Morphology, vegetative and reproductive development of the red alga *Portieria hornemannii* (Gigartinales: Rhizophyllidaceae)

Dioli Ann Payo [a,*], Hilconida Calumpong [b], Olivier De Clerck [a]

[a] Phycology Research Group, Ghent University, Krijgslaan 281, S8, 9000 Ghent, Belgium
[b] Institute of Environmental and Marine Sciences, Silliman University, Dumaguete City, Philippines

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ABSTRACT

Earlier descriptions of the Indo-Pacific red alga *Portieria hornemannii* lacked detailed information on its vegetative and reproductive development and morphology. An in-depth treatment is presented on the development of the uniaxial thallus and the formation of male, female and tetrasporangial nemathecia. Post fertilization events and carposporophyte development is described, confirming Kylin's presumption on the development of connecting filaments between the carpogonium and the auxiliary cell following fertilization. Phylogenetic analysis using chloroplast encoded *rbc*L and nuclear ribosomal LSU gene sequences, including members of Rhizophyllidaceae and their close relatives suggests a monophyletic family. *Contarinia* is resolved as the sister taxon of a clade uniting *Nesophila*, *Ochtodes* and *Portieria*. The relationships among the latter genera remain largely unresolved.

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1. Introduction

Portieria Zanardini is a small red algal genus with six currently accepted species that are widely distributed in tropical and subtropical waters of the Indo-West Pacific Ocean (Guiry and Guiry, 2010). *Portieria* belongs to the family Rhizophyllidaceae. The family, erected by Schmitz (1889), was based on the genus *Rhizophyllis*, which is currently regarded as a synonym of *Contarinia* (Denizot, 1968). Wiseman (1975) proposed the retention of the family Rhizophyllidaceae against Denizot's Contariniaceae on the basis that *Rhizophyllis* is a legitimate synonym of *Contarinia* and is the basionomic stem of the family. Its members are characterized with flattened or cylindrical thalli, uniaxial or biaxial, prominent or not so prominent central axis, large gland cells, spermatangia and tetrasporangia in sessile nemathecia and female globular nemathecia (Kylin, 1956; Wiseman, 1975; Millar, 1990; Abbott, 1999; Oliveira et al., 2005). Rhizophyllidaceae is among the nematheciabearing families of the order Gigartinales which now only includes 28 members as more recently, Peyssonnelliaceae, became an order of its own (Saunders et al., 2004; Krayesky et al., 2009; Verbruggen et al., 2010). The family includes 4 genera: *Contarinia*, *Ochtodes*, *Nesophila* and *Portieria*. The genera have interesting biogeographic distributions with each genus exhibiting a near exclusive distribu-

tion. The genus *Contarinia* is known with certainty only from the Mediterranean Sea and Atlantic coast of northwestern Morocco, Portugal, and Spain (Feldmann, 1939; Benhissoune et al., 2002; Berecibar et al., 2009; Peña and Bárbara, 2010). Two additional species, *C. okamurae* Segawa and *C. pacifica* (Børgesen) Denizot have been reported from Japan and in Easter Island respectively but their identity requires confirmation. *Ochtodes*, *Portieria* and *Nesophila* are endemic to the Caribbean, Indo-Pacific, and New Zealand, respectively. The generic concept of *Portieria* has been solely based on observations of structural characters (Zanardini, 1851).

The vegetative and reproductive developments have been studied in detail only for a few members of the Rhizophyllidaceae. Detailed treatments of the structure of *Ochtodes secundiramea* were provided by Joly and Ugadim (1966) and Wiseman (1976, 1977). *Contarinia squamariae* was studied by Denizot (1968) and more recently by Berecibar et al. (2009). All other species remain largely unstudied. This includes the genus *Portieria*. The earliest accounts on *Portieria* (often as *Desmia* and *Chondrococcus*; see Silva et al., 1987 for details on the generic synonymy) were limited to general descriptions (Lyngbye, 1819; Zanardini, 1851; Kützing, 1867; Agardh, 1876). Kylin (1930, 1956) provided a more detailed account of the vegetative and the reproductive structures of *Portieria* which included illustrations of the male reproductive structures, auxiliary filaments in the female nemathecia and tetrasporangia. Fine details of early post fertilization events, however, were not presented. The genus was studied in detail by D. Reid Wiseman but these results were never published.

* Corresponding author. Fax: +32 9 264 8599.
  E-mail address: dioli_20@yahoo.com (D.A. Payo).

In this paper, we provide a more detailed account of *Portieria hornemannii*'s vegetative and reproductive development, with an emphasis on post fertilization development. We will interpret the developmental relationships among genera and relate them to phylogenetic observations.

2. Methods

2.1. Morphological analysis

Reproductive and non-reproductive *P. hornemannii* samples were collected from different locations in the Indo-Pacific from 1980 to 1997. Observations were made on specimens preserved in 5% formalin–seawater solution. Fine cross sections of the thallus were made by hand using a single or double-edged blade. To observe reproductive structures, nemathecia-bearing tissues were squashed. Whole axial cells were observed by making a slight longitudinal cut along the axis of a piece of thallus and pressing it with cover slip to split the tissue which further reveals the cells. Whole-mount and sectioned materials were stained with 1% aniline blue solution, fixed with a drop of 10% HCl, rinsed with distilled water and mounted in Karo syrup for preservation. To reveal presence of nuclei, bleached tissue sections were stained with Wittmann's aceto-iron-haematoxylin-chloral-hydrate solution. Excess water was removed from tissue sections before application of staining solution. The stain was allowed to stay on the material for at least 30 min before adding 45% acetic acid on one edge of the cover slip to destain. Excess acid was drained from the opposite side of the cover slip using an absorbent paper. Hoyer's mounting medium (1:1 distilled water) was applied next from one side of the cover slip and was allowed to stand for about an hour. The mounting medium was removed with acetic acid and finally, mounted using Karo syrup. Further details of this procedure are described in Wittmann (1965) and Hommersand et al. (1992). Photographs were taken with an Olympus DP50 digital camera or Olympus Colorview IIIu digital color camera mounted on a Leitz Diaplan or BX51 Olympus compound microscope,or Leica Wild M10 stereo microscope. Specimens are housed at the herbarium of Ghent University (GENT).

2.2. Molecular analyses

DNA sequences were either retrieved from Genbank or generated for this study. DNA was extracted from silica dried material and informative loci amplified and sequenced according to De Clerck et al. (2005). Primers used for amplication were derived from Wang et al. (2000) and Wilkes et al. (2005) for *rbc*L gene and Harper and Saunders (2001) for the LSU nrRNA gene. Generated sequences were edited using BIOEDIT 7.0.9.0 (Hall, 1999) and were aligned together with Genbank sequences using MAFFT (Multiple Alignment using Fast Fourier Transform) (Katoh and Toh, 2008). The alignments included 7 taxa consisting four members of Rhizophyllidaceae and three genera of closely related families (Dumontiaceae, Kallymeniaceae, and Polyidaceae). The initial alignment of the LSU nrRNA gene contained 2817 bases but was finally reduced to 2659 bases, excluding 158 positions from regions difficult to align. The *rbc*L alignment included 1430 bases. The concatenated alignment of the two genes included 4088 positions. Phylogenetic analyses were performed on three datasets: DNA sequences of the LSU nrRNA gene, *rbc*L gene, and concatenated sequences of the two. The three datasets were exported for phylogenetic analysis to MEGA 4.0.2 (Tamura et al., 2007) for initial Neighbour Joining (NJ) analyses. The concatenated dataset was exported to PhyML (Guindon et al., 2009) for Maximum Likelihood (ML) analyses, and to MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) for Bayesian Inference (BI). The model of nucleotide substitution used for ML was General Time Reversible (GTR), determined using Modeltest 3.7 (Posada and Crandall, 1998) according to the Akaike information criterion (Posada and Buckley, 2004). PhyML was set to estimate the proportion of invariable sites, consider 4 substitution rate categories, estimate the gamma distribution parameter, use BIONJ as an input tree, and to conduct a non-parametric bootstrap analysis of 100 replicates. Bayesian analysis was performed using a GTR + I + Γ model. The data set was divided in two partitions, corresponding to the *rbc*L and LSU nrRNA genes, with all model parameters uncoupled between the partitions. Posterior probabilities were estimated using a Metropolis-coupled Markov chain Monte Carlo approach with sampling according to the Metropolis–Hastings algorithm. The analysis used four chains, one cold and three incrementally heated. Each run consisted of 1,000,000 generations and was sampled every 1000 generation. Burnin value was determined using TRACER V1.4 (Rambaut and Drummond, 2007) and was set at 100 generations.

3. Results

3.1. P. hornemannii (Lyngbye) P.C. Silva

Forty-two *P. hornemannii* specimens spanning the Indo-Pacific were morphologically examined for its vegetative and reproductive development (Table 1).

3.2. Description

Thallus erect, color varying from greenish pink to dark red, older specimens becoming orange to brown. Freshly collected specimens exhibiting a strong pungent smell. Thalli attached with a discoid holdfasts or with a crust like base, sometimes entangled among other seaweeds lacking a clear holdfast. One to several axes arising from the base, growing to a height of 3–15 cm (Fig. 1a–e). Main axes compressed, 300–1800 μm wide near the base, gradually narrowing towards the apices, up to 1100 μm thick near the base; alternately branched up to four to five orders. Apices typically incurved, sometimes straight in newly developing axes (Fig. 2a and b). Epiphytic specimens often with curled, entwined axes, secondarily attached to one another by hapteres (Fig. 2c).

Thallus uniaxial; apical cell conspicuous, dome-shaped, 5–6 μm × 4–7 μm dimensions. Growth of an indeterminate axis takes place by oblique division of the apical cell with the high side of successive axial cells alternating in a single plane (1/2 divergence). Each axial cell cuts off a first periaxial cell (the initial of a lateral filament) 1–2 cells below the apex, in an alternating distichous pattern (Fig. 2d and e). A second periaxial cell is cut off towards the dorsal surface (i.e. away from the inrolled apex) approximately 7–9 cells below the apex. Two additional periaxial cells, one situated below the first periaxial cell and another one at the ventral surface are then cut off. The first periaxial cell forms a lateral determinate filament. The other periaxial cells do not develop so extensively and contribute mostly to the thickness of the axes. There seems to be no strict pattern in the sequence of periaxial cell formation nor are there always four periaxial cells formed per segment. At least three periaxial cells are produced from each axial cell. The first periaxial cells divide to produce a lateral determinate filament, up to 10 cells long, which are largely responsible for the lateral expansion of the axes. Each of the cells of a lateral filament cuts off up a dorsal, ventral and abaxial cell. The abaxial cell forms a dominant filament, resulting to a secund pattern (Fig. 2e). The dorsal and ventral cell form comparatively shorter filaments, contributing only to the thickness of the thallus. Peripheral cells will differentiate to form a small-celled cortical layer up to 3 layers thick. Cortical cells measure 5 × 10.3 μm in surface view (Fig. 2f).

Table 1
List of specimens used in morphological analyses.

Specimen number	Place of collection	Date of collection	Habitat	Collector
DAP102, DAP210, DAP211, DAP212, DAP213, DAP299, DAP713	Dapdap, Siquijor, Siquijor, Philippines	17.02.2007; 14.04.2007	Found from 1 to 1.5 m depth on rocks and dead corals exposed to waves	D.A. Payo
DAP167	Daang-Lungsod, Alcoy, Cebu, Philippines	14.03.2007	Found in a site with patchy dead corals	D.A. Payo
DAP202, DAP203, DAP204, DAP378, DAP389	Takot Sawang, Tambisan, San Juan, Siquijor, Philippines	17.03.2007	In an offshore reef either epilithic, epiphytic or unattached	D.A. Payo
DAP247, DAP249, DAP251	Airport side, Silliman Beach, Dumaguete, Negros Oriental, Phlippines	30.03.2007	Epilithic on rocks by the airport	D.A. Payo
DAP285, DAP288	Pasig Reef, Maydolong, Eastern Samar, Philippines	08.04.2007	In an offshore reef exposed to strong waves	D.A. Payo
DAP333, DAP337, DAP338, DAP339, DAP342, DAP344, DAP345, DAP346	White Beach, Mahatao, Batanes, Philippines	21.04.2007	In a furrowed intertidal area exposed to strong waves	D.A. Payo
DAP363, DAP366, DAP368	Chanaryan, Basco, Batanes, Philippines	22.04.2007	In an intertidal area exposed to strong waves	D.A. Payo
HEC4217	Laing Island, Hansa Bay, Bogia, Madang Province, Papua New Guinea	05.1980	Found at the base of a coral, among *Halimedas* at 2 m depth	E. Coppejans
HV584	Logon Bay, Malapascua Is., Philippines	22.01.2004	Epiphytic located in an intertidal flat	H. Verbruggen
HV646	Olango, Cebu, Philippines,	25.01.2004	Intertidal flat, epiphytic	H. Verbruggen
KZN027	Zinkwazi, Black Rock Park, South Africa, KZN1485 Port O'Call, Trafalgar, Kwazulu-Natal, South Africa	23.12.1999	Intertidal rock pools	O. De Clerck
KZN2027	Palm Beach, Kwazulu-Natal, South Africa	07.02.2001	Intertidal	O. De Clerck
KZN2056	Trafalgar, Kwazulu-Natal, South Africa	08.02.2001	Intertidal	O. De Clerck
MAS264	Masirah Is., Oman		Epilithic at 2.5 m depth	T. Schils
ODC906	Kaalawai, Oahu, Hawaii	26.04.2003	Shallow subtidal	O. De Clerck
ODC1077	Mzamba, Eastern Cape Province	21.08.2005	Intertidal rock pools and shallow subtidal	O. De Clerck
ODC1160, ODC1164	Palm Beach, Kwazulu-Natal, South Africa	22.08.2005		O. De Clerck
SOC030	Nojid, Rhiy di-Qatanhin, Socotra Archipelago, Socotra, Yemen		Fossil reef rock platform, epilithic, shallow subtidal	F. Leliaert
SOC154	Rhiy di-Irisalepilithic, Socotra Archipelago, Socotra, Yemen		Epilithic, subtidal, and exposed	F. Leliaert

Internal cells enlarge and will form a medulla, up to 3 layers thick in the central part of the thallus (Fig. 2g). Medullary cells of are up to 160 μm in diameter. Gland cells, 8–25 μm in diameter, are formed from the subcortical cells and abound all throughout the thallus (Fig. 3a). They can also be found in the nemathecia. Gland cells are situated just below the surface and are surrounded by a ring of 5–7 cortical cells (Fig. 3a). The shape of the axial cell changes markedly in older portions of the thallus. About 3 segments below the apex, the axial cell elongates, becoming 3.5 times longer than broad. At the middle part of the thallus, the axial cell becomes barrel-shaped (210–270 μm × 50–90 μm), and the pit connections separating successive axial cells become very prominent (Fig. 3b). The enlarged axial contain up to 10 nuclei which stain prominently with aniline blue and haematoxylin solution (Fig. 3c). In the basal portions of the thallus, the midsection of the axial cell shrinks, becoming barbell-shaped (Fig. 3d). At the base of the thallus, multinucleated narrow rhizoidal filaments are issued from the axial cells, extending towards the medullary cells, finally passing between cortical cells towards the periphery of the thallus (Fig. 3e and f). Secondary pit connections are not formed. Indeterminate branches are formed every 2–10 mm. Similar to the formation of determinate laterals, indeterminate branches are also formed in the same plane. Indeterminate branches originate in a holoblastic manner, i.e. being formed in addition to the periaxial cells. The branching pattern of the newly formed axes repeats that of the main axes.

3.3. Reproductive morphology

Gametophytes are dioecious. Spermatangial branches are borne in sessile nemathecia which vary in size and shape (258–864 μm × 129–748 μm) and are produced on the thallus surface (Fig. 4a). Spermatangial filaments are compactly arranged in the nemathecia. Sterile paraphyses are absent but gland cells may be observed. Fully developed spermatangial branches are enveloped in a mucilaginous coating and reach 30–147 μm long. The spermatangial branches cut off at least 9–10 spermatia (3–5 μm). Development of the branch begins with a round basal cell cutting off two cells that elongate and divide into a 4 to 6-celled filament (Fig. 4b and c). The cells forming the new spermatangial filament serve as the initials for the spermatia. Up to 3 spermatia are formed per cell (Fig. 4d and e).

Globular cystocarps (300–500 μm) are borne on either side of the thallus at the base of the ultimate or penultimate lateral branchlets. Carpogonial and auxiliary branches occur in the same nemathecium (Fig. 5a–c). The carposporophyte development is non-procarpic (Fig. 5d–j). Early development of a nemathecium starts with cortical cells cutting off two initials from which auxiliary filaments, carpogonial branches and sterile paraphyses develop. Every mature auxiliary and carpogonial branch is paired with a sterile filament. Paraphyses, 60–80 μm long, are unbranched except for the distal end. In the development of the auxiliary branch, an apical initial undergoes two or three periclinal divisions to
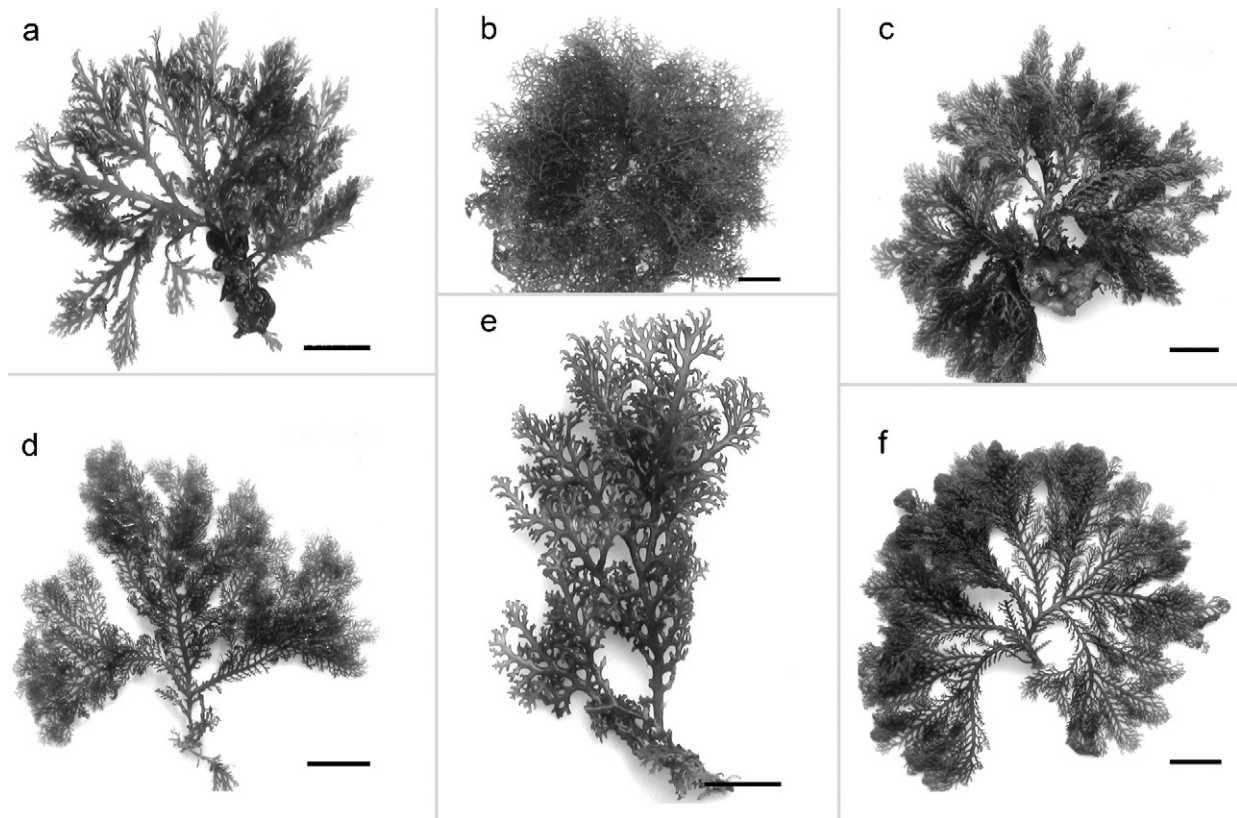
Fig. 1. General morphology of *P. hornemannii*. Scale bar: 10 mm. (a) DAP703, Dapdap, Siquijor, Siquijor. (b) Sawang, Siquijor. (c) DAP337, White Beach, Mahatao, Batanes (d) DAP345, White Beach, Mahatao, Batanes (e) DAP368, Chanaryan, Basco, Batanes (f) DAP336, White Beach, Mahatao, Batanes.

form a three or sometimes four-celled branch, consisting of a darkly staining basal cell (7–18 μm × 3–7 μm), an auxiliary cell (4–7 μm × 4–7 μm) and a terminal cell (6–8 μm × 4–6 μm). The basal cell is markedly broader and longer compared to the auxiliary and terminal cells. The basal cell and terminal cell may have nutritive purposes for the developing gonimoblasts after fertilization. Carpogonial branches were only rarely observed. Four-celled carpogonial branches which include a basal cell, a subhypogynous cell, a hypogynous cell, and a terminal carpogonium with trichogyne (133 μm long) reach a length of about 178 μm. The formation of the branch begins with periclinal division of an apical initial. The resulting two cells undergo substantial elongation with the upper cell subsequently forming a long extension. The initial further undergoes anticlinal division resulting to a 3 or 4-celled branch with the terminal cell becoming the carpogonium with a trichogynal extension. Only a short receptive portion of the trichogynal process projects above the rest of the nemathecial filaments. Hair cells can also be found in the nemathecium and can be mistaken as a trichogyne of a carpogonial branch (Fig. 5k). Following fertilization, subhypogynous and hypogynous cells fuse with the carpogonium and produce a non-septate connecting filament (Fig. 5d and e). The connecting filament fuses with an auxiliary cell which then begins to extend at its upper tip, reorienting obliquely evading the terminal cell. From the expanded auxiliary cell, several gonimoblast initials are issued from which carpospores develop (Fig. 5g). After fertilization and before fusing with the connecting filament of a carpogonial branch, the auxiliary cell appears completely stretched and loses its deep staining character. Even after the appearance of gonimoblast initials, auxiliary cell remains attached to the basal and terminal cells. The basal cell of the auxiliary branch forms a distinctive stalk and does not participate in the carpospore development. Succeeding divisions of carpospores occurs in all directions, which explains the irregularly globular structure of a nemathecium. A well documented post-fertilization pattern reported in other genera within Gigartinales (e.g. *Dudresnaya*, *Gigartina*, *Kallymenia*, and *Waernia*) (Robins and Kraft, 1985; Hommersand and Fredericq, 1990; Hommersand et al., 1992; Wilce et al., 2003; Rodriguez-Prieto and Hommersand, 2009) is suggested in *Portieria*. The presence of a conspicuous number of auxiliary branches within a nemathecium compared to a rather obscure presence of a carpogonial branch most likely suggests that a single carpogonial branch when fertilized, produces several copies of the now diploid nucleus, forms several connecting filaments, and deposits a nucleus to a number of auxilliary branches, such that a single fertilization can produce several gonimoblasts. As observed, a single connecting filament can form a continuous link among succeeding auxiliary cells forming a long chain. Within the nemathecium, clusters of carpospores are separated into pockets by a thin layer of sterile cells. The entire nemathecium is covered by a mucilaginous coating.

Tetrasporangial nemathecia are initially formed on the surfaces of the ultimate and penultimate branches and later spread out in the entire thallus with only small surfaces free of tetrasporangia (Fig. 6a). They are enclosed in a flat, sessile nemathecium (258–903 μm × 129–194 μm). Similar to the spermatangial nemathecium, sterile paraphyses are lacking. The entire nemathecium is also enclosed in a mucilaginous coating (Fig. 6b). Tetrasporangia (L – 25.6–32 μm) are zonate and are transversely to obliquely divided. They develop from a basal cell originating from the cortex, which cuts off two tetrasporangial mother cells. These undergo meiosis and giving rise to four (sometimes 3–6) haploid spores (Fig. 6b and c).

3.4. Molecular phylogenetics

The molecular data set consisted of *rbc*L (1430 bp) and LSU nrRNA gene (2659 bp) sequences consisting of 4 Rhizophylli-
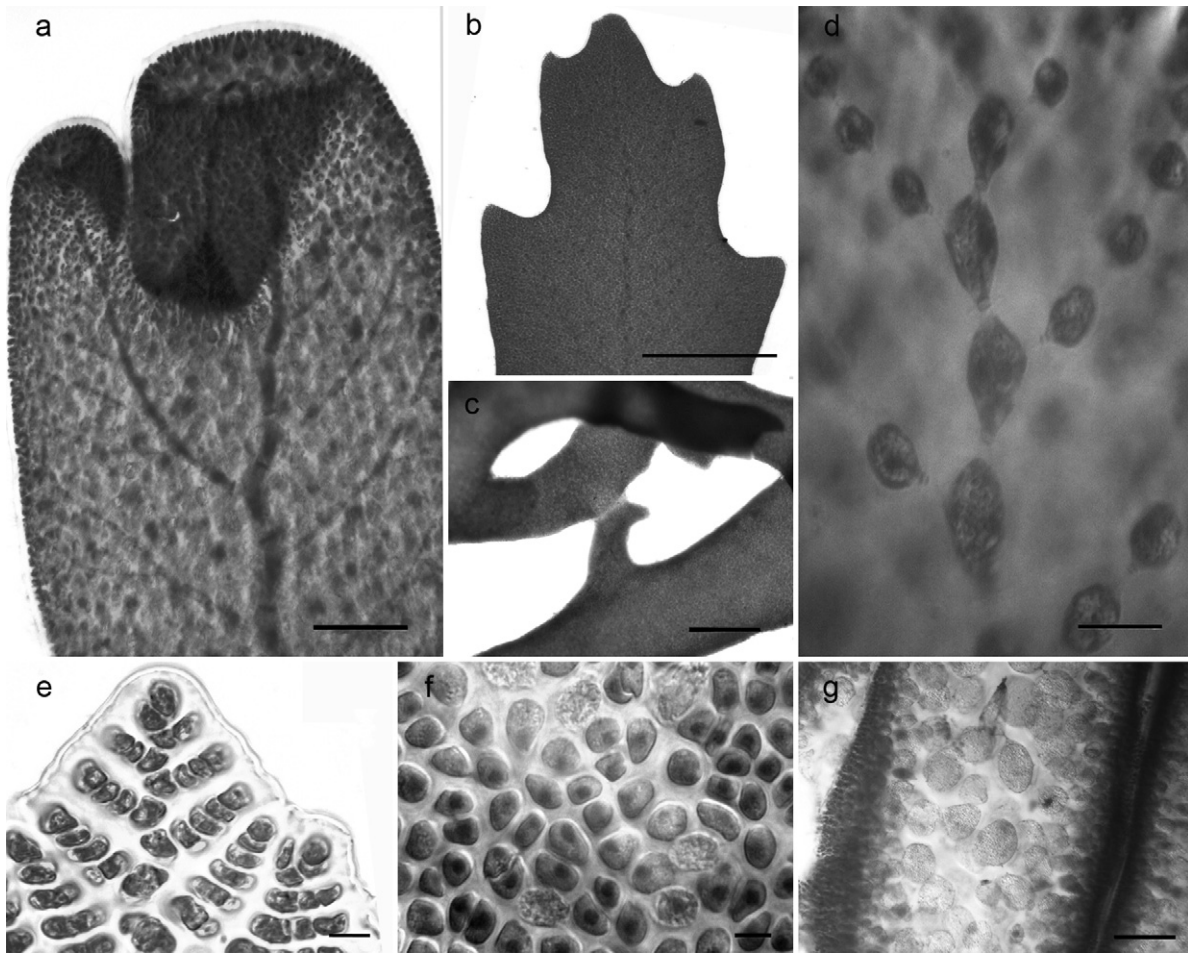
Fig. 2. Vegetative morphology of *P. hornemannii*. (a) Typically inrolled tip of a major axis. Scale bar: 100 μm. (b) Newly developing flat apex. Scale bar: 100 μm. (c) Detail of a haptere anastomosing separate branches. Scale bar: 0.5 mm. (d) Longitudinal optical section of the axial filament and the alternating distichous pattern of primary lateral periaxial cells. Scale bar: 100 μm. (e) Detail of the apex and the abaxial branching pattern of the distichous primary lateral filaments. Scale bar: 10 μm. (f) Surface view of cortical cells. Scale bar: 10 μm. (g) Medullary cells in cross sectional view. Scale bar: 100 μm.
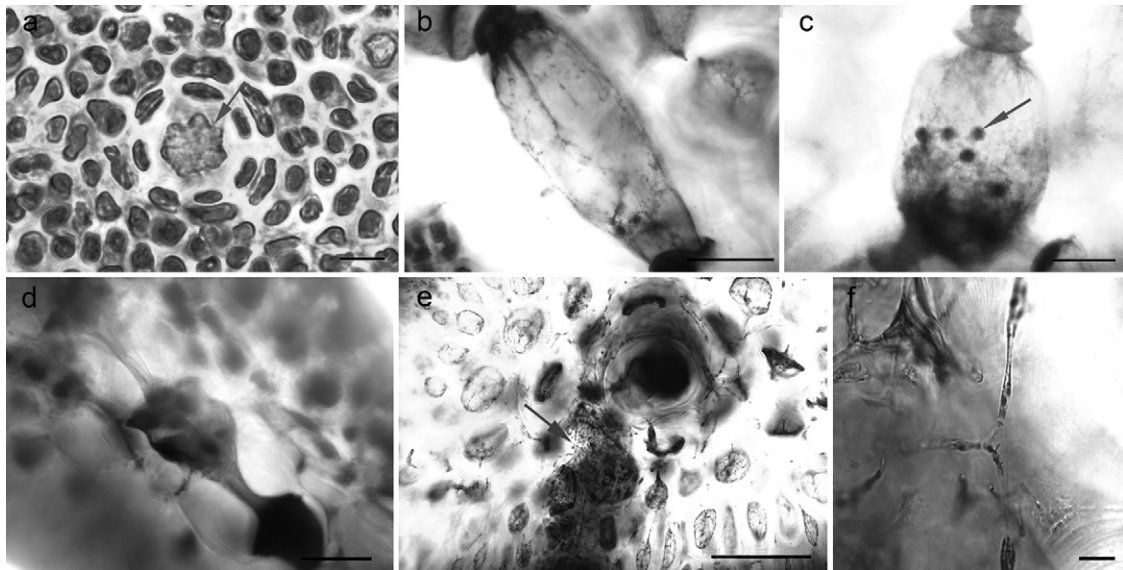

Fig. 3. Vegetative morphology of *P. hornemannii*. (a) Surface view of a conspicuously depressed, large gland cell surrounded by a ring of cortical cells. Scale bar: 10 μm. (b, c) Detail of a barrel-shaped, multinucleate axial cell. Scale bar: 100 μm. (d) Dumb-bell-shaped axial cells observed at the thallus base. Scale bar: 100 μm. (e) Rhizoidal filaments found at the thallus base. Scale bar: 100 μm. (f) Detail of the rhizoidal cells. Scale bar: 10 μm.
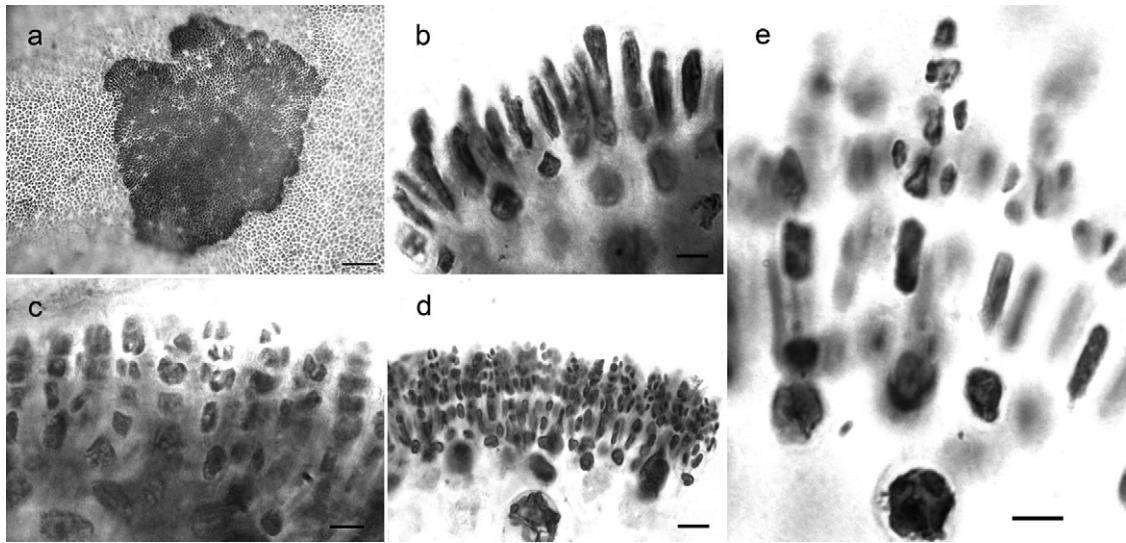
Fig. 4. Development of male reproductive structures in *P. hornemannii*. (a) Surface view of a sessile nemathecium. Scale bar: 100 μm. (b) Transverse section of elongated daughter cells originating from a basal cell during the early development of spermatangial filaments. Scale bar: 10 μm. (c) Daughter cells formed from subsequent peri- and anticlinal cell division of the elongated cells. Scale bar: 10 μm. (d) Fully developed spermatangial branches. Scale bar: 100 μm. (e) A mature spermatangial branch bearing spermatia. Scale bar: 10 μm.
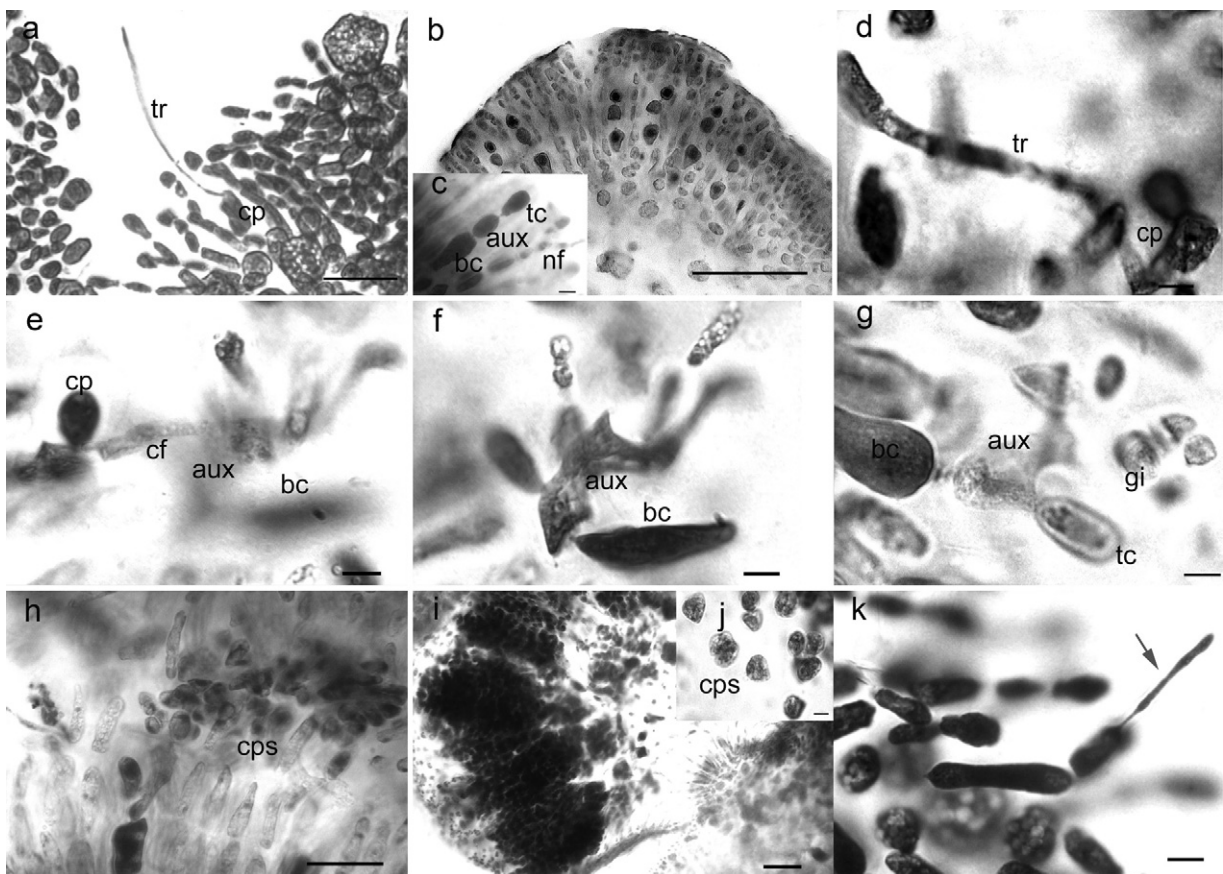

Fig. 5. Female reproductive structures and carposporophyte development in *P. hornemannii*. (a) Unfertilized carpogonial branch – trichogyne (tr) and carpogonium (cp). Scale bar: 50 μm. (b) Several auxiliary branches in a nemathecium. Scale bar: 50 μm. (c) Auxilliary branch – basal cell (bc), auxilliary cell (aux), and terminal cell (tc) and a sterile nemathecial filament (nf). Scale bar: 10 μm. (d) Fertilized carpogonium. Scale bar: 10 μm. (e) and (f) A connecting filament (cf) connects the carpogonium to the auxilliary cell. Scale bar: 10 μm. (g) Gonimoblast initial (gi) developing from auxilliary cell. Scale bar: 10 μm. (h) A developing carposporophyte. Scale bar: 50 μm. (i) Cross section of a cystocarp with fully developed gonimoblasts. Scale bar: 100 μm. (j) Carpospores (cps). Scale bar: 10 μm. (k) Hair cell from a sterile nemathecial filament. Scale bar: 10 μm.
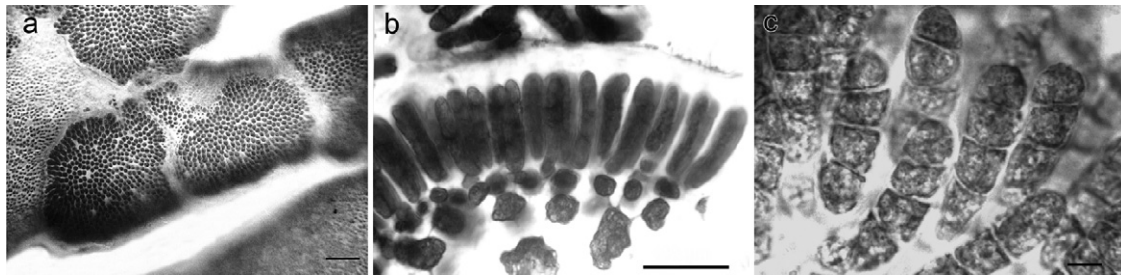
Fig. 6. Tetrasporore development in *P. hornemannii*. (a) Surface view of a tetrasporangial nemathecium. Scale bar: 100 μm. (b) Compact arrangement of tetrasporangia covered in transverse section. Scale bar: 100 μm. (c) Detail of zonately divided tetrasporangia. Scale bar: 10 μm.

dacean genera and 3 closely related families (Dumontiaceae, Kallymeniaceae, and Polyidaceae) (Table 2). The two datasets were concatenated to include a total of 4088 characters, 997 of which were parsimony informative. The placements of the different genera within the Rhizophyllidacean clade were also inconsistent in the datasets. BI and ML trees inferred from the concatenated datasets of *rbc*L and LSU nrRNA genes showed an identical topology with an exclusive Rhizophyllidacean clade (Fig. 7). Bootstrap and BI support values of the family were high (100% for both) with *Kallymenia cribrosa* as the closest relative. Bootstrap and BI support values for intrageneric relationships were high (99–100%) except for the low support on the node separating *Ochtodes* and *Nesophila* (46% and 75%, respectively). Branch lengths separating *Portieria*, *Nesophila* and *Ochtodes* are relatively short compared to the branch leading to *Contarinia*.

Fig. 7. Maximum likelihood phylogeny of family Rhizophyllidaceae based from combined *rbc*L and LSU nrRNA gene sequences. Node support values are given at each ramification (ML and BI). The log-likelihood value of the tree is −10542.80329. Base frequencies are A = 0.26209, C = 0.19951, G = 0.27892, T = 0.25949. The substitution rates are AC = 0.67945, AG = 2.29698, AT = 2.49003, CG = 0.88817, CT = 6.35790, GT = 1.0000. The proportion of invariable sites in the alignment is 0.504 and the shape parameter of the gamma distribution among site rate heterogeneity is 0.735.

4. Discussion

Detailed observations of the vegetative morphology and reproductive structures are presented for the genus *Portieria* (formerly *Chondrococcus* and *Desmia*). Despite considerable variation in external morphology, all genera of the Rhizophyllidaceae share a similar basic structure, differing only in minor details. All genera
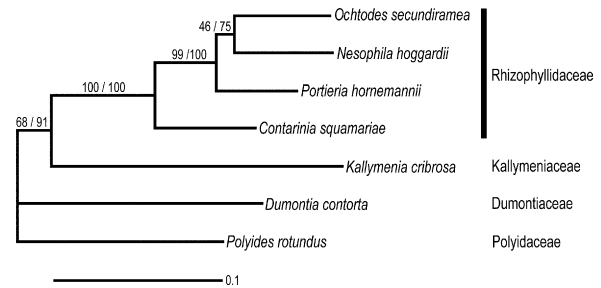
are pseudoparenchymatous with inner cells differentiated into a cellular medulla while peripheral cells form a small-celled, pigmented cortical layer. Thin rhizoidal filaments are interspersed in the medullary region of all four genera. Secondary pit connections, although reported for the Rhizophyllidaceae by Millar (1990) as

Table 2
List of species used in the *rbc*L and LSU nrDNA analyses with accession numbers.

Species	Gene	Location	Collector, date	Accession	Specimen number	Source
Portieria hornemannii (Lyngbye) P.C. Silva	LSU nrDNA	Dapdap, Siquijor, Siquijor, Philippines	D.A. Payo, 15.03.2007		DAP213	This study
	*rbc*L	South Africa	S. Fredericq	AF212185		Unpublished
Ochtodes secundiramea (Montagne) M.A. Howe	LSU nrDNA	Rocher du Diamant, Martinique	D. and M. Littler		DML30919	This study
	*rbc*L	Baie Olive, Guadeloupe	A. Renoux	EU349209		Krayesky et al. (2009)
Nesophila hoggardii W.A. Nelson and N.M. Adams	LSU nrDNA	Matu Kapiti I., New Zealand	W. Nelson, 20.12.1994	EU349089		Krayesky et al. (2009)
	*rbc*L	Matu Kapiti I., New Zealand	W. Nelson, 20.12.1994	EU349210		Krayesky et al. (2009)
Contarinia squamariae (Meneghini) Denizot	LSU nrDNA	Begur, Catalunya, Spain	O. De Clerck, 18.01.2008		ODC1498	This study
	*rbc*L	Begur, Catalunya, Spain	O. De Clerck, 18.01.2008		ODC1498	This study
Kallymenia cribrosa Harvey	LSU nrDNA	–	J.T. Harper and G.W. Saunders	AY171611		Harper and Saunders (2002)
	*rbc*L	Australia: Tarcoola Beach	M.H. and F. Hommersand, 21.09.1995	EU349216		Krayesky et al. (2009)
Polyides rotundus (Hudson) Gaillon	LSU nrDNA	Pointe du Nid de Corbet, Audresselles, Nord-Pas de Calais, France	O. De Clerck, 16.09.2004		ODC1014	This study
	*rbc*L	Penmaich, Brittany, France	D.W. Freshwater	U04214		Fredericq et al. (1996)
Dumontia contorta (S.G. Gmelin) Ruprecht	LSU nrDNA	Manomet Bluffs, Plymouth Co., MA, USA	M.H. Hommersand, 23.04.1993	EU349094		Krayesky et al. (2009)
	*rbc*L	USA: Manomet Bluffs, Plymouth Co, MA	M.H. Hommersand, 23.04.1993	AY294378		Unpublished

cited from Wiseman (1973), appear to be absent. Wiseman, however, did not mention presence of secondary pit connections in both *Ochtodes* and *Portieria*. Studies on *Nesophila* and *Contarinia* do not mention presence or absence of this character (Denizot, 1968; Nelson and Adams, 1996; Berecibar et al., 2009). Its absence might be a uniform characteristic of the family but this will need confirmation.

Another consistent vegetative character is the presence of large prominent gland cells located in the cortex. In *Portieria*, these cells are suspected to harbor halogenated monoterpenes (Meñez et al., 1996). The uniaxial growth pattern is shared with *Nesophila* and *Contarinia*, but not with *Ochtodes* in which growth is initiated by a pair of apical cells which give rise to a helicoid biaxis (Joly and Ugadim, 1966; Wiseman, 1976). A truly biaxial organization as observed in *Ochtodes* is unique within the red algae, but despite the fact that 2 axial filaments are formed the fundamental structure of the thallus is very similar to that observed in *Portieria*. The most important difference is presented by the number of periaxial cells which is cut off from each axial cell. In *Portieria* 3 or 4 periaxial cells are formed, compared to only 2 in *Ochtodes*. Unfortunately, this information is lacking for both *Contarinia* and *Nesophila*. The axial cells are large and prominent in all genera but the shape of the cells varies. In *Portieria*, the axial cells are barrel-shaped, multinucleate and possess conspicuously broad pit connections. The axial cells of *Nesophila*, *Ochtodes* and *Contarinia* appear long and cylindrical (Nelson and Adams, 1996; Berecibar et al., 2009).

Reproductive features are conserved in the Rhizophyllidaceae as suggested by Kylin (1956) and confirmed by the works of Wiseman (1977), Nelson and Adams (1996), Berecibar et al. (2009) and this study. Although Wiseman (1977) reports that *Ochtodes* can be procarpic and non-procarpic, members of the family are generally and perhaps, exclusively non-procarpic. The non-procarpic character as exhibited in *Portieria* and the rest of the family, which permits a series of diploid nuclei transfer from a single fertilization, increases the certainty of successful continuation of a set of genetic characteristics. This reproductive strategy may be partially limiting in terms of genetic diversity compared to a procarpic one where carporsporophytes are potentially offsprings of different male gametes. This limitation is however compensated by the possibility of increased genetic recombination by meiosis during sporangial development (Hawkes, 1990).

The presence of male, female and tetrasporangial nemathecia are consistent throughout Rhizophyllidaceae. In *Portieria*, nemathecia are borne in both surfaces of the thallus. The upright habit of *Portieria* probably permits its presence on both surfaces as opposed to the creeping habit of *Contarinia* where nemathecia are formed on dorsal surfaces (Berecibar et al., 2009). This is probably particularly important for female gametophytes in achieving a greater settling surface for spermatia. While for tetrasporophytes and male gametophytes, the increased surface also allow for a greater number of spores. Tetrasporangia and spermatia are formed in nemathecia lacking sterile paraphyses. Tetrasporangia are zonately to irregularly zonately divided. Spermatia are formed by means of anticlinal divisions from a 4 to 6-celled filament. The female reproductive structures are likewise very similar among all four genera. Auxiliary and carpogonial cell filaments are produced in the same nemathecia interspersed with sterile nutritive filaments. The auxiliary cell filaments are usually 3 to 4-celled with a large basal cell subtending the auxiliary cell. Kylin (1956) was unable to observe the early post fertilization events in *Portieria*. From the structural observations, however, he hypothesized that the transfer of the diploid nucleus from carpogonium to the auxiliary cell would be mediated by means of a connecting filament. Wiseman in his unpublished thesis, was also unable to follow the details of post-fertilization development with certainty in *Portieria*. Our observations clearly demonstrate that a connecting filament, issued from a fusion cell which includes the carpogonium, hypogynous and subhypogynous cell, fuses with a nearby auxiliary cell. These observations are congruent with those from *Ochtodes* (Wiseman, 1977) and *Contarinia* (Berecibar et al., 2009).

While the taxonomic position of the Rhizophyllidaceae in the order Gigartinales has been confirmed using small subunit (SSU) rDNA gene sequences (Tai et al., 2001; Saunders et al., 2004) and combined multi-gene datasets (Verbruggen et al., 2010), phylogenetic relationships within Rhizophyllidacean genera have not been examined. The close relationship of the genera of the Rhizophyllidaceae is confirmed by means of molecular sequence analyses, which resolves *Contarinia* as the sister taxon of a clade uniting *Nesophila*, *Ochtodes* and *Portieria*. The relationships among the latter genera remain largely unresolved. In this respect, it is important that the genus *Contarinia* was represented in the analysis by *C. squamariae* and not by the type of the genus *Contarinia peyssonneliaeformis* Zanardini. Especially the latter genus, with 5 currently species, remains highly understudied at present, at its monophyletic nature is all but certain. Future studies may reveal that *Contarinia* is present only in the Mediterranean Sea and warm temperate Eastern Atlantic Ocean, while all other species attributed to the genus belong to widely divergent lineages.

Acknowledgements

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