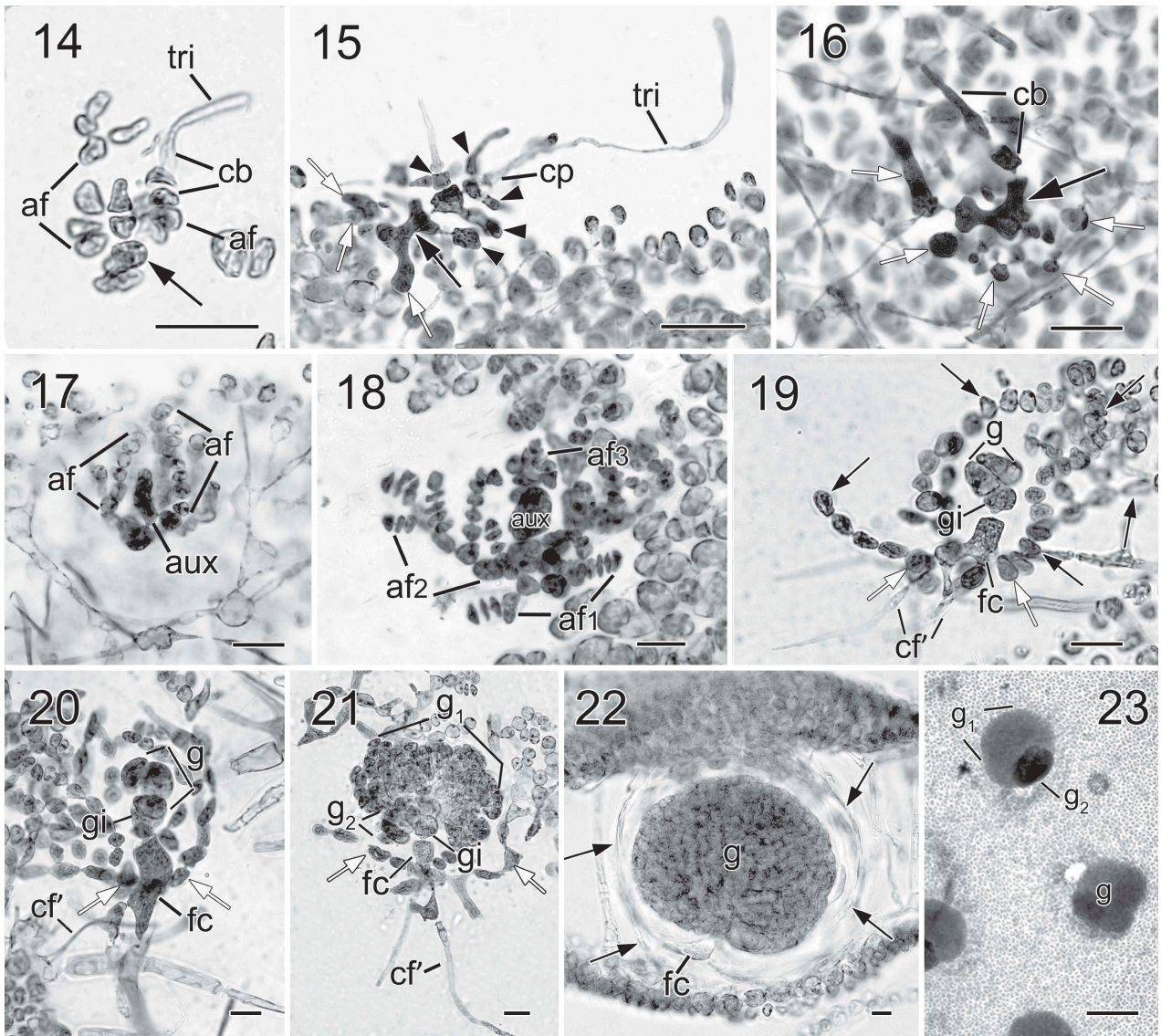
very soft and gelatinous in texture. Upper parts of blades are 90–345 µm thick, usually rounded, and with an entire margin, but upper parts of old blades are often broken. The cortex is composed of 2 cell layers in young parts and 4 layers in older parts (Fig. 4). Outer cortical cells are rounded to irregularly polygonal, not very compacted and up to 5.8–14.3 × 3.8–9.4 µm in surface view (Fig. 5), protruding above thallus surface and 6.5–11.6 µm broad in cross section (Fig. 6). Secondary pit-connections are absent between outer cortical cells (Fig. 5).

The subcortical cells in old thalli form a network comprised of three layers, one of rounded cells (Fig. 4), one of ovoid cells with short arms (Fig. 7), and a third layer of inner cortical stellate cells with relatively long arms (Fig. 8), all of them interconnected via secondary pit-connections. The medulla is either hollow or loosely filled with a network (Figs 9–10) of small stellate cells with long, thin and straight arms parallel to the thallus surface and situated just under the inner cortical cells, and elongated filaments (Fig. 4, white arrows), more-or-less anticlinal to the thallus surface, 83–340 µm long and 5–17 µm thick. Outermost cortical cells are more pigmented than inner cortical cells or medullary cells, but all of them have a reticulated plastid.

Reproductive structures. Tetrasporangia are initially produced from subcortical cells and scattered over the thallus surface (Fig. 11). Fully developed tetrasporangia are rounded or ovoid, cruciately divided, and 14.8–21.0 µm long by 11.5–19.0 µm wide (Fig. 12).

The species is monoecious. Spermatangial parent cells are initially produced from surface cells and formed in sori scattered over the fertile blade (Fig. 13). Mature spermatangia are 11.2–17.7 µm long by 2.9–5.4 µm wide. The fully developed carpogonial branch ampulla (Fig. 14) consists of at least one ampullar filament and a two-celled carpogonial branch, often bearing a long trichogyne with a distal, swollen end (Fig. 15). After fertilization, the basal cell of the carpogonial branch enlarges and fuses with neighbouring ampullar cells to form a darkly stained 'carpogonial' fusion cell (Figs 15–16 black arrows). Notably, a cluster of small cells, presumably functioning as nutritive cells, is produced at the basal part of the fertilized carpogonial branch ampulla (Fig. 16, white arrows). This cluster is similar to the one observed in the generitype, *H. floresii* (Rodríguez-Prieto *et al.*, 2018). Auxiliary cell ampullae are abundant, developing outwardly from inner stellate subcortical cells. The auxiliary cell is the basal cell of the third-order ampullar filament and enlarges when fully developed (Figs 17–18).

Very early diploidization stages were not found. After presumed diploidization, the diploidized auxiliary cell soon produces a gonimoblast initial (Fig. 19). In turn, several primary gonimoblast cells are cut off from the gonimoblast initial acropetally (Fig. 19). At



Figs 14–23. *Halymenia ballesterosii* Rodríguez-Prieto, S.-M. Lin, De Clerck & Huisman sp. nov. Morphology of carpogonial branch and auxiliary cell ampullae and development of cystocarp. **Fig. 14.** Close up of a carpogonial branch ampulla showing a 2-celled carpogonial branch with a short trichogyne and short branched ampullar filament (af) borne on a subcortical cell (arrow) (HGI-A 19485). **Fig. 15.** Early post-fertilization of carpogonial branch ampulla showing a 2-celled carpogonial branch with a fertilized carpogonium (cp) and a well-developed distally swelled trichogyne (tri). Note that the cells of carpogonial branch and the cells of the ampullar filaments cut off lateral branches (arrowheads) and the enlarged basal cell (black arrow) fused with neighbouring cells to form a fusion cell and became darkly stained. Few small derived cells (= nutritive cells, white arrows) were cut off from the basal part of the fusion cell (HGI-A 11077). **Fig. 16.** A later stage of Fig. 15, showing the carpogonial branch (cb) and the fusion cell (black arrow) cutting off more nutritive cells/filaments (white arrows) (HGI-A 11077). **Fig. 17.** A young auxiliary cell ampulla showing ampullar filaments (af) and the auxiliary cell (aux) (HGI-A 19490). **Fig. 18.** A fully developed auxiliary cell ampulla showing the three orders of branched ampullar filaments (af1, af2 & af3) and auxiliary cell (aux) (HGI-A 19490). **Fig. 19.** Early stage after diploidization showing two primary gonimoblast cells (g) just cut off from the gonimoblast initial (gi) borne on the newly formed fusion cell (fc), flanking ampullar filaments (black arrows), and two secondarily produced connecting filaments (cf'). Note that the ampullar cells close to the fusion cell (white arrows) enlarged slightly (HGI-A 19490). **Fig. 20.** A slightly later stage of Fig. 19, showing primary gonimoblasts (g) cut off from the gonimoblast initial (gi) borne on fusion cell (fc), cells of ampullar filaments (white arrows), and a secondarily produced connecting filament (cf') (HGI-A 19490). **Fig. 21.** Immature carposporophyte showing gonimoblast initial (gi), two gonimoblasts (g1 & g2) borne on a small fusion cell (fc), and a secondarily produced connecting filament (cf'). Note that most cells (white arrows) of ampullar filament near the fusion cell are darkly stained but do not branch further (HGI-A 19490). **Fig. 22.** Cross section of a fully developed cystocarp showing gonimoblasts (g) borne on a conspicuous basal fusion cell (fc) and ampullar filaments forming a filamentous pericarp (arrows) (HGI-A 19484). **Fig. 23.** Subsurface view of cystocarp-bearing blade showing gonimoblasts (g) and one of the mature cystocarps composed of two gonimolobes (g1 & g2) (HGI-A 19499). Aniline blue (Figs 14–21, 23); Not stained (Fig. 22). Scale bars: Figs 14, 16–22 = 20 μ m; Figs 15, 23 = 50 μ m.

the same time, the auxiliary cell remnant fuses with the ampullar cells nearby to form a fusion cell and several secondary connecting filaments are cut off from basal ampullar cells near the fusion cell (Figs 19–20). As gonimoblast development continues, the fusion cell fuses with more basal ampullar cells and most ampullar cells elongate slightly but do not branch further (Fig. 21). Fully developed, mature carposporangia-bearing gonimoblasts are composed of at least two different-sized gonimolobes and are 60–195 µm in diameter (Figs 22–23). Most cells of the gonimolobes are transformed into pyriform or rounded carposporangia, 12.5–15.0 µm in diameter. At this stage, the fusion cell remains distinguishable at the base of mature gonimoblasts (Fig. 22). Fully developed cystocarps are ostiolate, scattered over the thallus surface, and slightly protruding (Figs 22–23).

***Nesoia hommersandii* Rodríguez-Prieto, S.-M. Lin, Afonso-Carrillo, De Clerck & Huisman sp. nov.**
(Figs 24–46)

DIAGNOSIS: Thalli erect, rose to dark red in colour, foliose, consisting of a simple, oval or lobate blade, 16–20 cm long and 10–14 cm wide, arising from short stipes, soft and gelatinous in texture. Blades 100–260 µm thick in upper parts, cortex made up of 2–3 cell layers, outer cortical cells not secondarily pit-connected, flattened or irregularly polygonal, 8.0–15.9 µm wide by 6.0–12.1 µm long in surface view, 5.5–16.4 in height by 6.49–13.6 µm in width in longitudinal view, subcortex composed of a network of rounded or ovoid cells with short arms, subtended by a network of stellate cells with long arms. Medulla composed of a network of stellate cells with long arms, and sparse filaments, anticlinal to thallus surface, 57–126 µm long and 8–20 µm thick. Tetrasporangia cruciately, cruciately-decussately, or (rarely) irregularly divided, 14.7–17.7 µm long by 10.9–14.5 µm wide. Gametophytes monoecious. Carpogonial branches two-celled; auxiliary cell ampullae abundant and composed of three orders of ampullar filaments, up to 11–15 cells long when fully developed; carposporophyte composed of two gonimolobes, up to 126–209 µm in diameter; carposporangia 7.6–13.4 µm in diameter; mature cystocarps ostiolate, protruding above the thallus surface.

ETYMOLOGY: The species name is dedicated to Professor Max H. Hommersand, for his immense contribution to the systematics of red algae, and especially to the knowledge of female reproduction in red algae.

TYPE COLLECTION: Holotype, a tetrasporic plant (Fig. 24) growing in a mixed sand-rock bottom at La Rapadura, Santa Úrsula, Tenerife, Canary Islands, Spain, 01 October 2016, collected by R. Martel, at

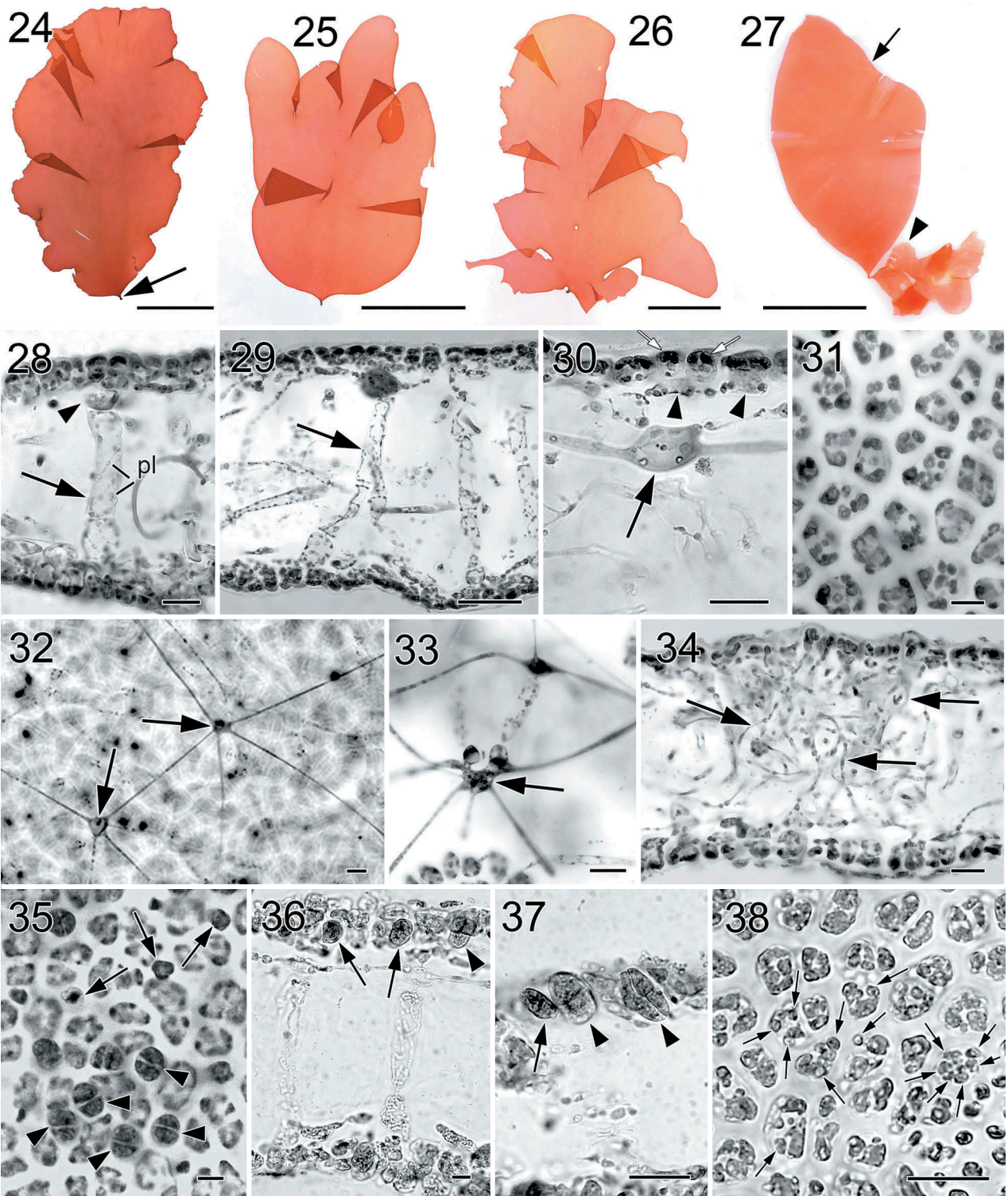
a depth of 40 m. The specimen is deposited at the algae section of the Herbarium of the University of La Laguna (TFC Phyc 15296). Isotypes: TFC Phyc 15297 (Fig. 25, tetrasporic), TFC Phyc 15294 (Fig. 26, tetrasporic), and TFC Phyc 15300 (Fig. 27 containing tetrasporic, male and female).

TYPE LOCALITY: La Rapadura, Santa Úrsula, Tenerife, Canary Islands, Spain (Macaronesia). **DISTRIBUTION:** This species has only been found in Santa Úrsula (Tenerife, Canary Islands, Spain).

HABITAT AND SEASONALITY: The species was found in October at a depth of 40 m, on a mixed sand-rock bottom.

SPECIMENS EXAMINED: La Rapadura, Santa Úrsula, Tenerife, Canary Islands, Spain, –40 m, 01.x.2016, coll. R. Martel, TFC Phyc 15296 (tetrasporic); TFC Phyc 15297 (tetrasporic), TFC Phyc 15294 (tetrasporic), TFC Phyc 15300 (tetrasporic, male and female), TFC Phyc 15301 (tetrasporic) and TFC Phyc 15299 (tetrasporic).

Habit and vegetative structure: Thalli are foliose, 16–20 cm long and 10–14 cm wide, composed of simple or lobate blades, arising from small discoid holdfasts with short stipes (Figs 24–27). Blades are soft and gelatinous in texture, rose to dark red with a thin darker margin. Blades are 100–260 µm thick, composed of two thin layers of cortex and a large lax medulla (Figs 28–29). The cortex is made up of 2 cell layers in young thalli and becomes 3 cell layers in older parts (Fig. 29). Outer cortical cells are 8.0–15.9 µm wide by 6.0–12.1 µm long in surface view and 5.5–16.4 µm high by 6.49–13.6 µm wide in longitudinal view, not protruding at thallus surface (Fig. 30), and are irregularly polygonal and compactly arranged in surface view (Fig. 31). Surface cells lack secondary pit-connections (Fig. 31). The subcortex in fully developed thalli is composed of two networks of secondarily connected stellate cells: an outer layer of rounded or ovoid cells with short arms (Fig. 30, arrowheads) and the innermost layer with sometimes darkly stained stellate cells possessing relatively long arms (Fig. 30, arrow). However, the network of stellate cells with short arms is lacking in the young subcortex. The medulla is composed of a network of stellate cells parallel to the thallus surface and situated mostly just under inner cortical cells (Fig. 32, arrows), and some sparsely arranged anticlinal filaments of elongated cells (Figs 28–29, arrows) that are produced from innermost cortical cells, and that can be up to 57–126 µm long and 8–20 µm thick at the apex of the thallus. Medullary cells bearing long arms cannot be easily observed in transverse section but can be seen in subsurface view after treated with haematoxylin or aniline blue stain. Occasionally, some clusters of rhizoidal filaments are found in the old medulla (Fig. 34).



Figs 24–38. *Nesoia hommersandii* Rodríguez-Prieto, S.-M.Lin, Afonso-Carrillo, De Clerck & Huisman sp. nov. Thallus morphology, vegetative, and reproductive structure. **Fig. 24.** Holotype, a sporophyte with a single, simple blade and a short stipe (arrow) (TFC Phyc 15296). **Figs 25–26.** Isotypes, sporophytes bearing lobed margins or bladeletes (TFC Phyc 15297 and 15294). **Fig. 27.** Isotypes, a small monoecious gametophyte (arrowhead) attached to the basal part of a big sporophyte (arrow). Note that the gametophyte bears many cystocarps that can be distinguished at naked eye (= red dots) (TFC Phyc 15300). **Fig. 28.** Transverse section of a young blade showing a thin cortex, subcortical cell (arrowhead) and a large medulla with anticlinal medullary filaments (arrow). Note that plastids (pl) are network-like (TFC Phyc 15300). **Fig. 29.** Transverse section of another young blade showing a thin cortex and broadened medullary filaments (arrow) (TFC Phyc 15300). **Fig. 30.** Cross section of a young blade showing flattened surface cells (white arrows), ovoid subcortical pit-connected cells with short arms (arrowheads), and inner cortical cell with long arms (black arrow) (TFC Phyc 15300). **Fig. 31.** Polygonal outer cortical cells in surface view (TFC Phyc 15300). **Fig. 32.** Subsurface view of thallus blade showing stellate cells of medullary network (arrows) interconnected by long and thin arms (TFC Phyc 15301). **Fig. 33.** Transverse section showing cells of the anticlinal medullary filaments (arrow) (TFC Phyc 15300). **Fig. 34.** Transverse section of an old blade showing many rhizoidal filaments (arrows) within the medulla (TFC Phyc 15301). **Fig. 35.** Subsurface view of a tetrasporangial sorus showing immature tetrasporangia (arrows) and fully divided tetrasporangia (arrowheads) (TFC Phyc 15301). **Fig. 36.** Cross

Outer cortical cells have more pigmented plastids than cells in the subcortex and medulla but all of them have reticulated plastids (Fig. 28, pl).

Reproductive structures. Tetrasporangial sori are scattered over the thallus surface (Fig. 35). Tetrasporangial initials are cut off from subcortical cells and different ages of developing tetrasporangia can be found simultaneously (Figs 35–37). Fully developed tetrasporangia are rounded or ovoid, cruciately, cruciately-decussate or irregularly divided, 14.7–17.7 µm long by 10.9–14.5 µm wide (Figs 36–37).

Gametophytes are monoecious. Spermatangial sori are scattered over the thallus surface (Fig. 38). The carpogonial branch ampulla is composed of a two-celled carpogonial branch (Fig. 39) with a distally swollen trichogyne and two ampullar filaments when fully developed (Fig. 40). Auxiliary cell ampullae are abundant, developing outwardly from inner stellate, subcortical cells with short arms. The auxiliary cells are always the basal cell of the third-order ampullar filament (Fig. 41).

Direct diploidization was not observed. After presumed diploidization via a connecting filament, the auxiliary cell elongates, its nucleus divides once (Fig. 42), cutting off a gonimoblast initial at its distal end (Fig. 43). Soon after, the remnant of the auxiliary cell fuses with several basal ampullar cells to form a primary fusion cell (Fig. 43) while the distal cells of first- and second-order ampullar filaments produce lateral branches. At the same time, the fusion cell cuts off one or several secondary connecting filaments (Fig. 45). As gonimoblast development continues, several secondary connecting filaments are issued from enlarged, multinucleate fusion cells (Figs 44–45). Fully developed, mature carposporangia-bearing gonimoblasts are composed of at least two different sized gonimolobes and are 126–209 µm in diameter (Fig. 46). Most cells of the gonimolobes are transformed into pyriform or rounded carposporangia, 7.6–13.4 µm in diameter (Fig. 46). At this stage, the fusion cell remains distinguishable at the base of mature gonimoblasts and fully developed cystocarps are ostiole and slightly protruding and scattered over the thallus surface (Fig. 46).

Discussion

Based on *rbcL* sequence analyses (Fig. 1), most described species of *Halymenia* from the Mediterranean Sea, Atlantic and Pacific oceans, together with two monotypic genera, *Gelinaria ulvoidea* and *Epiphloea bullosa*,

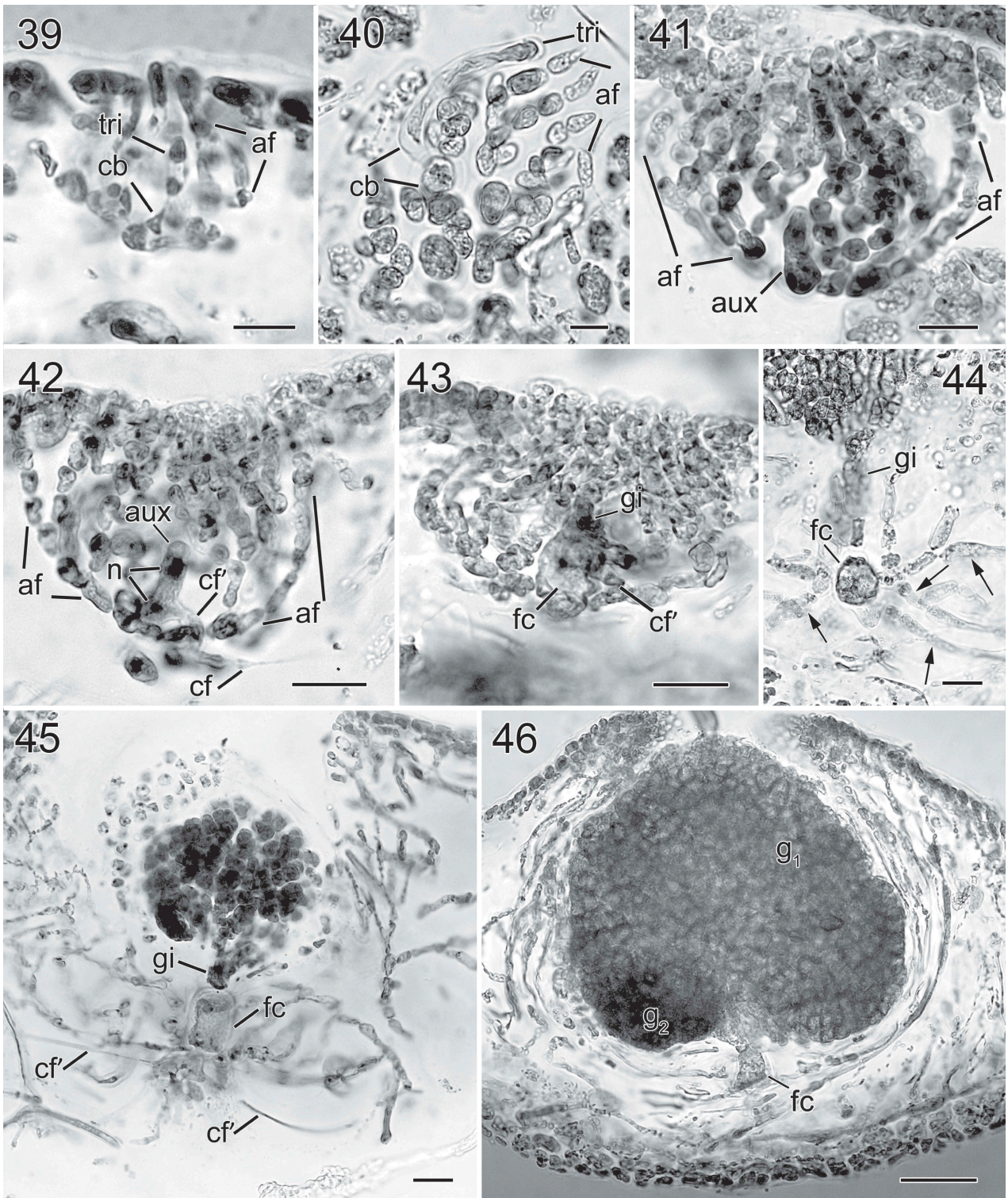
from Western Australia, are clustered into a large clade (*Halymenia* sensu stricto herein). Moreover, foliose Halymeniaceae from Europe and the Mediterranean Sea are separated into five distinct species: *Halymenia ballesterosii* (a new species described in this study), *Neofolia rosea* (Rodríguez-Prieto et al., 2018), *Nesoia hommersandii* (a new species described in this study), *Nesoia mediterranea* (Rodríguez-Prieto et al., 2019) and *N. latifolia* Lee & Kim (2019). The former four species were previously confused with *Nesoia latifolia*, which was previously known as ‘*Halymenia*’ *latifolia* and was once regarded as having a wide distribution in both the Atlantic and Pacific oceans and the Mediterranean Sea (D’Archino et al., 2014; Rodríguez-Prieto et al., 2019).

In the field, *H. ballesterosii* is difficult to separate from *Nesoia latifolia* and *N. mediterranea* as the three species share a similar thallus morphology (i.e. blade colour and texture). *Halymenia ballesterosii* is also very similar to *Neofolia rosea*, although the latter is a little darker and tougher than the other three taxa (Rodríguez-Prieto et al., 2018, 2019). However, the vegetative and female reproductive structure of *H. ballesterosii* is similar to the generitype, *H. floresii*, in terms of having protruding surface cells, two-celled carpogonial branches, and possessing basal nutritive cell cluster in the carpogonial branch ampullae, secondary connecting filaments issued from basal cells of ampullar filaments as well as mature cystocarps immersed in the medulla (Rodríguez-Prieto et al., 2018, 2019, this study). Moreover, the surface cells in *Nesoia* and *Neofolia* are level with the thallus surface and both genera lack a nutritive cellular cluster at the base of the carpogonial branch ampullae (Rodríguez-Prieto et al., 2018, 2019). A morphological comparison of the five foliose species of *Halymenia*, *Neofolia* and *Nesoia* from Europe is given in Table 1.

Nesoia hommersandii can be confused with *Nesoia latifolia* in having a similar thallus morphology. However, *N. hommersandii* (lacking swollen arms) differs from *N. latifolia* (having swollen arms) in the morphology of medullary stellate cells. On the other hand, young or smaller plants of *N. hommersandii* (2-celled carpogonial branches) are sometimes indistinguishable from *Neofolia rosea* (having swollen arms in medullary cells, 3-celled carpogonial branches) in habit, but they differ in vegetative and female reproductive morphology (see Table 1).

The observations on female reproductive structures in the two newly described species corroborate

section through a tetrasporangial sorus showing immature tetrasporangia (arrows) and nearly mature tetrasporangia (arrowhead) (TFC Phyc 15301). **Fig. 37.** Close up of immature tetrasporangia (arrows) and mature tetrasporangia (arrowhead) (TFC Phyc 15300). **Fig. 38.** Subsurface view of a spermatangial sorus showing mature spermatangia (arrows) (TFC Phyc 15300). Haematoxylin (Figs 28, 36, 37). Aniline blue (Figs 29–35, 38). Scale bars: Figs 24–27 = 5 cm; Figs 28, 30, 33, 34, 35, 37, 38 = 20 µm; Figs 29 = 50 µm; Figs 31, 32, 36 = 10 µm.



Figs 39–46. *Nesoia hommersandii* Rodríguez-Prieto, S.-M.Lin, Afonso-Carrillo, De Clerck & Huisman sp. nov. Morphology of carposporophyte and auxiliary cell ampullae and cystocarp development. **Fig. 39.** Close up of a carposporophyte ampulla showing a young carposporophyte (cb) prolonged by a short trichogyne (tri) surrounded by short branched ampullar filaments (af) (TFC Phyc 15300). **Fig. 40.** Subsurface view of a fully developed carposporophyte ampulla showing an enlarged trichogyne (tri) and highly branched ampullar filaments (af) (TFC Phyc 15300). **Fig. 41.** Close up of a developing auxiliary cell ampulla showing ampullar filaments (af) and the auxiliary cell (aux) (TFC Phyc 15300). **Fig. 42.** A fully developed auxiliary cell ampulla showing connecting filament (cf), ampullar filaments (af) and an enlarged auxiliary cell (aux) with stained nuclei (n). Note that a secondary connecting filament (cf') is cut off from the basal part of the auxiliary cell (aux) (TFC Phyc 15300). **Fig. 43.** Diploid auxiliary cell ampulla showing a gonimoblast initial (gi), a secondary connecting filament (cf') cut off from basal part of newly formed fusion cell (fc) (TFC Phyc 15300). **Fig. 44.** Close up of a young carposporophyte showing an elongated gonimoblast initial (gi) and a fusion cell (fc) that cut off several secondarily connecting filaments (cf') (TFC Phyc 15300). **Fig. 45.** An immature carposporophyte showing a gonimoblast initial (gi) and a fusion cell (fc) which is sending out two secondarily connecting filaments (cf') (TFC Phyc 15300). **Fig. 46.** A fully developed carposporophyte showing fusion cell (fc) and two gonimolobes (g_1 & g_2) (TFC Phyc 15300). Aniline blue (Figs 39, 40, 44, 46); Haematoxylin (Figs 41–43, 45). Scale bars: Figs 39, 40 = 10 μm ; Figs 41–45 = 20 μm ; Fig. 46 = 50 μm .


Table 1. Morphological comparison among foliose species of *Halymenia*, *Neofolia* and *Nesoaia* from Europe.

	<i>Halymenia floresii</i> (genitype)	<i>Halymenia ballesterosii</i>	<i>Neofolia rosea</i> (genitype)	<i>Nesoaia latifolia</i>	<i>Nesoaia mediterranea</i>	<i>Nesoaia hommersandii</i>
Thallus morphology	Foliose, irregularly pinnate blades	Foliose, simple or occasionally bifurcate	Foliose, simple, oval to lanceolate blades, sometimes bearing subdichotomous branches	Foliose, simple, oval to lanceolate blades. Occasionally bifurcate	Foliose, simple, oval to lanceolate blades	Foliose, simple or lobate
Blade sizes	9–28 cm long by 0.3–3.0 cm wide	3–13 cm long by 1.4–4.0 cm wide	6.0–8.8 cm long by 1.5–2.2 cm wide	2–27 cm long by 1.0–6.5 cm wide	2.5–5.5 cm long by 1.4–3.2 cm wide	16–20 cm long by 1.0–14 cm wide
Blade thickness at apical thallus	No data	90–345 µm	107–207 µm	135–185 µm	280–310 µm	100–260 µm
Cortical cell layers	3	2–4	3	3–4	2–3	2–3
Surface cells	Compactly arranged in fresh specimens, but laxly arranged in fixed or stained specimens	Compactly arranged in fresh specimens, but more laxly arranged in fixed or stained specimens	Compactly arranged in fresh, fixed or stained specimens	Compactly arranged in fresh specimens, but laxly arranged in fixed or stained specimens	Compactly arranged in fresh specimens, but laxly arranged in fixed or stained specimens	Compactly arranged in fresh specimens, but more laxly arranged in fixed or stained specimens
Outer cortical cells	Protruding thallus surface, 6.0–20.0 µm long by 5.0–13.0 µm wide	Protruding thallus surface, 6.5–11.6 µm long by 3.8–14.3 µm wide	Level with thallus surface, 8.0–22.5 µm long by 6.5–15.5 µm wide	Level with thallus surface, 7.5–18.0 µm long by 5.0–14.5 µm wide	Level with thallus surface, 14.5–34.0 µm long by 8.5–26.5 µm wide	Level with thallus surface, 6.49–13.6 µm long by 6.0–15.9 µm wide
Presence of darkly staining outer stellate medullary cells	Often	Often	Not often	Often	Not often	Often
Presence of swollen arms of medullary stellate cells	Often	No	Often	Often	Occasionally	No
Medullary filaments cells at upper blades	795–805 µm long by 17–19 µm wide	83–340 µm long by 5–17 µm wide	115–120 µm long by 12–14 µm wide	85–150 µm long by 6–11 µm wide	110–240 µm long by 9–25 µm wide	57–126 µm long and 8–20 µm thick
Tetrasporangia	15–28 µm long by 10.0–23.5 µm wide	14.8–21.0 µm long by 11.5–19.0 µm wide	12–26 µm long by 10.5–18.0 µm wide	14–20 µm long by 10.5–19.0 µm wide	12.0–19.5 µm long by 10.0–16.5 µm wide	14.7–17.7 µm long by 10.9–14.5 µm wide
Carpogonial branch	2 celled	2 celled	3 celled	Not observed	2 celled	2 celled
Basal nutritive cell cluster	Present	Present	Absent	Not observed	Absent	Absent
Number & diameter of mature gonimolobes	3; 225–235 µm	2; 60–95 µm	2–3; 145–274 µm	2; 90–420 µm	2; 175–370 µm	2; 126–209 µm
Cystocarps morphology	Immersed in the medulla	Slightly protruding at thallus surface	Protruding thallus at surface at maturity	Immersed in the medulla	Immersed in the medulla	Slightly protruding at thallus surface
Carpoporangia in diameter	11–17 µm	12.5–15.0 µm	9–17 µm	8–14 µm	8–15 µm	7.6–13.4 µm
Secondarily connecting filaments	Issued from the basal cells of the ampullar filaments	Issued from the basal cells of the ampullar filaments	Issued from the fusion cell	Not observed	Not observed	Issued from the fusion cell
Distribution	Mediterranean, Atlantic Europe, and Macaronesia	Western Mediterranean (Catalonia, The Eastern Pyrenees, and Balearic Islands)	North-eastern Atlantic Ocean and western Mediterranean Sea	Atlantic and Mediterranean coasts of Europe	Western Mediterranean (Catalonia, Corsica and Balearic Islands)	Macaronesia (Canary Islands)
References	Rodríguez-Prieto et al. (2018)	This study	Rodríguez-Prieto et al. (2018)	Rodríguez-Prieto et al. (2019)	Rodríguez-Prieto et al. (2019)	This study

the diagnostic characters proposed to describe or redefine *Halymenia*, *Neofolia* and *Nesoia* (Rodríguez-Prieto *et al.*, 2018). Traditionally, all species in these genera were considered to possess fairly uniform female reproductive and post-fertilization structures. Careful observations however revealed a subtle but distinct set of characters that allow separation at the genus level. The structures reported for the new *Halymenia* and *Nesoia* species agree with the defining characteristics of both genera. From a more general point of view, the combined molecular-morphological approach taken in our studies is promising to redefine the classification and biogeography of many of the remaining *Halymenia* species worldwide. As seen in the literature, the name '*Halymenia latifolia*' was widely used for large, unbranched foliose plants occurring in Europe (Maggs & Guiry, 1982; Loiseaux-de Goër & Noailles, 2008; Rodríguez-Prieto *et al.*, 2013; Athanasiadis, 2016). The exact biogeographic ranges of *H. ballesterosii* and *Nesoia hommersandii* are difficult to delineate at present as many records of '*Halymenia latifolia*' need to be reexamined. Nevertheless, *H. ballesterosii* has been currently confirmed only for Banyuls-sur-Mer, Catalonia and the Balearic Islands (Western Mediterranean) while *N. hommersandii* is present only in the Canary Islands (Macaronesia).

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No potential conflict of interest was reported by the author(s).

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Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at <https://doi.org/10.1080/09670262.2020.1749888>.

Supplementary table 1. List of species used in *rbcL* analysis and accession numbers in GenBank. Accession numbers in bold were determined for this study.

Author contributions

C. Rodríguez-Prieto & S.-M. Lin: original concept and drafting manuscript; C. Rodríguez-Prieto: light microscopy; C. Rodríguez-Prieto & J. Afonso-Carrillo: field investigations; S.-M. Lin & O. De Clerck: analysis of molecular data; C. Rodríguez-Prieto, Julio Afonso-Carrillo, Olivier De Clerck, John M. Huisman & S.-M. Lin all review & approved the final manuscript.

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