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amb el títol

"El gènere Bonnemaisonia (Bonnemaisoniales, Rhodophyta) a la Península Ibèrica i les illes Balears: taxonomia, cicles vitals, corologia i aplicacions"

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Characterization of two frequently confused species, Bonnemaisonia asparagoides and Bonnemaisonia clavata (Bonnemaisoniales, Rhodophyta), on the basis of morphological and molecular evidence.

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Estudi morfoanatòmic de Bonnemaisonia asparagoides i *Bonnemaisonia clavata* (*Bonnemaisoniales*, *Rhodophyta*), *dues espècies sovint confoses*.

L'escassa informació vegetativa i reproductora de *Bonnemaisonia clavata* Hamel i la seva semblança morfològica amb *Bonnemaisonia asparagoides* (Woodward) C. Agardh han propiciat que s'hagi qüestionat l'estatus taxonòmic de la primera espècie i que durant molt temps aquestes espècies sovint s'hagin confós entre elles, com queda demostrat amb la revisió dels plecs d'espècimens d'aquestes espècies dels principals herbaris europeus i nacionals.

L'estudi morfoanatòmic de *B. asparagoides* i *B. clavata*, tant dels gametòfits com de les seves generacions "Hymenoclonium" corresponents, ha permès descriure nous caràcters distintius per diferenciar aquests tàxons. En els gametòfits aquestes diferències es manifesten en l'estructura i la forma del tal·lus, la longitud de les cèl·lules axials dels filaments principals, la forma de les cèl·lules corticals internes, el tipus de tricògina, la simetria del pericarpi i el nombre de carposporangis per cistocarp. Quant a la generació "Hymenoclonium", *B. asparagoides* i *B. clavata* presenten com a caràcters inèdits distintius la forma del tal·lus, el seu tipus de ramificació oposada, la presència o absència de rizoides, la forma de les cèl·lules de primer i tercer ordres, i les dimensions de les cèl·lules de primer i segon ordres. És important destacar que en cap de les dues espècies no es van observar mai estructures reproductores en aquesta generació.

El seguiment dels cultius de les carpòspores també proporcionà informació sobre la fixació, la segmentació i la germinació de les carpòspores de *B. asparagoides* i *B. clavata.* En tots aquests processos les carpòspores de les dues espècies van tenir comportaments diferents i aquests processos van ser sempre més ràpids en *B. clavata* que en *B. asparagoides*. El manteniment dels cultius dels "Hymenoclonium" al llarg de l'any també ens va permetre observar diferències en les taxes de creixement, així com en els patrons de desenvolupament.

El gen *rbc*L va ser amplificat, mitjançant PCR, i directament seqüenciat per calcular el percentatge de divergència entre les seqüències de les dues espècies, i es va obtenir un 7,66 % de divergència genètica.

Aquest treball aporta la primera descripció detallada de les morfologies i anatomies vegetatives del gametòfit i l' "Hymenoclonium" de *B. clavata*. Els nous caràcters descriptius aportats, així com les dades moleculars obtingudes, ens permeten confirmar la seva validesa taxonòmica.

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Characterization of two frequently confused species, *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata* (Bonnemaisoniales, Rhodophyta), on the basis of morphological and molecular evidence

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The similar morphology of *Bonnemaisonia clavata* and *Bonnemaisonia asparagoides* and the rarely reported vegetative and reproductive characters of the former have resulted in considerable difficulty distinguishing these two taxa as well as uncertainty concerning the taxonomic status of *B. clavata*. We have reassessed the relationship between these two species using both morpho-anatomical and molecular data. Observations on gametophytes confirm a suite of distinguishing features including structure of the thallus (monopodial vs sympodial), axial cell size, presence or absence of a spiral trichogyne, pericarp symmetry and number of carposporangia per cystocarp. Investigation of the 'Hymenoclonium' prostrate phase developed from cultured carpospores provided additional distinguishing characters including carpospore germination pattern and morphology of 'Hymenoclonium' developmental stages. The characteristics utilized by previous authors to distinguish *B. asparagoides* and *B. clavata* are discussed and evaluated. Partial chloroplast-encoded *rbc*L sequences for *B. clavata* and *B. asparagoides* were 7.66% different. This level of divergence supports the morphological evidence that *B. clavata* and *B. asparagoides* are distinct species.

KEY WORDS: Algal culture, *Bonnemaisonia*, Bonnemaisoniales, 'Hymenoclonium' phase, Rhodophyta, *rbc*L, Taxonomy

INTRODUCTION

The genus *Bonnemaisonia* C. Agardh (1822: 196) (Bonnemaisoniaceae, Bonnemaisoniales) includes erect gametangial plants that are densely branched and distichous or spirally arranged with cylindrical or slightly compressed uniaxial axes; each axial cell supports two opposite periaxial cells; one periaxial cell initiates a longer determinate branchlet opposite a short branchlet that produces the sexual structures or an indeterminate branch. Numerous vesicular cells are located in the outermost cortex. Plants are monoecious or dioecious with a three-celled carpogonial branch and a spermatangial branch forming clusters of spermatangia. The triphasic life history of this genus is heteromorphic, including macroscopic gametophytes and erect (='Trailliella') or prostrate (='Hymenoclonium') tetrasporangial phases (Chihara & Yoshizaki 1972; Dixon & Irvine 1977).

Bonnemaisonia currently includes seven species (Guiry & Guiry 2007), mostly from temperate and subtropical regions of the world. The similar morphology of *Bonnemaisonia clavata* and *Bonnemaisonia asparagoides* and the rarely reported vegetative and reproductive characters of the former have frequently resulted in extensive taxonomic confusion. Consequently, incorrect descriptions and illustrations have been published and herbarium specimens routinely misidentified (see Discussion).

Bonnemaisonia asparagoides (Woodward) C. Agardh (1822: 197), the type species, was described as *Fucus asparagoides* Woodward (1794: 29). According Dixon & Irvine (1977), the lectotype is an original illustration by Woodward (1794, Pl. 6). *Bonnemaisonia clavata* Hamel (1930: 104) was described as a dioecious species on the basis of Schousboe's material from Marseille conserved in the PC herbarium (lectotype TA22350; Dixon 1962). This material, which included only male specimens with long spermatangial branches, was referred to as *Ceramium alternum* var. *clavatum* Schousboe (Bornet 1892). However, Schousboe's epithet was never published (Dixon 1962). Later, Derbès & Solier (1856) and Crouan & Crouan (1867) considered *B. asparagoides* to be dioecious, citing male specimens from Marseille and Brest respectively, with long spermatangial branches that could correspond to *B. clavata*, in our opinion. Chemin (1928) considered material collected in Britain to be an anomalous form of *B. asparagoides* with overly developed spermatangial branches and lacking female structures. Hamel (1930) concluded that two different species of *Bonnemaisonia* had been confused: *B. asparagoides* (monoecious with small spermatangial branches) and *B. clavata* (dioecious with long spermatangial branches and unknown female specimens). Subsequently, Feldmann & Feldmann (1942) found female specimens of *B. clavata* on the Algerian coast, corroborating Hamel's hypothesis.

The morphology of *B. asparagoides* (gametangial phase) has been the subject of numerous studies (Woodward 1794; Harvey 1846; Preda 1908–1909; Hamel 1930; Feldmann & Feldmann 1942; Funk 1927; Kylin 1956; Gayral 1966; Dixon & Irvine 1977; Rueness & Åsen 1982; Coppejans 1983). In contrast, only a small number of studies concern

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the morphology of *B. clavata* or describe both taxa simultaneously (Hamel 1930; Feldmann & Feldmann 1942; Dixon & Irvine 1977). These authors distinguished the species by the monoecious or dioecious character of the plant, shape and size of the spermatangial branches and cystocarps, carposporangial size and features regarding the habit of the plant such as the colour, height and branching pattern. However, since some of these features are only present in fertile specimens and others could vary with the age of the plant or the environmental conditions, Dixon & Irvine (1977) considered sterile gametangial plants of *B. asparagoides* and *B. clavata* to be essentially indistinguishable. Guiry (2007) speculated that, since the monoecious or dioecious character is the only observable difference between these taxa, the two entities could be the same species.

Feldmann & Mazoyer (1937) were the first to recognize that the alga described as *Hymenoclonium serpens* (P. Crouan & H. Crouan) Batters (=*Callithamnion serpens* P. Crouan & H. Crouan) corresponded to the prostrate phase developed from the carpospore germination of *B. asparagoides*. Rueness & Åsen (1982) completed the life history of this taxon in culture from carpospores, studying its germlings to maturity. Similarly, Feldmann & Feldmann (1942) obtained the prostrate phase of *B. clavata*, which was described as a 'Hymenoclonium' with a ramification pattern and cell dimensions distinct from those observed in *H. serpens*. However, *B. clavata* germlings never have been grown to maturity in culture to provide a more detailed and reliable morphological description, and the life history of *B. clavata* remains unknown. Dixon & Irvine (1977) considered the prostrate (='Hymenoclonium') phases of *B. asparagoides* and *B. clavata* to be indistinguishable, and Rueness & Åsen (1982) suggested that the 'Hymenoclonium' phases of *Bonnemaisonia* are similar to the prostrate filaments produced by developing carpospores of other red algae with a heteromorphic life cycle, such as members of Nemaliales and Cryptonemiales.

The present study was initiated to clarify the taxonomic status of *B. asparagoides* and *B. clavata*. We have reassessed the proposed diagnostic characters of these two species on the basis of an extensive morphological investigation of the vegetative and reproductive structures of gametangial plants, the vegetative anatomy of their 'Hymenoclonium' phases as well as carpospore germination and development. This morphological study was complemented by a molecular analysis of the *rbc*L gene that encodes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Recent studies confirm the good resolution at the species level of this marker (Harper & Saunders 2001; Saunders & Lehmkuhl 2005; Wilkes *et al.* 2005, 2006).

MATERIAL AND METHODS

Morphological studies

Bonnemaisonia asparagoides and *B. clavata* were collected by scuba along the Atlantic and Mediterranean coasts of Spain. Collections were made during spring–summer (2003, 2004 and 2005) from the sublittoral zone at various localities on the Catalonian coast (Girona) and Balearic Islands (Majorca, Minorca) of the Mediterranean Sea. Additional specimens of *B. asparagoides* were collected in 2005 from the eulittoral zone of the Basque coast (Guipúzcoa) of the Atlantic Ocean. Samples were preserved in 4% formalin–seawater and pressed as herbarium material, and were deposited in the BCN-Phyc. Herbarium (Documentation Center of Plant Biodiversity, Barcelona University, Spain). Iberian specimens of these taxa held at the main national herbaria (ABH-Algae, BCN-Phyc., HGI-A, MA-Algae, MGC-Phyc., SANT-Algae, VAL-Algae) were studied. Material from other geographical areas held at various European herbaria such as the Muséum National d'Histoire Naturelle de Paris (PC), the Botanical Museum of Göteborg (GB), the Herbarium Universitatis Florentinae of Florence (FI) and the Naturhistorisches Museum of Wien (W) were also reviewed for comparison. The lectotypes of both taxa were reviewed. Herbarium abbreviations follow Holmgren *et al.* 1990.

For morphological observations, *c.* 50 specimens of each taxon were examined using both freshly collected and dried material, whereas for anatomical studies, 25 specimens of each taxon were examined using material both freshly collected and liquid preserved. All measurements were obtained only from vegetative structures at the base of the plant and from mature reproductive structures to standardize the data.

For anatomical studies, hand sections were cut with a razor blade and stained in a solution of 1 g of aniline blue, 100 ml of distilled water and 1 ml of acetic acid. The observations were made with a light microscope Nikon Optiphot-2. Line drawings were made using a camera lucida and photographs were taken with a Nikon Coolpix 4500.

Iberian specimens of *B. asparagoides* studied

ATLANTIC OCEAN: Guipúzcoa: Zumaya, 15 May 1987, HGI-A 1273; Ondarreta, 25 May 2005, BCN-Phyc. 1621. Lugo: Rinlo, −3/−5 m, 21 July 1993, cystocarps, SANT-Algae 3904. A Coruña: Fornelos point, −10/−15 m, 07 July 1986, cystocarps, MA-Algae 2492; *ibid.*, SANT-Algae 2768; *ibid.*, −3/−5 m, 08 July 1986, cystocarps, SANT-Algae 594; Ría de Ferrol, −10 m, 13 July 1991, cystocarps and spermatangial branches, SANT-Algae 381; Gaboteira point, −7 m, 26 June 2001, SANT-Algae 13382; Bastiagueiro, −8 m, 23 July 1992, cystocarps, MA-Algae 5079; *ibid.*, drifted, 11 July 1985, cystocarps, SANT-Algae 3311; *ibid.*, cystocarps, SANT-Algae 4390; *ibid.*, −8 m, 23 July 1992, cystocarps, SANT-Algae 13049; Lires, 15 June 1987, cystocarps, SANT-Algae 3312. Pontevedra: Cangas, Borneira point, −5 m, 15 April 1997, cystocarps, SANT-Algae 4998.

MEDITERRANEAN SEA: Almería: Isle of Terreros, June 1984, cystocarps, MGC-Phyc. 1478. Alicante: Isle of Tabarca, 04 May 2004, cystocarps, ABH-Algae 324; Jávea, 22 May 1983, VAL-Algae 40; Portitxol, −8 m, 16 June 1989, VAL-Algae 45; Penyal d'Ifac, −8 m, 11 July 1984, VAL-Algae 646B. Castellón de la Plana: La Ferrera (Columbrets Isles), −5 m, 31 July 04, BCN-Phyc. 1635.

Girona: Blanes, −5 m, 04 May 2005, cystocarps, BCN-Phyc. 1619; Sant Feliu de Guíxols, −8 m, 21 March 1996, cystocarps and spermatangial branches, HGI-A 2545; Calonge, drifted, 07 April 1985, cystocarps, HGI-A 3198; *ibid.*, HGI-A 3197; *ibid.*, 17 April 1988, cystocarps and spermatangial branches, HGI-A 3201; *ibid.*, cystocarps, HGI-A 3200; Palamós, 24 May 1987, cystocarps, HGI-A 3199; Roses, −5/−8 m, 24 April 1996, cystocarps, HGI-A 2541; *ibid.*, cystocarps, HGI-A 3846; Llançà, 14 May 2005, cystocarps, BCN-Phyc. 1615. Majorca: Es Cavalls, −15 m, 02 June 2004, cystocarps, BCN-Phyc. 1623; *ibid.*, −13 m, 02 June 2004, cystocarps, BCN-Phyc. 230; Cala Figuera, −15/−20 m, 05 June 2004, cystocarps, BCN-Phyc. 1620. Minorca: Isle of Aire, −6 m, 20 June 2003, BCN-Phyc. 1636; Cala Piques, −10/−20 m, 24 June 2003, BCN-Phyc. 1637.

Iberian specimens of *B. clavata* studied

MEDITERRANEAN SEA: Alicante: Les Rotes, −8 m, 10 May 1993, VAL-Algae 1168B; Portitxol, drifted, 30 May 1982, BCN-Phyc. 1626; Cova Tallada, drifted, 20 June 2004, cystocarps, ABH-Algae 335. Girona: Palamós, −17 m, 03 May 1993, cystocarps HGI-A 3202, as *B. asparagoides*; *ibid.*, cystocarps, HGI-A 3203 as *B. asparagoides*; *ibid.*, cystocarps, HGI-A 3204 as *B. asparagoides*; *ibid.*, cystocarps, HGI-A 3205 as *B. asparagoides*; *ibid.*, −25 m, 03 May 1993, one specimen with cystocarps the other with spermatangial branches, HGI-A 3206, as *B. asparagoides*; Blanes, BCN-Phyc. 1633; *ibid.*, −5 m, 04 May 2005, BCN-Phyc. 1631; *ibid.*, 23 May 2005, cystocarps, BCN-Phyc. 1616; *ibid.*, −2/−7 m, BCN-Phyc. 1632; Begur, −5 m, 14 April 2005, BCN-Phyc. 1634; *ibid.*, cystocarps, BCN-Phyc. 1617; *ibid.*, −15 m, 14 May 2000, cystocarps, BCN-Phyc. 1628; Llançà, −3 m, 14 May 2005, cystocarps, BCN-Phyc. 1618. Majorca: Faralló d'Aubarca, 06 June 2004, cystocarps, BCN-Phyc. 1622; Cala Bona, 03 June 2004, one specimen with cystocarps and other with spermatangial branches, BCN-Phyc. 229.

Cultures

'Hymenoclonium' phases of *B. asparagoides* and *B. clavata* were cultured from carpospores obtained in the laboratory. Cystocarpic specimens of both species were collected in the Sant Francesc Cove (Girona, Spain) in spring (23 May 2005) and transported in a small refrigerator to the laboratory. Cystocarpic specimens were submerged in small seawater aquaria, and released carpospores were collected on slides over 24 h. Pairs of slides with attached carpospores were transferred to vessels with 200 ml of culture medium prepared from filtered seawater (filter pore size of 0.22 μm), sterilized with a microwave oven (900 W, 10 min) and enriched using half-strength modification of von Stosch's medium (Guiry & Cunningham 1984). GeO_2 (5 mg l^{-1}) and penicillin-G (4 mg l^{-1}) were added to the medium to control diatom and bacterial growth (Vergés *et al.* 2004). Potassium tellurite hydrate [$K_2TeO_3 \cdot H_2O$, 0.01% (M/V = kg/l)] was used when necessary to control cyanobacteria (Ducker & Willoughby 1964). Cultures were maintained under conditions simulating ambient spring (15°C, 15 μmol photons m^{-2} s^{-1}, 12 : 12 light : dark), were agitated daily and the medium was changed weekly. Epiphytes were controlled by cleaning cultivated algae with a paintbrush weekly. For each species, triplicate cultures were carried out.

Carpospore settling time was determined by observations carried out at 3–6 h intervals. Carpospore development was studied by observations of 30 carpospores after 12, 24 and 48 h of culture and was followed by observations every 48 h during the following week. The length of the main filaments and the total surface area of selected germlings were recorded in photographs and line drawings at 8, 10, 12, 19 and 26 days of culture. It should be noted that the germlings were observed weekly during 1 year of culture to determine the life history of these taxa. These observations will be published separately.

Molecular analysis

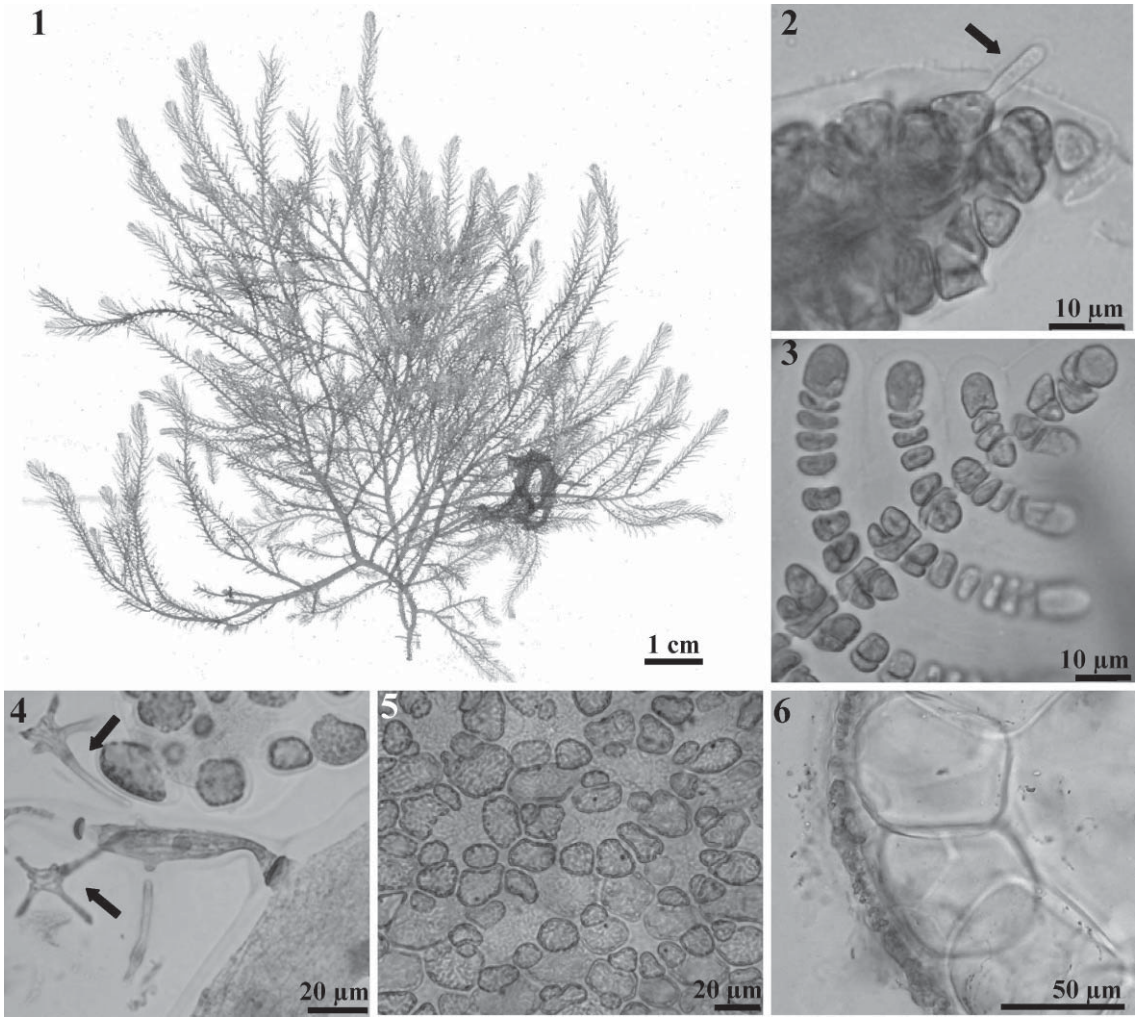
Samples of *B. asparagoides*, *B. clavata* (Girona, Spain, 23 May 2006 and 16 May 2006) and *Bonnemaisonia hamifera* (Guipúzcoa, Spain, 12 May 2006) were collected and dried in silica gel. DNA was extracted according to Saunders (1993), with slight modifications; instead of the final agarose gel cleaning procedure, the DNA was purified through the QIAshredder mini spin columns and DNeasy mini spin columns (DNeasy Plant Mini Kit, QIAGEN, Italy), according to manufacturer instructions. The *rbc*L gene was amplified using either a single primer pair (F57 and R1150), a set of two (F57 and R646, F577 and R1381/F57 and R1150, F765-i/-ii and rbcL-rev) or three pairs of primers (F57 and R646, F481 and R1150, F765-i/-ii and R1381-ii) according to previous authors (Freshwater & Rueness 1994; Wang *et al.* 2000). The purified polymerase chain reaction products were agarose-gel purified (Saunders 1993) and sequenced by an external company (MWG Biotech, Ebersberg, Germany).

Nucleotide sequences were edited and aligned visually by sequential pairwise comparison (Swofford & Olsen 1990) with BioEdit 5.0.9 (Hall 1999). The pairwise distances were calculated with PAUP version 4.0b10 (Swofford 2002) as the percentage of uncorrected nucleotide substitution ('p distance').

RESULTS

Bonnemaisonia asparagoides (Woodward) C. Agardh

Gametangial thalli are erect, brownish-red in colour, sympodial, palmate, 4–10.5 (12) cm long and attached by a small basal disc (Fig. 1). Main axes are highly branched with a distichous pattern, except some specimens collected from 30 m depth that show some branchlets arranged in different planes (irregular distichous pattern). The apical zone of young branches, some with unicellular hairs (Fig. 2), has a single apical cell 8.2–10.2 μm long and 6.1–8.2 μm wide (Fig. 3). Axial filaments are comprised of cells (524–) 736 (–961) μm long and (74–) 84 (–100) μm in diameter (6 to 11 times longer than wide) at the base of the plant, with each axial cell bearing a pair of opposite periaxial cells. Both axial and periaxial cells are uninucleate.

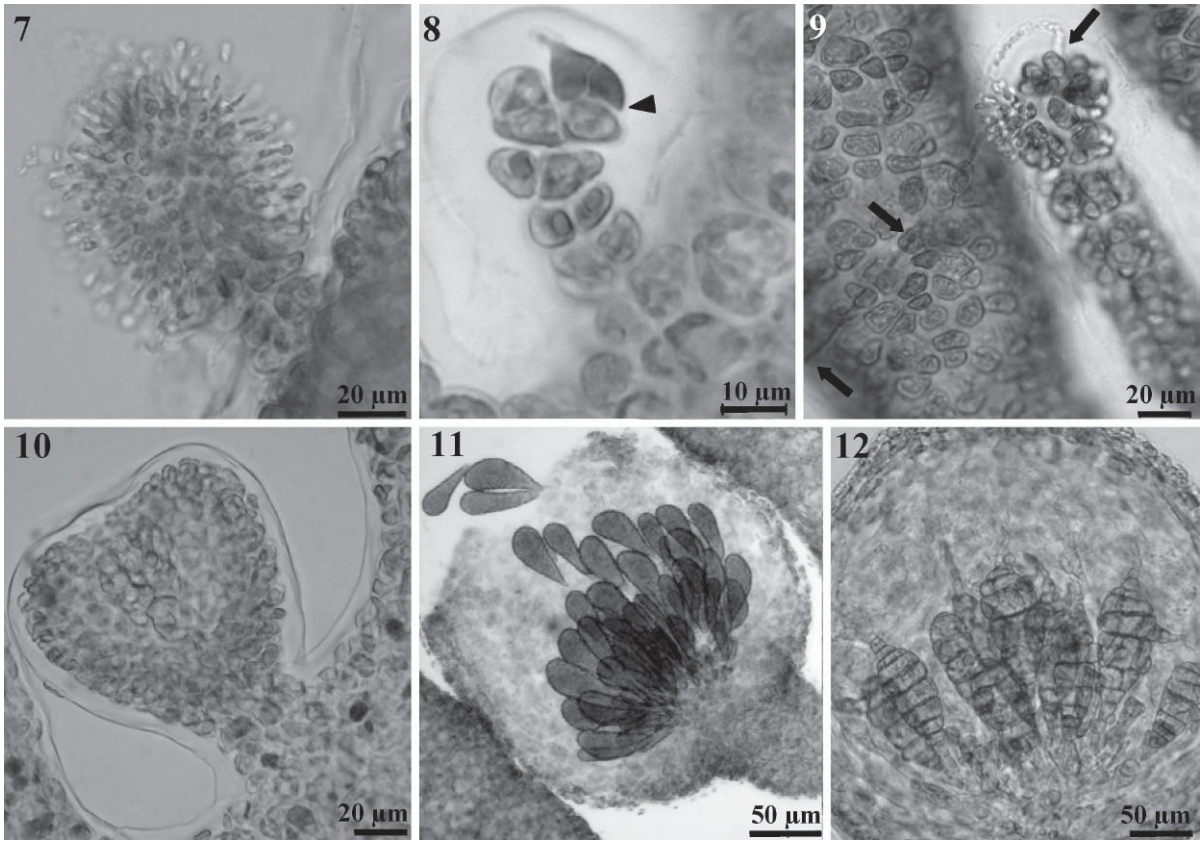


Figs 1–6. Vegetative characters of *Bonnemaisonia asparagoides* gametangial plant.
Fig. 1. Habit (BCN-Phyc. 1623).
Fig. 2. Unicellular hair (arrow) at apical zone of young branch (VAL-Algae 646B).
Fig. 3. Detail of apex (VAL-Algae 646B).
Fig. 4. Stellate cells (arrows) from a periaxial cell (VAL-Algae 646B).
Fig. 5. Outer cortical cells in surface view (VAL-Algae 646B).
Fig. 6. Cross-section showing inner cortical cells (VAL-Algae 646B).

Each periaxial cell forms three stellate cells (Fig. 4); each one bears additional cells that form the cortex. This cortex is three-cell layered and separated from the axial filament by a space produced by the periaxial cells. Outer cortical cells are irregular in surface view and radially arranged around the subcortical cells (Fig. 5). Inner cortical cells are spherical and uninucleate, with a large central vacuole and peripheral cytoplasm (Fig. 6). Vesicle cells are apparent in the outermost cortex, especially in the youngest branches. Elongated cortical cells were observed in some specimens covering damaged parts or assuming the aspect of long rhizoids.

Plants are monoecious. Spermatangial branches are (40–) 100 (–180) µm long and (40–) 64 (–100) µm in diameter, short stalked and clavate (Fig. 7). Carpogonial branches are three-celled, with a long trichogyne (57–) 152 (–214) µm long (Figs 8–9). Pericarp is symmetric (Fig. 10). Cystocarps are stalked and globular, (286–) 393 (–572) µm long and (200–) 387 (–646) µm in diameter (Fig. 11). Each cystocarp contains fewer than 35 pear-shaped carposporangia measuring (80–) 117 (–155) µm long and (20–) 49 (–71) µm in diameter. Occasionally, germination of carpospores within the cystocarp was observed (Fig. 12).

Carpospore attachment to the substratum occurs at 36 h. At 12 h after settling, 56% of carpospores had not divided, 38% had divided once in a perpendicular plane to the substratum, and the remaining 6% had secondary divisions (Figs 13, 14). At 24 h, 50% had divided once, and the rest showed secondary divisions in planes parallel to the first division (Fig. 15). At 48 h, 40% had divided only once, whereas the remaining 60% showed new perpendicular divisions, resulting in germlings with up to 10 cells (Figs 13, 16). At this time, the initial cells of uniseriate filaments originated as protuberances from carpospores with one or more divisions. Of these germlings, 45% showed protuberances, 10% with one and 90% with two. Between the third and fourth day of culture, the number of protuberances initiated from the outer border cells increased (4–10) and



Figs 7–12. Reproductive characters of *Bonnemaisonia asparagoides* gametangial plant.
Fig. 7. Short-stalked spermatangial branch (BCN-Phyc. 1620).
Fig. 8. Three-celled carpogonial branch (arrowhead) (BCN-Phyc. 1620).
Fig. 9. Carpogonial branch with long trichogyne (arrows) (BCN-Phyc. 1620).
Fig. 10. Symmetric pericarp (BCN-Phyc. 1620).
Fig. 11. Stalked cystocarp with carposporangia (BCN-Phyc. 1620).
Fig. 12. Germinated carpospores within the cystocarp (BCN-Phyc. 1615).

began to divide by transverse walls, developing crusts of uniseriate, radially arranged filaments recognizable as 'Hymenoclonium' (Fig. 17).

Observations of selected germlings of *B. asparagoides* in the first month of growth (Figs 17–20) showed that main filaments reached a mean length of 283 µm after 26 days of culture, with a uniform growth rate during this time ranging between 7 and 11 µm day^{-1} (Figs 22, 23). The total surface area of these filaments reached an average of 0.21 mm^2 after 26 days, with a range of 0.010–0.015 mm^2 day^{-1} for the first five days and 0.043–0.11 mm^2 day^{-1} for the remaining days (Figs 24, 25).

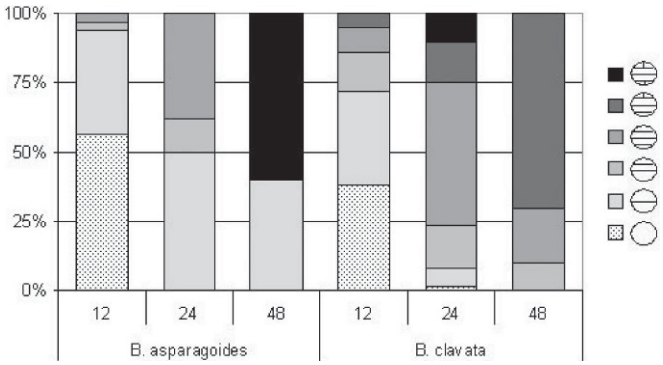
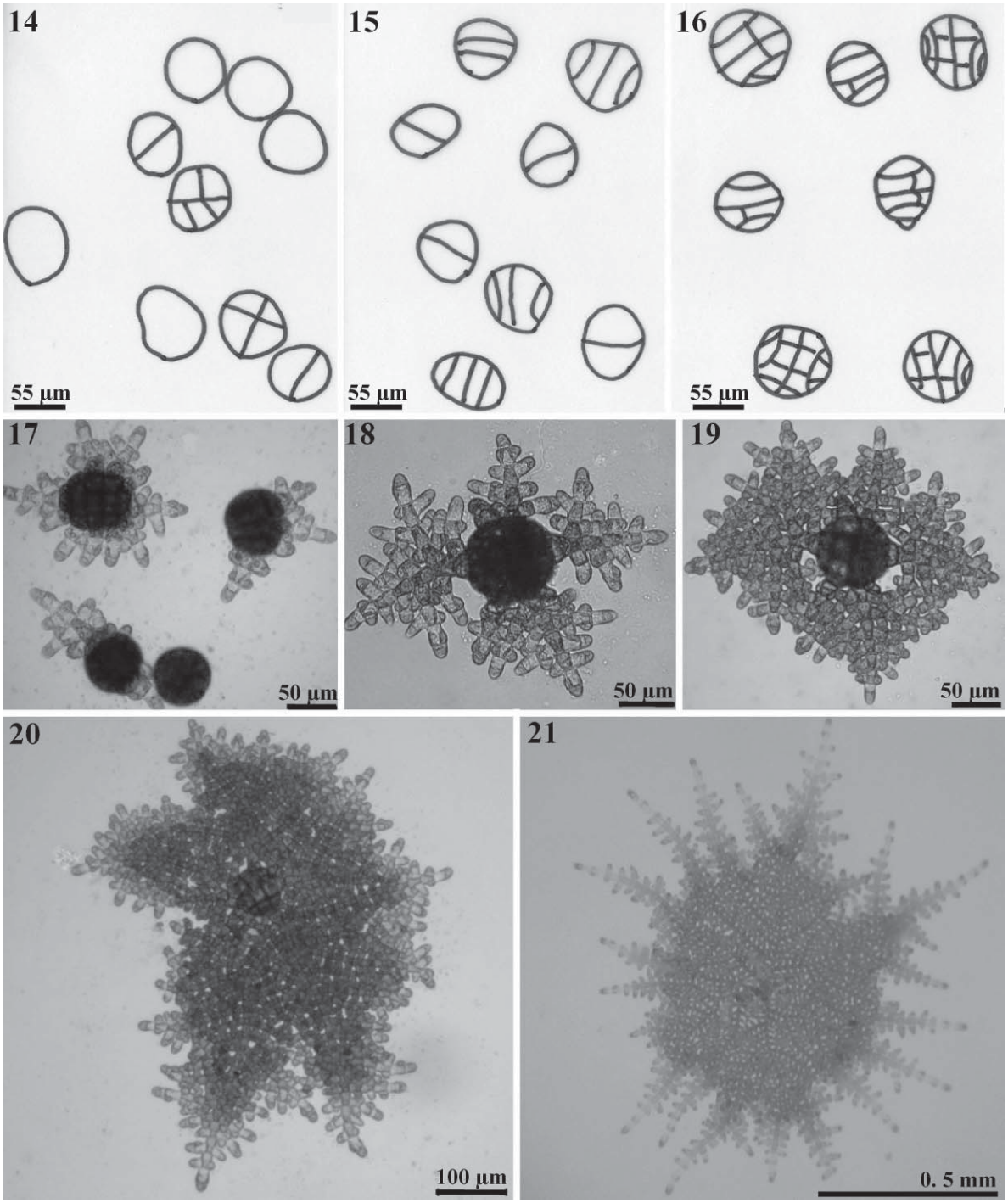


Fig. 13. Comparison of percentage of carpospore segmentation stages at 12, 24 and 48 h.

'Hymenoclonium' filaments, initially unbranched, began to divide after five days of culture (Fig. 17), producing up to fourth-order branching in well-developed specimens (Fig. 21). Main axes were formed by elongated cells (41–) 50 (–61) µm long and (20–) 22 (–24) µm wide, branched in two opposite, unequal and alternate components (Fig. 26). Branches included elongated cells (26–) 36 (–45) µm long that formed hemispheric cells or pluricellular rhizoids (Fig. 27). All cells, except the hemispheric cells, produced vesicular cells in their distal part (Fig. 28). The resulting thallus (=*H. serpens*) after *c.* 2 months of culture is a red disc-shaped crust 1.2–1.4 cm in diameter without reproductive structures (Fig. 21).

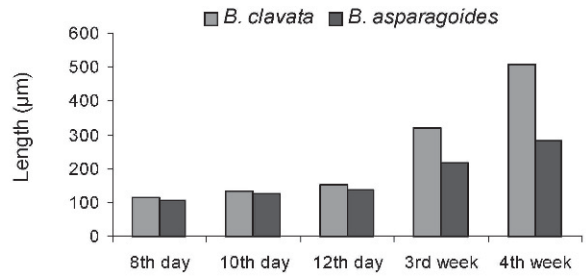
Bonnemaisonia clavata Hamel

Gametangial thalli are erect, reddish-pink in colour, monopodial, triangular, 5–15 (19) cm long and attached by a small basal disc (Fig. 29). The main axis is highly branched with some lateral branches growing in different planes, resulting in an irregularly distichous branching pattern. The apical zone of young branches, some with



Figs 14–21. Germination and developmental stages of the 'Hymenoclonium' phase of *Bonnemaisonia asparagoides*.
Fig. 14. Segmentation stages of carpospores at 12 h after settling on slides.
Fig. 15. Segmentation stages at 24 h.
Fig. 16. Segmentation stages at 48 h.
Fig. 17. 'Hymenoclonium' phase after 5 days.
Fig. 18. 'Hymenoclonium' phase after 8 days.
Fig. 19. 'Hymenoclonium' phase after 13 days.
Fig. 20. 'Hymenoclonium' phase after 4 weeks.
Fig. 21. Disc-shaped crust after 2 months.

unicellular hairs (Fig. 30), has a single apical cell 8.2 µm long and 6.1 µm wide (Fig. 31). Axial filament are comprised of cells (850–) 1338 (–1513) µm long and (49–) 57 (–155) µm in diameter (15 to 30 times longer than wide) at the base of the plant. Each axial cell bears a pair of opposite periaxial cells. Both axial and periaxial cells are uninucleate. Each periaxial cell forms three stellate cells (Fig. 32); each one bears additional cells that form the



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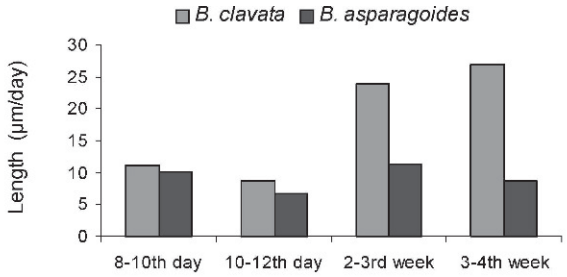

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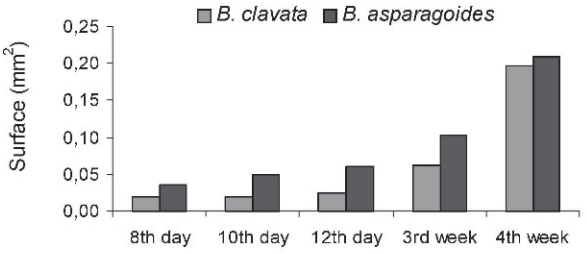

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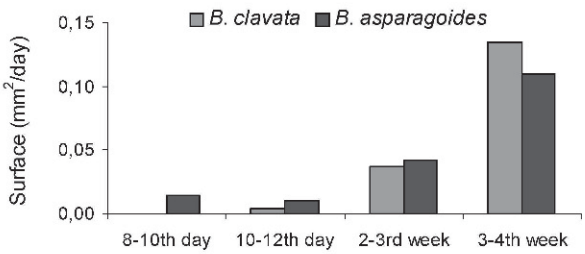

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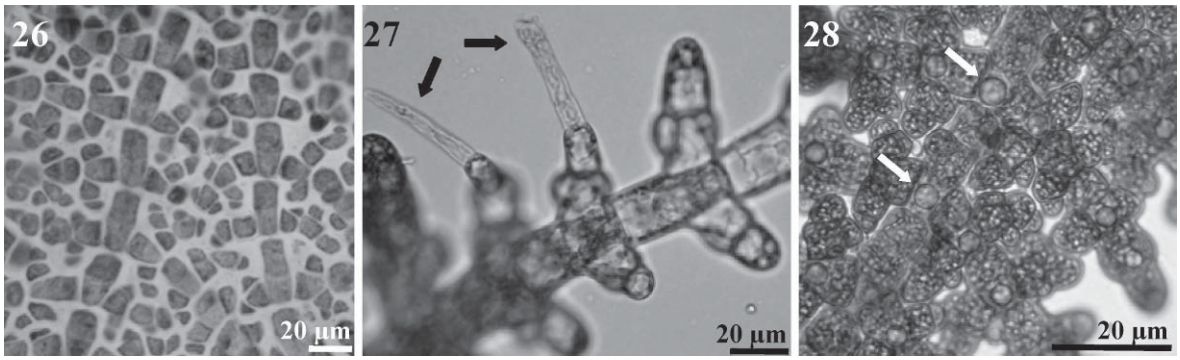
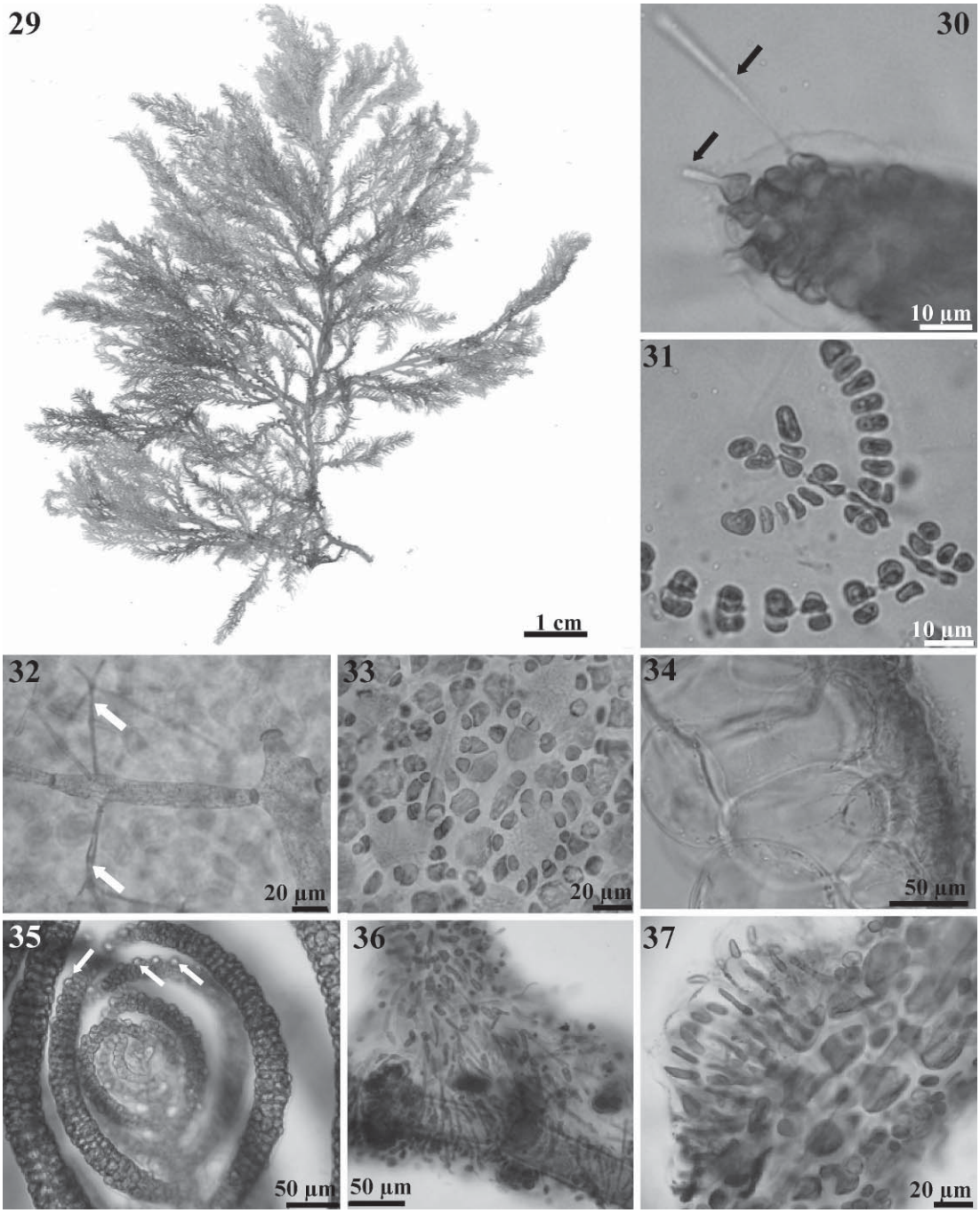
Figs 22–25. 'Hymenoclonium' growth during 1 month.
Fig. 22. Mean length of main filaments.
Fig. 23. Growth rate in length of main filaments.
Fig. 24. Mean surface of total area.
Fig. 25. Growth rate in total surface area.

cortex. This cortex is three-cell layered and separated from the axial filament by a space produced by the periaxial cells. Outer cortical cells are irregular in surface view and radially arranged around subcortical cells (Fig. 33), whereas inner cortical cells are ovoid and uninucleate, with a large central vacuole and peripheral cytoplasm (Fig. 34). The thallus bears vesicle cells in the outermost cortex, which are most abundant in the youngest branches (Fig. 35). Some specimens showed elongated cortical cells covering damaged parts or adhering to some objects (Figs 36, 37).

Plants are dioecious. Spermatangial branches are (140–) 757 (–981) µm long and (140–) 295 (–360) µm in diameter, long stalked and clavate (Fig. 38). Carpogonial branches are three-celled with a spiral trichogyne (92–) 107 (–122) µm long (Figs 39, 40). Pericarp is slightly asymmetric, with one side wider than the other (Fig. 41). Cystocarps are stalked



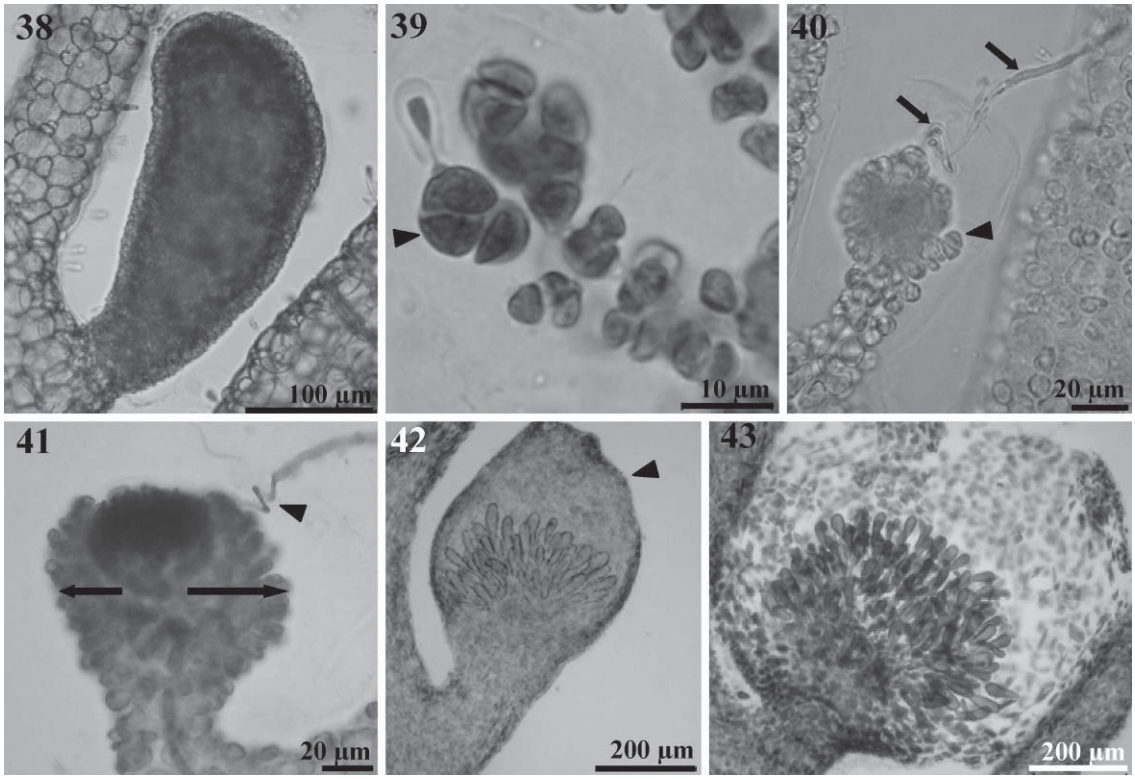
Figs 26–28. Vegetative characters of *Bonnemaisonia asparagoides* prostrate phase ('Hymenoclonium') (BCN-Phyc. 2735).
Fig. 26. Morphology of 'Hymenoclonium' phase.
Fig. 27. Pluricellular rhizoids (arrows) on distal part of crust.
Fig. 28. Vesicular cells (arrows).



Figs 29–37. Vegetative characters of *Bonnemaisonia clavata* gametangial plant.
Fig. 29. Habit (BCN-Phyc. 1618).
Fig. 30. Unicellular hairs (arrows) of apical zone of young branch (BCN-Phyc. 1616).
Fig. 31. Detail of apex (BCN-Phyc. 1616).
Fig. 32. Stellate cells (arrows) from a periaxial cell (BCN-Phyc. 1616).
Fig. 33. Outer cortical cells in surface view (BCN-Phyc. 1616).
Fig. 34. Cross-section of inner cortical cells (BCN-Phyc. 1616).
Fig. 35. Vesicular cells (arrows) in outermost cortex (BCN-Phyc. 1616).
Fig. 36. Elongated cortical cells adhering to an object (BCN-Phyc. 1631).
Fig. 37. Elongated cortical cells covering damaged cortex (BCN-Phyc. 1631).

and spherical to ovoid, (491–) 604 (–859) µm long and (449–) 614 (–969) µm in diameter (Figs 42, 43). Each cystocarp contains more than 60 pear-shaped carposporangia measuring (57–) 75 (–102) µm long and (20–) 36 (–70) µm in diameter. Occasionally, germination of carpospores within the cystocarp was observed.

Carpospore attachment to the substratum occurs at 12 h. At 12 h after settling, 38% of carpospores had not divided,



Figs 38–43. Reproductive characters of *Bonnemaisonia clavata* gametangial plant.
Fig. 38. Long-stalked spermatangial branch (BCN-Phyc. 1632).
Fig. 39. Three-celled carpogonial branch (arrowhead) (BCN-Phyc. 1616).
Fig. 40. Carpogonial branch with spiral trichogyne (arrows) and lateral branch (arrowhead) (BCN-Phyc. 1616).
Fig. 41. Asymmetric pericarp (arrows) with spiral trichogyne (arrowhead) (BCN-Phyc. 1617).
Fig. 42. Ovoid cystocarp with asymmetric side (arrowhead) and carposporangia (BCN-Phyc. 1631).
Fig. 43. Spherical cystocarp with carposporangia (BCN-Phyc. 1616).

34% divided by means of a plane perpendicular to the substratum, 14% showed a secondary division in a parallel or perpendicular plane to the first division, 9% showed two secondary divisions, and the remaining 5% of carpospores showed three secondary divisions (Figs 13, 44). At 24 h of culture, 1% had not divided, 7% showed the first perpendicular division, 15% another division, 52% two secondary divisions, 15% three secondary divisions and the remaining 10% more than three secondary divisions (Figs 13, 45). At the same time, 70% of carpospores developed one (24%), two (74%) or three (2%) protuberances (Fig. 45). At 48 h, 70% of carpospores showed more than two secondary divisions producing germlings with up to nine cells, 20% showed two secondary divisions and only 10% still showed the first secondary division. In this time, the number of protuberances per germling increased: 43% had two protuberances, 27% four, 13% three, 10% five and 7% six (Figs 13, 46). Although all of these were cut off by transverse walls, only the two opposite protuberances formed uniseriate filaments, developing 'Hymenoclonium' crusts with two opposite main axes (Figs 47, 48).

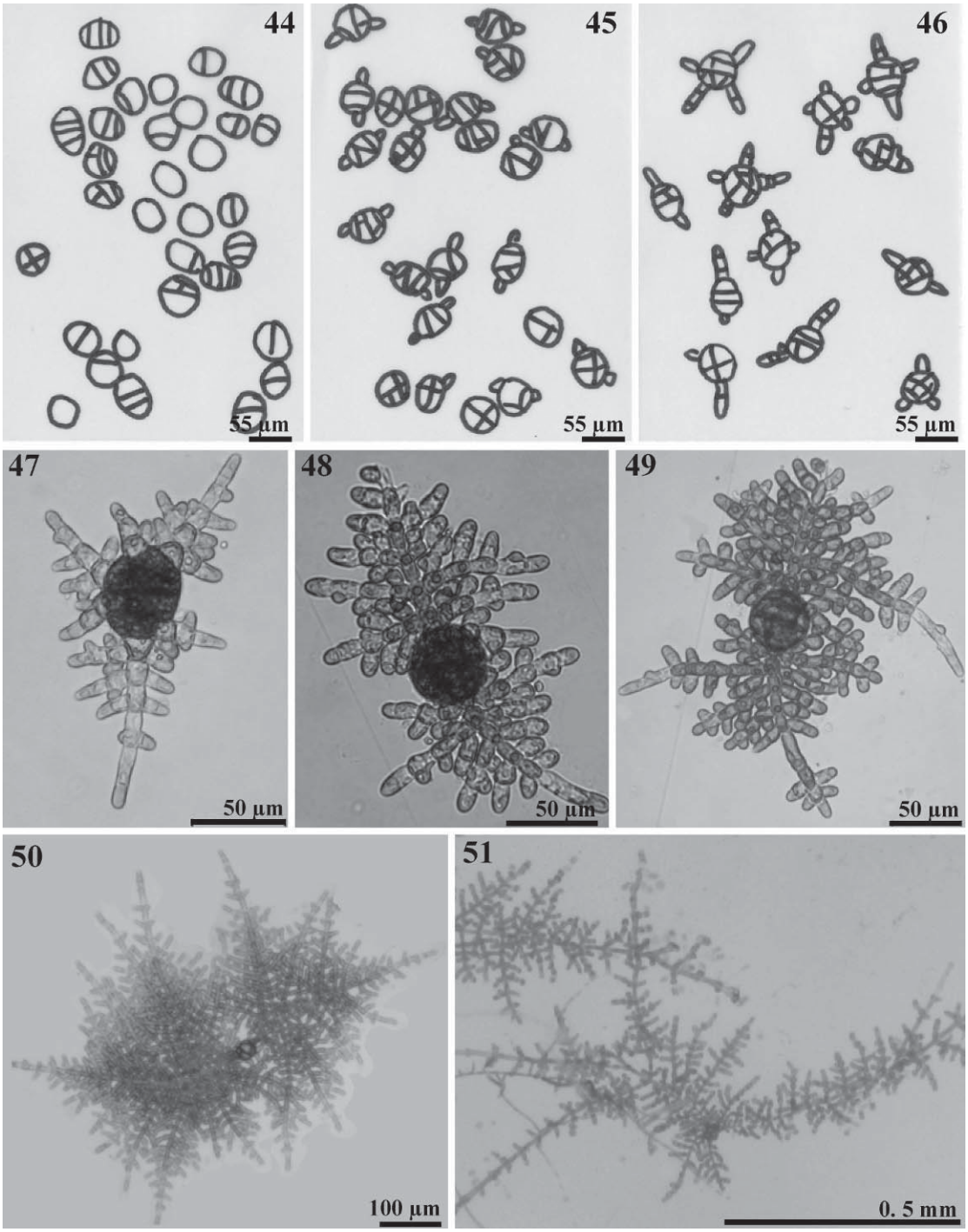
Observations made on selected germlings of *B. clavata* until *c.* the first month of growth (Figs 47–50) showed that the polar filaments reached a mean length of 509 µm after 26 days of culture, with a growth rate of 9–11 µm day^{-1} during the first five days and 24–27 µm day^{-1} in the following days (Figs 22, 23). The total surface reached an average of 0.20 mm^2 after 26 days, with an insignificant growth rate during the first days and 0.14 mm^2 day^{-1} in the last week (Figs 24, 25).

'Hymenoclonium' filaments, initially unbranched, began to divide after 5 days of culture (Fig. 47), resulting in up to fourth-order branching in well-developed specimens (Fig. 51). Main axes were formed by elongated cells (51–) 66 (–82) µm long and (10–) 12 (–14) µm wide, with two opposite branches composed of elongated cells (14–)18 (–20) µm long and terminating in spherical cells (Fig. 52). Vesicular cells were observed in the distal parts of elongated branches (Fig. 53). The resulting thallus after *c.* 2 months of culture is an elongated crust, red in colour, without reproductive structures (Fig. 51).

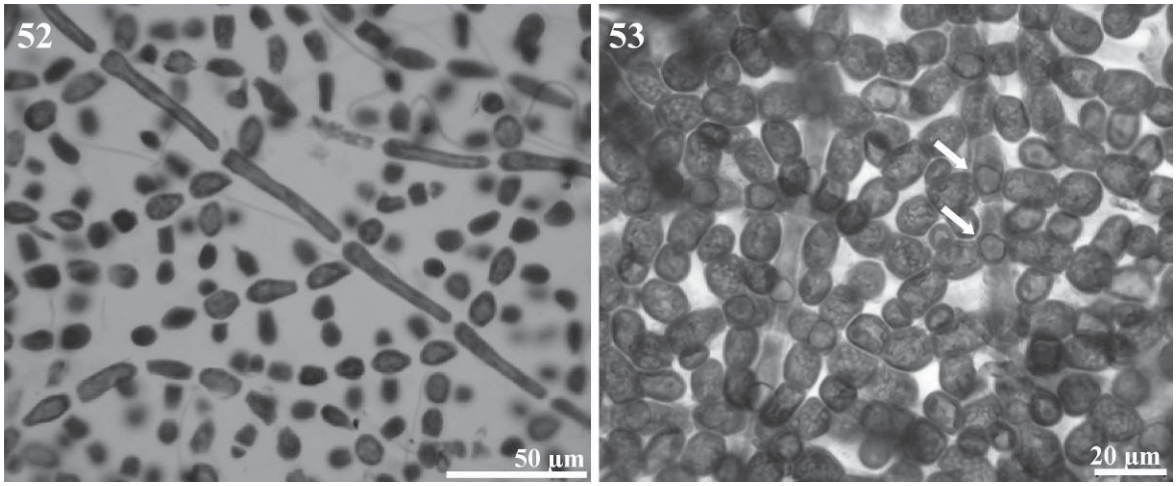
The diagnostic characters used to distinguish the gametangial plants of *B. asparagoides* and *B. clavata*, as well as their prostrate 'Hymenoclonium' phases, are summarised in Tables 1 and 2, respectively.

Distance analysis

Partial *rbc*L sequences of 1335 to 1366 were generated for the studied *B. clavata* and *B. asparagoides* specimens. No sequence of *B. hamifera* was obtained despite repeated attempts. Previously published Bonnemaisoniaceae



Figs 44–51. Germination and developmental stages of 'Hymenoclonium' phase of *Bonnemaisonia clavata*.
Fig. 44. Segmentation stages of carpospores at 12 h after settling on slides.
Fig. 45. Segmentation stages at 24 h.
Fig. 46. Segmentation stages at 48 h.
Fig. 47. 'Hymenoclonium' phase after 5 days.
Fig. 48. 'Hymenoclonium' phase after 9 days.
Fig. 49. 'Hymenoclonium' phase after 14 day.
Fig. 50. 'Hymenoclonium' phase after 4 weeks.
Fig. 51. Elongated and irregular crust after 2 months.



Figs 52–53. Vegetative characters of the *Bonnemaisonia clavata* prostrate phase ('Hymenoclonium') (BCN-Phyc. 2736).
Fig. 52. Morphology of 'Hymenoclonium' phase.
Fig. 53. Vesicular cells (arrows).

sequences were obtained from GenBank and included in the alignment for analysis. No insertions or deletions were found, making the alignment unambiguous. Pairwise sequence divergences were calculated from two different permutations of the alignment: (1) the data set was contracted to 666 base pairs, the length of the shortest included sequence (*Asparagopsis armata*, GenBank accession U04043); (2) the shortest sequence was included in the full alignment for pairwise sequence divergence calculations.

Sequence divergences ranged from 5.28% (*Delisea flaccida* vs *Delisea pulchra*) to 12.54% (*D. pulchra* vs *B. hamifera*) between the included Bonnemaisoniaceae. Interspecific sequence divergences between *Bonnemaisonia* species ranged from 7.66% (*B. clavata* vs *B. asparagoides*) to 12.17% (*B. asparagoides* vs *B. hamifera*).

DISCUSSION

Gametangial plants

In the specimens studied, *B. asparagoides* appears redder than *B. clavata*, which is a reddish-pink colour, as observed by Dixon & Irvine (1977) in material from the British Isles. However, this character is only apparent when the algae are submerged in seawater, whereas this difference is not so clear in herbarium specimens. Dixon & Irvine (1977) reported the height of *B. asparagoides* and *B. clavata* specimens up to 40 cm and 15 cm, respectively. In contrast, *B. clavata* specimens were taller than those of *B. asparagoides*. The branching pattern of *B. asparagoides* observed in this study was consistently distichous, in contrast to the irregular branching pattern of *B. clavata* (Table 1), in agreement with Feldmann & Feldmann (1942). However, as was mentioned in the results, this character could vary with the depth. Given that the three aforementioned characters (related to the habit of the plant) are variable, it is possible to conclude that they have little taxonomic value. In this study, we suggest two new diagnostic characters related to the habit: shape and structure of the thallus. In *B. asparagoides* the thallus is palmate and sympodial, whereas in *B. clavata* it is triangular and monopodial (Figs 1, 26).

Dixon & Irvine (1977) proposed several anatomical characters for distinguishing between these species, including the main axis diameter (*B. asparagoides* 800 µm and *B. clavata* 500 µm), the length of the longer branchlet (to 3 and 15 mm, respectively) and the orientation angle of the lateral branchlet with respect to the main axis (45–60° and 80–100°, respectively). The specimens studied herein showed similar values for all of these characters, suggesting that they cannot be used to differentiate the two species. However, we propose using the size of the axial cell as a new vegetative anatomical character for species recognition. *Bonnemaisonia asparagoides* has axial cells 6 to 11 times longer than wide, whereas *B. clavata* has axial cells 15 to 30 times longer than wide (Table 1).

The monoecious (*B. asparagoides*) or dioecious character (*B. clavata*) has been used the most to differentiate these species (Hamel 1930; Feldmann & Feldmann 1942; Dixon & Irvine 1977), and our examinations of herbarium specimens confirms that this character is species specific. In addition, we found the spermatangial branch of *B. asparagoides* to be smaller than in *B. clavata*, as previously reported (Hamel 1930; Dixon & Irvine 1977). In fact, the large size and shape of the spermatangial branch were the primary diagnostic characters used by Hamel (1930) to recognize *B. clavata* as a distinct species. Other characters commonly used include the cystocarp and carposporangia sizes. Some authors described (Svedelius 1933; Dixon & Irvine 1977) or illustrated (Coppejans 1983) ovoid and spherical cystocarps in *B. clavata* and *B. asparagoides,* respectively. However, in Iberian specimens of *B. clavata*, both spherical and ovoid mature cystocarps were observed. Carposporangial size was greater in *B. asparagoides* than in *B. clavata* as reported by Dixon & Irvine (1977) and Feldmann & Feldmann (1942). These observations suggest that the smaller carposporangial size indicated by Hamel (1930) for *B. asparagoides* actually corresponds to *B.*

Table 1. Distinguishing features for gametangial plants of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*. Newly proposed characters are highlighted in bold print.

Features	Species	
	B. asparagoides	*B. clavata*
Colour when fresh	brownish-red	reddish-pink
Thallus structure	sympodial	monopodial
Thallus shape	palmate	triangular
Branching	distichous (except specimens from 30 m depth)	irregularly distichous (always)
Axial cell size (μm)	6–11 length/width	15–30 length/width
Inner cortical cells	spherical	ovoid
Monoecious/dioecious	monoecious	dioecious
Spermatangial branch length × width (μm)	(40–) 100 (–180) × (40–) 64 (–100)	(140–) 757 (–981) × (140–) 295 (–360)
Trichogyne shape	straight	spiral
Pericarp	symmetric	asymmetric
Cystocarp		
Shape	spherical	spherical to ovoid
Length × width (μm)	(286–) 393 (–572) × (200–) 387 (–646)	(491–) 604 (–859) × (449–) 614 (–969)
Carposporangia		
Number	< 35	> 60
Length × width (μm)	(80–) 117 (–155) × (20–) 49 (–71)	(57–) 75 (–102) × (20–) 36 (–70)

clavata, demonstrating again the historic confusion between these taxa. Concerning the reproductive features, we propose new characters to distinguish the taxa: the length and shape of the trichogyne, the symmetry or asymmetry of the pericarp and the number of carposporangia per cystocarp. In *B. asparagoides* the trichogyne is long and straight, whereas in *B. clavata* it is shorter and spiral. Therefore, the descriptions and illustrations of the spiral trichogyne of *B. asparagoides* (Kylin 1916; Svedelius 1933) must refer to *B. clavata*. The pericarp of *B. asparagoides* is symmetric, in contrast to the asymmetric pericarp of *B. clavata.* This asymmetry is due to the development, on the basal part of the pericarp, of a lateral branch that later fuses to the pericarp. Concerning the number of carposporangia per cystocarp, there is an important difference: *B. asparagoides* exhibits fewer than 35, whereas *B. clavata* has more than 60 (Table 1).

Some original observations for *B. clavata* are provided in this study. The hyaline unicellular hairs, observed by Rueness & Åsen (1982) in culture specimens of *B. asparagoides*, were found in most of the collected specimens of both *B. asparagoides* and *B. clavata*. These hairs could be associated with the absorption of nutrients during active growth, and their presence may fluctuate with seasonal and environmental conditions (Dixon 1973; Ribera Siguan & Soto Moreno 1992). *In situ* germination of carposporangia within the cystocarp was observed in some specimens of *B. clavata*, as previously described for *B. asparagoides* (Feldmann & Feldmann 1942; De Valera & Falan 1964). Finally, in some specimens of *B. asparagoides* and *B. clavata*, elongated rhizoid-like cells were observed for the first time. These cells covered damaged parts of the cortex or they were intricately entangled with other algae and objects in the water. A similar tissue also was described in *A. armata* (Svedelius 1933) and in the hook-like branchlets of *B. hamifera* (Chemin 1928).

Prostrate phases

In *B. asparagoides*, carpospore segmentation (Golenkin 1894; Kylin 1917; Chemin 1937; Feldmann & Feldmann 1942) and germination (Feldmann & Feldmann 1942) had previously been studied, but for *B. clavata* these processes were only briefly mentioned (Feldmann & Feldmann 1942).

Table 2. New diagnostic features of 'Hymenoclonium' phase in *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*.

Features	Species	
	B. asparagoides	*B. clavata*
Germination	4–10 protuberances	2–6 protuberances
Shape	discoidal	elongated
Branching	unequal and alternate	opposite
Cells (first order)		
Shape	barrel-shaped	elongated
Width (μm)	(20–) 22 (–24)	(10–) 12 (–14)
Cells (second–third order)		
Length (μm)	(26–) 36 (–45)	(14–) 18 (–20)
Cells (fourth order)		
Shape	hemispherical	spherical
Rhizoids	pluricellular	absent

In our observations, carpospores of *B. clavata* attached more quickly compared with those of *B. asparagoides* (12 and 24 h, respectively), perhaps as a result of the difference in the length of time required for the release of carpospores from the cystocarp. Similarly, carpospore segmentation occurred faster in *B. clavata* than in *B. asparagoides* (Fig. 13), and protuberances appeared in the former (at 24 h) before they did in the latter (after 48 h). The germination process corresponds to the *Naccaria* type (Chemin 1937).

The two taxa exhibited a different pattern of 'Hymenoclonium' phase development. *Bonnemaisonia asparagoides* forms 4–10 protuberances that produce radially arranged filaments with similar lengths (stellate pattern), whereas *B. clavata* forms 2–6 protuberances, although only two opposite main filaments ultimately develop (bipolar pattern). Consequently, the 'Hymenoclonium' phase of *B. asparagoides* is a disc-shaped crust, whereas *B. clavata* is an irregular elongated crust (Figs 21, 51). A different growth rate of the germlings of both taxa was also observed. During the first 2 weeks, the growth rate in length was similar for both taxa, whereas in the third week *B. clavata* doubled, compared with *B. asparagoides* (24 and 11 μm day^{-1}, respectively), and in the fourth week tripled (27 and 8 μm day^{-1}, respectively). Thus, the average length of the main filaments of *B. clavata* (509 μm) doubled compared with *B. asparagoides* (283 μm) after a month of culture (Figs 22, 23). In contrast, in all the observations recorded, the surface area of *B. asparagoides* was greater than in *B. clavata*. However, the surface growth rate after 1 month of culture was greater in *B. clavata* than in *B. asparagoides* as a result of the elevated growth of the main filaments of *B. clavata* during the third and fourth weeks (Figs 24, 25). The differences observed in growth rate can be related to the different development patterns of these taxa. In addition to the different shapes of the prostrate crusts, other noted morphological differences observed include branching type, presence or absence of rhizoids, shape of fourth-order cells and size of first-, second- and third-order cells. It should be noted that the greater size of the cells in the prostrate phase of *B. clavata* compared with *B. asparagoides* coincides with the size observed in the axial cells of their gametophytes. Moreover, a relationship seems to exist between the developmental pattern of the 'Hymenoclonium' phase and the structure of its gametophyte; the monopodial structure of *B. clavata* corresponds to a bipolar pattern of the prostrate phase and the sympodial structure of *B. asparagoides* corresponds to a stellate pattern.

Molecular analysis

The *rbc*L sequence divergence between *B. asparagoides* and *B. clavata* is relatively high at 7.66% and within the range of interspecific sequence divergences seen in the Bonnemaisoniaceae (this study) and other red algal families such as Halymeniaceae (Wang *et al.* 2000; De Clerck *et al.* 2005) and Rhodymeniaceae (Wilkes *et al.* 2006). The level of divergence supports the recognition of *B. asparagoides* and *B. clavata* as separate species.

In conclusion, we affirm that *B. asparagoides* and *B. clavata* are two well-characterized species. In addition to the monoecious/dioecious character and the size of the spermatangial branches, features traditionally used to differentiate both species, we propose the monopodial/sympodial thallus structure and the length/width ratio of the axial cells as the clearest and most useful new diagnostic characters. The review of herbarium material has allowed us to document the frequent confusion between *B. asparagoides* and *B. clavata*, to give new citations of *B. clavata* on the Iberian Peninsula as well as to provide a more accurate distribution of both taxa in this geographical area (Salvador *et al.* 2006). It will be necessary to review all the herbarium specimens upon which previously published citations are based to provide an accurate worldwide distribution.

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Estudio taxonómico del género Bonnemaisonia C. Agardh en la Península Ibérica.

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ESTUDIO TAXONÓMICO DEL GÉNERO *BONNEMAISONIA* C. AGARDH EN LA PENÍNSULA IBÉRICA

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INTRODUCCIÓN

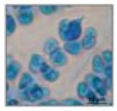
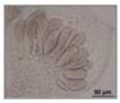
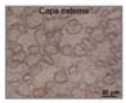
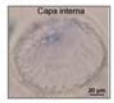
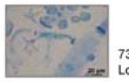
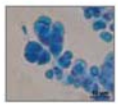
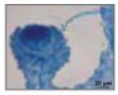
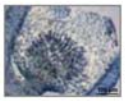
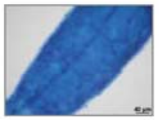
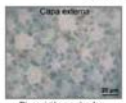
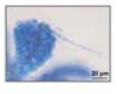
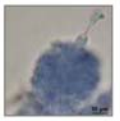
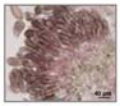
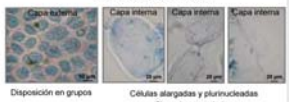
La familia *Bonnemaisoniaceae* Schmitz in Engler (Bonnemaisoniales, Rhodophyta) está ampliamente distribuida en las regiones templadas, tropicales y subtropicales de todos los océanos y comprende los géneros *Asparagopsis* Montagne, *Bonnemaisonia* C. Agardh, *Delisea* Lamouroux, *Leptophyllis* J. Agardh y *Ptilonia* J. Agardh. Esta familia ha sido objeto de numerosos estudios referentes a su gran poder de dispersión (Chemin 1928), a su ciclo biológico heteromórfico (Feldmann y Feldmann 1942; Chihara 1961, 1962) y al contenido de sus células vesiculares y a la bioactividad de sus metabolitos secundarios (Wolck 1968; McConell y Fenical 1979).

El género *Bonnemaisonia* está representado a nivel mundial por siete especies (http://www.algaebase.org) de las cuales *B. asparagoides* (Woodward) C. Agardh, *B. clavata* G. Hamel y *B. hamifera* Hariot están presentes en las costas de la Península Ibérica y las islas Baleares. El objetivo de este trabajo ha sido realizar la revisión taxonómica de este género para el proyecto "Flora phycologica iberica". Este estudio ha permitido establecer los principales caracteres morfológicos y anatómicos de las estructuras vegetativas y reproductoras distintivas de las tres especies, realizar una clave dicotómica para su identificación y actualizar sus mapas de distribución en el área estudiada.

MATERIAL Y MÉTODOS

El estudio se ha realizado a partir del material recogido a lo largo de las costas de la Península Ibérica y las Islas Baleares. Este material ha sido conservado en agua de mar y formol al 4%, así como en pliegos de herbario depositados en el herbario BCN-Phyc (Universitat de Barcelona). Asimismo ha sido revisado todo el material correspondiente a estos táxones, de esta zona geográfica, conservado en los principales herbarios españoles (ABH-Algae, BCN-Phyc., HGI-A, MA-Algae, MAF-Algae, MGC-Phyc., SANT-Algae, VAB-Phyc.) y en el Muséum national d'histoire naturelle de París (PC). La tinción de los ejemplares con azul de anilina ha permitido una mejor visualización de algunas estructuras de difícil observación tales como sinapsis, núcleos y rama carpogonial. Los mapas de distribución han sido realizados con el programa MapInfo Professional Version 4.

RESULTADOS

ESPECIES	ESTRUCTURAS REPRODUCTORAS			ESTRUCTURAS VEGETATIVAS	MAPAS DE DISTRIBUCIÓN
B. asparagoides  Marrón-rojizo Palmatiforme Dística SANT-Algae 4998	 **Rama carpogonial** 3 células Sin papila  **Tricógina** 152 ± 83 µm No espiralada	 **Cistocarpo** Desarrollo postfecundación 406 ± 91µm x 387 ± 131µm  **Carpósporas** 117 ± 19 µm x 49 ± 17 µm Nº por cistocarpo inferior a 35	**Rama anteridial**  101 ± 38 µm x 63 ± 18 µm	**Células corticales**  Disposición en circulos  Células redondas y uninucleadas Ausencia de sinapsis secundarias Sin rizinas  **Eje axial** 736 ± 208 µm x 84 ± 13 µm Longitud/diámetro = 9 ± 8,9	 Citas bibliográficas Materia de herbario
B. clavata  Rojo-rosado Porte triangular Generalmente dística MGC-Phyc. 3745	 **Rama carpogonial** 3 células Sin papila  **Tricógina** 107,6 ± 13,5 Espiralada	 **Cistocarpo** Desarrollo postfecundación 623 ± 122 µm x 614 ± 177 µm  **Carpósporas** 75 ± 20 µm x 36 ± 18 µm Nº por cistocarpo superior a 60	**Rama anteridial**  756 ± 161µm x 295 ± 68 µm	**Células corticales**  Disposición en circulos  Células redondas y uninucleadas Ausencia de sinapsis secundarias Sin rizinas  **Eje axial** 1338 ± 294 µm x 57 ± 8 µm Longitud/diámetro = 24 ± 7,9	 Citas bibliográficas Material de herbario
B. hamifera  Rojo-cereza Porte triangular Helicoidal	 **Rama carpogonial** 3 células Con papila  **Tricógina** 97 ± 34 µm No espiralada	 **Cistocarpo** Desarrollo prefecundación 514 ± 123 µm x 446 ± 118 µm  **Carpósporas** 74 ± 12 µm x 28 ± 8 µm Nº por cistocarpo superior a 60	**Rama anteridial**  516 ± 179 µm x 257 ± 164 µm	**Células corticales**  Disposición en grupos Células alargadas y plurinucleadas Sinapsis secundarias Presencia de rizinas  **Eje axial** 2182 ± 275 µm x 90 ± 76 µm Longitud/diámetro = 36 ± 21	 Citas bibliográficas Materia de herbario

CONCLUSIONES

El estudio del material de las especies del género *Bonnemaisonia* en la Península Ibérica ha permitido:

- **Proponer nuevos caracteres taxonómicos para separar *B. asparagoides* de *B. clavata*** tales como la forma de la tricógina, el número de carpósporas por cistocarpo, el tamaño de las células del eje axial y algunas características del hábito como son el color, el porte y la organización del talo.
- **Corregir y actualizar los mapas de distribución de ambas especies**. La difícil determinación de *B. clavata*, debido principalmente a la escasa información descriptiva sobre su estructura vegetativa incluida en la bibliografía (Feldmann y Feldmann 1942; Dixon e Irvine 1977), es la razón por la que esta especie ha sido generalmente confundida con *B. asparagoides*. En base a estos nuevos caracteres taxonómicos se ha llevado a cabo la revisión del material de herbario que ha permitido establecer la actual distribución de dichas especies.
- **Aportar caracteres taxonómicos inéditos para *B. hamifera* que la diferencian claramente de las otras dos especies**, tales como la disposición de las células corticales externas, el número de núcleos de las células corticales internas y sus sinapsis secundarias, la presencia de rizinas, la presencia de una papila mucilaginosa que envuelve la tricógina y el desarrollo del cistocarpo anterior a la fecundación.
- **Destacar la proximidad de *B. hamifera* con el género *Asparagopsis***. Todos los caracteres anteriormente expuestos son característicos del género *Asparagopsis*, hecho que pone en duda la pertenencia de esta especie al género *Bonnemaisonia* y que por el contrario refuerza la propuesta de Okamura (1921) de combinar esta especie como *Asparagopsis hamifera* (Hariot) Okamura.

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CLAVE DE ESPECIES

1. Planta de color rojo-cereza, organización helicoidal, pleuridios en ocasiones sustituidos por unas ramitas curvadas en forma de garfio. Células corticales externas formando grupos. Células corticales internas alargadas, plurinucleadas y con sinapsis secundarias. Tricógina envuelta por papila mucilaginosa. Cistocarpo desarrollado antes de la fecundación ..... ***B. hamifera***

1. Planta de otro color, organización dística, sin garfios. Células corticales externas distribuidas formando círculos. Células corticales internas redondas, uninucleadas y sin sinapsis secundarias. Tricógina sin papila mucilaginosa. Cistocarpo desarrollado después de la fecundación 2

2. Planta monoica, palmatiforme, de color marrón-rojizo. Células del eje axial cortas (8-10 veces más largas que anchas). Tricógina no espiralada. Número de carpósporas por cistocarpo inferior a 35 ..... ***B. asparagoides***

2. Planta dioica, de forma triangular, de color rojo-rosado. Células del eje axial largas (23-25 veces más largas que anchas). Tricógina espiralada. Número de carpósporas por cistocarpo superior a 60 ..... ***B. clavata***

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Estudio taxonómico del género Bonnemaisonia C. Agardh en la Península Ibérica.

INTRODUCCIÓN

La familia *Bonnemaisoniaceae* Schmitz *in* Engler (*Bonnemaisoniales*, Rhodophyta) está ampliamente distribuida en las regiones templadas, tropicales y subtropicales de todos los océanos y comprende los géneros *Asparagopsis* Montagne, *Bonnemaisonia* C. Agardh, *Delisea* Lamouroux, *Leptophyllis* J. Agardh y *Ptilonia* J. Agardh. Esta familia ha sido objeto de numerosos estudios referentes a su gran poder de dispersión (Chemin 1928), a su ciclo biológico heteromórfico (Feldmann & Feldmann 1942; Chihara 1961, 1962) y al contenido de sus células vesiculares y a la bioactividad de sus metabolitos secundarios (Wolk 1968; McConell & Fenical 1979).

El género *Bonnemaisonia* está representado a nivel mundial por siete especies (http://www.algaebase.org) de las cuales *B. asparagoides* (Woodward) C. Agardh, *B. clavata* Hamel y *B. hamifera* Hariot están presentes en las costas de la Península Ibérica y las islas Baleares. El objetivo de este trabajo ha sido realizar la revisión taxonómica de este género para el proyecto *Flora phycologica iberica*. Este estudio ha permitido establecer los principales caracteres morfológicos y anatómicos de las estructuras vegetativas y reproductoras distintivas de las tres especies, realizar una clave dicotómica para su identificación y actualizar sus mapas de distribución en el área estudiada.

MATERIAL Y MÉTODOS

El estudio se ha realizado a partir del material recogido a lo largo de las costas de la Península Ibérica y las Islas Baleares. Este material ha sido conservado en agua de mar y formol al 4%, así como en pliegos de herbario depositados en el herbario BCN-Phyc (Universitat de Barcelona).

Asi mismo ha sido revisado todo el material correspondiente a estos táxones, de esta zona geográfica, conservado en los principales herbarios españoles (ABH-Algae, BCN-Phyc., HGI-A, MA-Algae, MAFAlgae, MGC-Phyc., SANT-Algae, VAB-Phyc.) y en el Muséum national d'histoire naturelle de París (PC). La tinción de los ejemplares con azul de anilina ha permitido una mejor visualización de algunas estructuras de difícil

observación tales como sinapsis, núcleos y rama carpogonial. Los mapas de distribución han sido realizados con el programa MapInfo Professional Version 4.

CONCLUSIONES

El estudio del material de las especies del género *Bonnemaisonia* en la Península Ibérica ha permitido:

• Proponer nuevos caracteres taxonómicos para separar B. asparagoides de B. clavata tales como la forma de la tricógina, el número de carposporangios por cistocarpo, el tamaño de las células del eje axial y algunas características del hábito como son el color, el porte y la organización del talo.

•Corregir y actualizar los mapas de distribución de ambas especies. La difícil determinación de *B*. *clavata*, debido principalmente a la escasa información descriptiva sobre su estructura vegetativa incluida en la bibliografía (Feldmann & Feldmann 1942; Dixon & Irvine 1977), es la razón por la que esta especie ha sido generalmente confundida con *B*. *asparagoides*. En base a estos nuevos caracteres taxonómicos se ha llevado a cabo la revisión del material de herbario que ha permitido establecer la actual distribución de dichas especies.

•Aportar caracteres taxonómicos inéditos para *B*. *hamifera* que la diferencian claramente de las otras dos especies, tales como la disposición de las células corticales externas, el número de núcleos de las células corticales internas y sus sinapsis secundarias, la presencia de filamentos medulares, la presencia de una papila mucilaginosa que envuelve la tricógina y el desarrollo del cistocarpo anterior a la fecundación.

•Destacar la proximidad de *B*. *hamifera* con el género *Asparagopsis*. Todos los caracteres anteriormente expuestos son característicos del género *Asparagopsis*, hecho que pone en duda la pertenencia de esta especie al género *Bonnemaisonia* y que por el contrario refuerza la propuesta de Okamura (1921) de combinar esta especie como *Asparagopsis hamifera* (Hariot) Okamura.

CLAVE DE ESPECIES

1. Planta de color rojo-cereza, organización helicoidal, rámulos cortos en ocasiones sustituidos por unas ramitas curvadas en forma de garfio. Células corticales externas formando grupos. Células corticales internas alargadas, plurinucleadas y con sinapsis secundarias. Tricógina envuelta por papila mucilaginosa. Cistocarpo desarrollado antes de la fecundación…….…..…….. *B. hamifera*

1. Planta de otro color, organización dística, sin garfios. Células corticales externas distribuidas formando círculos. Células corticales internas redondas, uninucleadas y sin sinapsis secundarias. Tricógina sin papila mucilaginosa. Cistocarpo desarrollado después de la fecundación…… 2

2. Planta monoica, palmatiforme, de color marrón-rojizo. Células del eje axial cortas (8-10 veces más largas que anchas). Tricógina no espiralada. Número de carposporangios por cistocarpo inferior a 35 …….. *B. asparagoides*

2. Planta dioica, de forma triangular, de color rojo-rosado. Células del eje axial largas (23-25 veces más largas que anchas). Tricógina espiralada. Número de carposporangios por cistocarpo superior a 60 …….……..…… *B. clavata*

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Molecular phylogeny of the Bonnemaisoniaceae (Bonnemaisoniales, Rhodophyta) from the Iberian Peninsula.

Noemi Salvador Soler, Maria Antonia Ribera Siguan, Amelia Gómez Garreta & Marina Morabito. 2009*b*.

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Filogènia molecular de la família Bonnemaisoniaceae (Bonnemaisoniales, Rhodophyta) a la península Ibèrica

A la península Ibèrica la família *Bonnemaisoniaceae* està representada per tres espècies del gènere *Bonnemaisonia* i dues del gènere *Asparagopsis*. Aquests gèneres estan ben caracteritzats tant per caràcters vegetatius com pels reproductius. No obstant això, una espècie present a la península Ibèrica, *Bonnemaisonia hamifera* Hariot, comparteix nombrosos caràcters presents en els dos gèneres. Per aquesta raó, aquest tàxon s'ha inclòs en els gèneres *Asparagopsis* o *Bonnemaisonia* depenent dels autors i de les seves observacions i, per tant, la seva posició taxonòmica restava sense aclarir.

Les seqüències obtingudes a partir del gen *rbc*L i del gen *SSU* s'han utilitzat principalment per aclarir la posició taxonòmica dels membres d'aquesta família presents a la península Ibèrica, així com la d'altres tàxons de l'ordre *Bonnemaisoniales*. Aquest estudi representa la primera estimació de les relacions filogenètiques dins de la família *Bonnemaisoniaceae*.

Només les anàlisis filogenètiques a partir del gen *SSU* van confirmar l'estreta relació entre els gèneres *Asparagopsis* i *Bonnemaisonia*. Això no obstant, els arbres generats no van permetre determinar la posició taxonòmica de *B. hamifera*. Respecte al gen *SSU*, en cap anàlisi les espècies del gènere *Bonnemaisonia* van quedar agrupades en una mateixa clada i, a més, *B. asparagoides* va ser espècie germana a la clada del gènere *Asparagopsis*. Per contra, les espècies del gènere *Bonnemaisonia* es van resoldre amb claredat en els arbres obtinguts a partir del gen *rbc*L, en què *B. asparagoides* i *B. clavata* van formar un fort agrupament i es va confirmar la distinció entre aquestes espècies.

L'evidència molecular dóna suport a la idea que la inclusió dels tàxons en les famílies *Bonnemaisoniaceae* i *Naccariaceae* s'hauria de revisar. Les relacions filogenètiques dins l'ordre *Bonnemaisoniales* són discutibles en vista dels resultats.

Molecular phylogeny of the Bonnemaisoniaceae (Bonnemaisoniales, Rhodophyta) from the Iberian Peninsula.

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Abstract

The Bonnemaisoniaceae are represented in the Iberian Peninsula by three species of *Bonnemaisonia* and two species of *Asparagopsis*. Sequence data from the plastid-encoded *rbc*L gene and SSU ribosomal RNA gene have been used to assist in clarifying the taxonomic position of these genera and other members of the Bonnemaisoniales. This represents the first estimate of the phylogenetic relationship within the family Bonnemaisoniaceae. Molecular phylogenetic analyses confirmed the close relationship between the genera *Asparagopsis* and *Bonnemaisonia*, which was recognizable in all analyses. However, our phylogenies were weak at resolving the taxonomic position of *B. hamifera*. In all SSU analyses, *Bonnemaisonia* species failed to cluster and *B. asparagoides* was sister to the long-branched clade *Asparagopsis*. Conversely, *Bonnemaisonia* species were clearly resolved in the *rbc*L trees and *B. asparagoides* and *B. clavata* formed a strong group, confirming the taxonomic distinction between *B. asparagoides* and *B. clavata*.

Molecular evidence supports the need for a re-evaluation of both Bonnemaisoniaceae and Naccariaceae as presently circumscribed. Phylogenetic relationships within the Bonnemaisoniales are discussed in light of the results.

Key words: *Asparagopsis, Bonnemaisonia,* Bonnemaisoniaceae, Florideophyceae, Iberian Peninsula, Mediterranean Sea, Naccariaceae, phylogeny, *rbc*L gene, small subunit rDNA gene.

Introduction

The family Bonnemaisoniaceae Schmitz *in* Engler (Schmitz, 1892) comprises a group of algae distributed in a range of habitats throughout the temperate, tropical and subtropical marine waters. This family includes the genera *Asparagopsis* Montagne, *Bonnemaisonia* C. Agardh, *Delisea* Lamouroux, *Leptophyllis* J. Agardh, *Pleuroblepharidella* Wynne and *Ptilonia* J. Agardh.

The Bonnemaisoniaceae exhibit a uniformity in both thallus and reproductive morphology (Chihara & Yoshizaki, 1972). This family has been a matter of much interest since the discovery of an alternation of heteromorphic phases in some of its members (Desikarachy *et al.*, 1990). Feldmann & Feldmann (1942) were the first to link *Bonnemaisonia hamifera* Hariot with *Trailliella intricata* Batters (then included in the Ceramiaceae), *Asparagopsis armata* Harvey with *Falkenbergia rufolanosa* (Harvey) Schmitz (then included in the Rhodomelaceae) and *Bonnemaisonia asparagoides* (Woodward) C. Agardh with *Hymenoclonium serpens* (P. Crouan *et* H. Crouan) Batters (then included in the Ceramiaceae). Since then, numerous studies were carried out to describe the life history patterns observed in the Bonnemaisoniaceae (Feldmann & Feldmann, 1942; Chihara, 1961; Chihara, 1962; Boillot, 1967; Bonin & Hawkes, 1988a; Bonin & Hawkes, 1988b; Salvador *et al.*, 2008). The Bonnemaisoniaceae have been of interest to biochemists for their polyhalogenated compounds (McConnell & Fenical, 1977b; McConnell & Fenical, 1977a) with demonstrated antimicrobial and antifungal activity (Burkholder *et al.*, 1960; Olesen *et al.*, 1964; Hornsey & Hide, 1974; McConnell & Fenical, 1979; Cabañes *et al.*, 1984; Nylund *et al.*, 2005; Salvador *et al.*, 2007) and therapeutic properties in humans (Sugano *et al.*, 1990; Haslin *et al.*, 2001).

Within the Rhodophyta, the order Bonnemaisoniales is well defined on the basis of morphological characters (Feldmann & Feldmann, 1942; Chihara, 1961; Chihara, 1962; Womersley, 1996), the ultrastructure of its pit-plugs and cap layers (Pueschel, 1989) and plastids (Chihara & Yoshizaki, 1972) as well as molecular studies (Freshwater *et al.*, 1994; Le Gall & Saunders, 2007). However, Womersley (1996), citing anatomical observations of the carposporophyte, suggested that the Naccariaceae might not be related to the Bonnemaisoniaceae. Saunders & Kraft (1997) recommended that molecular systematists re-investigate the ordinal affinities of members of the Naccariaceae which may not be monophyletic (Schils *et al.*, 2003). Within the

Bonnemaisoniaceae, molecular studies have focused mainly on the invasive genus *Asparagopsis*, firstly to demonstrate the taxonomic validity of its two unique species *Asparagopsis taxiformis* (Delile) Trevisan and *Asparagopsis armata* Harvey (Andreakis *et al.*, 2004; Ní Chualáin *et al.*, 2004) and secondly to describe the global phylogeographic patterns of these taxa (Andreakis *et al.*, 2007; Sherwood, 2008). On the *Bonnemaisonia* genus, only one molecular study was carried out until now. That study together with morpho-anatomic observations demonstrated that *Bonnemaisonia clavata* and *B. asparagoides* had been often confused (Salvador *et al.*, 2008) A similar lack of certainty concerning circumscription of *Asparagopsis* species was recently described (Andreakis *et al.*, 2007).

In the Iberian Peninsula, the Bonnemaisoniaceae are represented by *A. taxiformis*, *A. armata*, *B. hamifera*, *B. clavata* and *B. asparagoides*. The morpho-anatomical comparison of the Iberian *Bonnemaisonia* species (Salvador *et al.*, 2005) revealed taxonomic characters for *B. hamifera* which are a combination of features shared by the genera *Asparagopsis* and *Bonnemaisonia*. Our observations were in agreement with those of Chihara & Yoshizaki (1972) who highlighted the diversity of the genus *Bonnemaisonia* and the similarities of *B. hamifera* with *Asparagopsis* taxa. Although *B. hamifera* was initially described as a *Bonnemaisonia*, Okamura (1921, p. 131) later transferred it to the genus *Asparagopsis* because it has filiform and penicillate branches (Chihara, 1961). Feldmann & Feldmann (1942) re-assigned this taxon to the genus *Bonnemaisonia* based on criteria diagnostic for the Bonnemaisoniaceae including a) the presence of ramuli with more than three pericentral cells, b) the colorless refringent content and position in the thallus of their vesicle cells, c) the position of their reproductive structures, d) the origin of the nutritive cells, e) the low number of carpospores, which do not completely fill the cystocarp and f) the presence of hook branches in other species of this genus, *Bonnemaisonia californica*. Despite this circumscription, the taxonomic position of this taxon remains unclear.

The aim of this study was to provide a better understanding of the intergeneric relationship of members of the Bonnemaisoniaceae and to verify the systematic position of *B. hamifera* using *rbc*L and SSU gene sequence analysis.

Materials and methods

DNA was extracted from silica gel dried thalli, ground in liquid nitrogen, with a modified Proteinase K protocol (Saunders, 1993). Instead of the final agarose gel cleaning procedure, the DNA was purified with the DNeasy® Plant mini kit (Qiagen spa, Italy). Undiluted DNA was used as template for PCR or diluted in sterile bi-distilled water up to 1:40, depending on each template. Voucher herbarium specimens are housed at the BCN-Phyc Herbarium (Documentation Center of Plant Biodiversity, University of Barcelona, Spain). The *rbc*L gene was PCR amplified from isolated DNA in one to three overlapping fragments using primers available from literature (Freshwater & Rueness, 1994; Wang *et al.*, 2000). PCR products were purified with QIAquick® PCR purification kit (Qiagen spa, Italy), according to the manufacturer instructions, or gel purified by 0.8% agarose gel electrophoresis and then recovered from the gel slice by centrifugation through a home-made dimethyldichlorosilane (DMCS)-treated glass wool column (Saunders, 1993). The DNA was then ethanol precipitated (Sambrook *et al.*, 1989). Sequencing reactions were performed by an external company (MWG Biotech AG, Ebersberg, Germany). Individual nucleotide sequences were assembled and aligned by eye, with the assistance of the program ChromasPro 1.34 (Technelysium Pty Ltd, Australia) and MacClade 4.08 for MacOSX (Maddison & Maddison, 2000). Sequence data generated for *rbc*L gene were submitted to GenBank and accession numbers together with collection information are given in table 1.

Additional *rbc*L sequences from species of Bonnemaisoniales and representative Rhodymeniophycidae were downloaded from GenBank (Benson *et al.*, 2008, browsed 25 June 2008) and finally the alignment included 21 sequences with 1233 nucleotide positions.

The SSU rDNA was PCR amplified form isolated DNA in two to four overlapping fragments using primers available from the literature (Saunders *et al.*, 2004). PCR products were sequenced as above. Sequence data generated for SSU gene were submitted to GenBank and accession numbers together with collection information are given in table 1. Additional sequences from species of Bonnemaisoniales and representative Rhodymeniophycidae were downloaded from GenBank (Benson *et al*.,

2008). Prior to phylogenetic inference, ambiguously aligned areas were removed from the alignment, which finally included 24 sequences with 1675 nucleotide positions.

All phylogenetic analyses were performed in PAUP* 4b10 for the Macintosh and in MrBayes 3.1.2 (serial version for the Macintosh and MPI versions for Unix clusters; Ronquist & Huelsenbeck, 2003; Altekar *et al.*, 2004).

Different models of nucleotide substitutions were tested as implemented in Modeltest 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004). The model selected was used in distance and maximum likelihood (ML) analyses.

Distance phylogenies were constructed with a NJ algorithm. ML analyses were performed under a heuristic search, with 10 random addition sequence replicates, tree bisection and reconnection (TBR) as branch-swapping algorithm, saving all minimal trees (MulTrees). Parsimony analyses were conducted under a heuristic search, with 2000 random addition sequence replicates, with TBR branch-swapping algorithm and MulTrees option in effect, using only parsimony informative characters. Distance and parsimony analyses were subjected to bootstrap re-samplings to estimate robustness of the internal nodes (Felsenstein, 1985), basing on 2000 replicates, but with 200 random addition sequence replicates in heuristic searches. Bootstrap re-sampling was not performed on maximum likelihood analysis, due to computational limitations.

Bayesian inferences (BI) were performed utilizing the GTR+G model of sequence evolution (Lanave *et al.*, 1984) combined with the covarion-like model (Huelsenbeck, 2002). The data set was partitioned according to codon positions, the prior for the site specific rates in the phylogenetic model was set as "variable" and parameter estimations (shape, statefreq, revmat, tratio, switchrates, brlens) were unlinked among partitions. Each analysis consisted of two parallel runs, each run using four chains, one cold and three incrementally heated (temp= 0.10), and consisting of 10 million generations sampled every 1000th tree. Burn-in values were set for each analysis when likelihood values stabilized and a majority rule consensus tree was calculated with the corresponding posterior probability distribution.

In all phylogenetic analyses unrooted trees were constructed, subsequently rooted with reference to the outgroup taxa.

In SSU analyses, additional data set were examined under the maximum likelihood criterion in order to check possible long branch artefacts effecting tree topologies (see results).

The Shimodaira-Hasegawa test (SH test, Shimodaira & Hasegawa, 1999) as implemented in PAUP was used to test two taxonomic hypotheses: a) *B. hamifera, B. clavata* and *B. asparagoides* are monophyletic; b) *B. hamifera, A. taxiformis* and *A. armata* are monophyletic. The differences of likelihood scores between the ML tree obtained for the SSU alignment and the ML trees obtained forcing the constraints outlined above were compared in a one-tailed bootstrap test using 5000 replications (test distribution FULLOPT) to determine whether scores were significantly different at a P value of 0.05.

Results

*rbc*L gene

Among the 1233 nt analysed (positions 160–1392, 85.88% of the entire length of the gene), 517 were variable characters, of which 409 were parsimony-informative.

The model of nucleotide substitution selected in accordance with the Akaike Information Criterion, not using branch lengths as parameters, was GTR+I+G (Akaike weight= 1.0000; base frequencies: A= 0.3126, C= 0.1314, G= 0.2096, T= 0.3464; substitution rate matrix: A-C= 4.4745, A-G= 10.8171, A-T= 11.2867, C-G= 4.9644, C-T= 30.3285, G-T= 1.0000; proportion of invariable sites= 0.5135, gamma distribution shape parameter= 1.5156). It was used both in distance and likelihood analyses.

Parsimony analyses resulted in 4 most parsimonious (MP) trees (tree length 1873, consistency index 0.394554, retention index= 0.328597), not shown. All MP trees agreed for ingroup taxa positions and were similar to the ML trees with one exception presented below. Distance analyses resulted in a NJ tree, not shown, similar to the MP trees with a few differences (see below). Bayesian analyses resulted in a majority-rule consensus tree not shown, similar to MP trees. Likelihood analyses resulted in a ML tree (ln likelihood= - 9526.62880, Fig. 1, shown with bootstrap proportion values

obtained for NJ and parsimony analyses and obtained Bayesian posterior probabilities superimposed at internal branches).

Presented Bonnemaisoniales species clearly resolved in a clade with full to moderate support, according to analyses (77/76/1.00, NJ/parsimony bootstrap proportion values/Bayesian posterior probabilities). All genera were unresolved in at least one analysis or generally resolved with weak support.

A fully resolved clade in all analyses (100/99/1.00) included all *Delisea* species and *Ptilonia magellanica*, but in ML tree *Delisea* species were paraphyletic with *P. magellanica*, sister of *D. pulchra*; differently in NJ, MP and Bayesian trees *D. pulchra*, *D. flaccida* and *D. japonica* were monophyletic, although with moderate to low support (90/75/0.54), with sister relationship to *P. magellanica*. Sisterships within *Delisea* were uncertain with all possibilities being resolved in one of the three analyses.

In ML and MP trees a clade including *Bonnemaisonia* and *Asparagopsis* species was resolved as sister to *Delisea/Ptilonia*, although it gained no support in any analyses, with genera weakly resolved (71/-/0.55 and -/-/0.76, respectively). Conversely, Bayesian tree indicated *Bonnemaisonia* species as monophyletic with *Delisea/Ptilonia* (69/-/0.52) and an *Asparagopsis* clade as sister of all the remaining Bonnemaisoniales. Distance analyses differed from all others in failing to resolve a monophyletic *Asparagopsis*. *Bonnemaisonia asparagoides* and *B. clavata* clustered strongly in all analyses (100/88/1.00).

Two sequences previously deposited in GenBank as *B. asparagoides* (AF212188, U26813) differed from the sequence of *B. asparagoides* included in present analyses by 84-86 nt (6.81-6.97%), a difference comparable to that between two separate species within *Bonnemaisonia*, while they differed only by 9-11 nt (0,73-0.89%) with the *B. clavata* sequence of the present paper. These results are consistent with GenBank AF212188, U26813 (*B. asparagoides)* being conspecific with *B. clavata*.

SSU gene

Among the 1675 nt analysed, 362 were variable characters, of which 221 were parsimony-informative.

The model of nucleotide substitution selected in accordance with the Akaike Information Criterion, not using branch lengths as parameters, was GTR+I+G (Akaike weight= 0.3808; base frequencies: A= 0.2452, C= 0.2110, G= 0.2950, T= 0.2489; substitution rate matrix: A-C= 0.6213, A-G= 2.6906, A-T= 0.6717, C-G= 0.6696, C-T= 3.9835, G-T= 1.0000; proportion of invariable sites= 0.6296, gamma distribution shape parameter= 0.5591). It was used both in distance and likelihood analyses.

Parsimony analyses resulted in 6 most parsimonious (MP) trees (tree length 883, consistency index 0.538, retention index = 0.529), not shown. Relationships resolved within ingroup taxa were very volatile, with trees failing to resolve also the order Bonnemaisoniales. Distance analyses resulted in a NJ tree (not shown) with few supported clades similar to the MP trees. Bayesian analyses resulted in a majority-rule consensus tree (not shown). Likelihood analyses resulted in a ML tree (ln likelihood= -6785.41237, Fig. 2, shown with bootstrap proportion values obtained for NJ and parsimony analyses and obtained Bayesian posterior probabilities superimposed at internal branches).

The order Bonnemaisoniales was not resolved as monophyletic in any analyses because a sequence of *Atractophora hypnoides* from North Wales (AY772728, Ní Chualáin *et al*. 2004) clustered in all analyses with gigartinalean taxa, although with variable support (-/-/0.85 with *Peyssonnelia* sp., 65/-/0.99 with *Peyssonnelia* sp. and *Solieria robusta*). Assessment of the position of *Atractophora* was beyond the aim of the present paper, so the clarification of the apparent incongruence was not pursued further.

Excluding *Atractophora*, Bonnemassoniales taxa formed a weak clade (84/66/1.00), in which three lineage were recognizable. A group including *Bonnemaisonia* and *Asparagopsis* species (83/-/1.00) showed a fully supported *Asparagopsis* with a very long branch, and a paraphyletic *Bonnemaisonia*, whose species failed to cluster in all analyses. *Bonnemaisonia asparagoides* positioned as sister of *Asparagopsis* in distance, Bayesian and likelihood analyses (68/-/0.87). A second clade included *Delisea pulchra*

and *Ptilonia australasica* with weak support (56/64/0.67). The last resolved group (100/98/1.00) included *Naccaria wiggii*, *Reticulocaulis mucosissimus*, both belonging to the family Naccariaceae, and *Delisea hypnoides*, which failed to join the other congeneric in all analyses, but rather joining *Reticulocaulis mucosissimus* (99/94/0.92).

Several combinations of taxa were further analysed under the maximum likelihood criterion in order to remove long branches and check for possible artefacts effecting tree topologies. No differences were observed in the new trees in regards to relationships of included taxa in comparison with the original ones (data not shown).

The monophyly of the genus *Bonnemaisonia* was further tested and, according to the Shimodaira-Hasegawa test, cannot be rejected. Similarly, the inclusion of *B. hamifera* in the same clade with *Asparagopsis* species was tested and again the hypothesis cannot be rejected.

Discussion

Initially included in the order Rhodymeniales, the family Bonnemaisoniaceae was later incorporated in the Nemaliales, as Nemalionales (Kylin, 1916), until Feldmann & Feldmann (1942) created the new order Bonnemaisoniales. They based the segregation on the observation that the families Bonnemaisoniaceae included species with a diplohaplontic life history with a heteromorphic alternation of generations, distinct from the presumably haplontic Nemaliales. This notion of the Nemaliales was based on the assumed absence of a sporophyte in its life history and the supposed occurrence of meiosis in fertilized carpogonia (Garbary & Gabrielson, 1990). Later, despite suggestions that the Nemaliales also include species with diplobiontic life histories (Magne, 1967), the Bonnemaisoniales continued to be considered an order distinct from the Nemaliales based on many morphological characters as well as the ultrastructure of its pit-plugs and plastids (Chihara & Yoshizaki, 1972; Pueschel, 1989).

The Bonnemaisoniales were traditionally considered related to the Ceramiales (Feldmann & Feldmann, 1942; Gabrielson & Garbary, 1987). However, phylogenies of the Rhodophyta based on both *rbc*L and SSU sequence data do not support a close association between these orders (Freshwater *et al.*, 1994; Ragan *et al.*, 1994; Saunders

& Bayley, 1997). The molecular study of Ní Chualáin *et al*. (2004), which included several Bonnemaisoniales species in a SSU alignment, grouped the Bonnemaisoniales as sister to the Gelidiales. Later, molecular phylogenies of the Florideophyceae using different markers, as the elongation factor 2 (EF2), small subunit (SSU) and large subunit (LSU) ribosomal DNA, confirmed that the Bonnemaisoniales are monophyletic (Harper & Saunders, 2001; Le Gall & Saunders, 2007) in disagreement with some authors who suggested that the Naccariaceae be placed close to the Gigartinales because of the differences observed in vegetative structure and carposporophyte anatomy with the Bonnemaisoniaceae (Gabrielson & Garbary, 1987; Abbott, 1999). Molecular phylogenies suggested a possible relationship between the Bonnemaisoniales and the Gigartinales (Harper & Saunders, 2001; Le Gall & Saunders, 2007). However, the combined SSU and LSU analyses of Withall & Saunders (2006) showed that the position of Bonnemaisoniales within Rhodymeniophycidae is unclear because of attraction artifacts. Our results do not address the monophyly of the order as our alignment was refined to investigate infraordinal rather that interordinal relationships. Nevertheless, an interesting point emerges related to placement of *Atractophora hypnoides* with gigartinalean taxa. These results suggest that the Naccariaceae may not be monophyletic, as was suggested previously (Schils *et al.*, 2003). However, we did not have the opportunity to examine samples, so this remains an open question waiting to be solved.

Excluding *Atractophora*, three lineage are recognizable within the clade Bonnemaisoniales: a) a group including *Bonnemaisonia* and *Asparagopsis* species, both belonging to the family Bonnemaisoniaceae, recognizable in almost all *rbc*L and SSU analyses; b) a group including *Delisea* and *Ptilonia* species, also belonging to the family Bonnemaisoniaceae, represented in both *rbc*L and SSU; c) a group, only recognizable in SSU analyses due to the absence of available *rbc*L GenBank sequences of Naccariaceae which included *Naccaria wiggii*, *Reticulocaulis mucosissimus*, both belonging to the family Naccariaceae, and *Delisea hypnoides*, which failed to join the other congeneric.

Relationships among the three lineages are not clear. In fact the *Delisea-Ptilonia* clade grouped with the Naccariaceae clade, although with weak support, in our SSU analyses whereas in the molecular results of Ní Chualáin *et al*. (2004) it joined the *Bonnemaisonia-Asparagopsis* clade but without support. The position of *Delisea*

hypnoides is also doubtful because in our analyses it groups together the Naccariacean *Reticulocaulis mucosissimus* with nearly full support, while in the phylogenetic tree based on a combined analysis (EF2+SSU+LSU) of Le Gall & Saunders (2007), this taxon joined with *B. hamifera*. Depending on analyses, *Delisea* species were monophyletic or paraphyletic in a clade including *P. magellanica* in *rbc*L contest, and polyphyletic in SSU contest. We cannot say whether the unexpected association of the *Delisea* and *Ptilonia* species is an artifact of low taxon sampling. Unfortunately, because of the absence of available *rbc*L GenBank sequences of representative Naccariaceae and sequences of *Delisea* species disproportionately represented, a fine comparison with *rbc*L analyses is not possible. In our opinion, all these associations should be considered as interesting working hypotheses for subsequent investigations.

Therefore, relationships among genera remain poorly resolved and equivocal among analyses with the exception of a strongly monophyletic *Asparagopsis* in SSU analyses with very long branches.

The *Bonnemaisonia-Asparagopsis* group was resolved but with variable support according to analyses. *Bonnemaisonia* species failed to cluster in all SSU analyses, being paraphyletic with *Asparagopsis*, and with *B. asparagoides* positioned as sister of the long-branched *Asparagopsis*. Conversely, *Bonnemaisonia* species were clearly resolved in the *rbc*L trees, although only weakly supported. *B. asparagoides* and *B. clavata* formed a strongly supported group, confirming their taxonomic distinction proposed by Salvador *et al*. (2008). Moreover, Shimodaira-Hasegawa tests performed on the SSU set do not exclude the monophyly of *Bonnemaisonia* and the presumed sistership of *B. hamifera* rather that of *B. asparagoides* with *Asparagopsis*, as suggested by previous studies (Salvador *et al.*, 2005).

The diversity of the genus *Bonnemaisonia* was previously described by Chihara & Yoshizaki (1972) who separated Bonnemaisoniaceae in two groups, splitting *Bonnemaisonia* species. *Bonnemaisonia hamifera* was grouped with *A. armata*, *A. taxiformis* and *Bonnemaisonia nootkana* (Esp.) Silva based on its small carpospore diameter, its 'bipolar erect' type germination and its '*Trailliella'* type tetrasporophyte whereas *B. asparagoides* was grouped with *Delisea pulchra* (Greville) Montagne and *Ptilonia magellanica* (Montagne) Agardh (as *Delisea fimbriata* (Lamouroux) Montagne and *Ptilonia okadai* Yamada, respectively) based on its larger carpospores, its 'direct

disc' type germination and its presumptive '*Hymenoclonium'* type tetrasporophyte. Similarly, Salvador *et al*. (2005) noted that *B*. *hamifera* exhibits a mixture of characters described for the genera *Asparagopsis* and *Bonnemaisonia*. According the authors, *B*. *hamifera* shares some reproductive and vegetative features with the genus *Asparagopsis* such as a trichogyne sheathed by a mucilaginous papilla, a pericarp of prefertilization development, a uniform distribution of the outer cortical cells in surface view, presence of medullar filaments, long axial cells, a '*Ceramium'* type carpospore germination, an erect diploid phase, production of tetraspores and inner cortical cells with secondary pit connections (Salvador *et al.*, 2005). In the light of these observations, the Chihara & Yoshizaki's proposal (1972) to divide *Bonnemaisonia* into two genera should be suspended until more informative DNA sequences are available for the Bonnemaisoniaceae family to clarify the taxonomic position of its genera.

This study represents the first estimate of the phylogenetic relationships within the family Bonnemaisoniaceae, based on the analyses of chloroplast encoded *rbc*L gene and SSU ribosomal RNA gene sequences. Although our phylogenies were weak at resolving genera, and in particular the taxonomic position of *B*. *hamifera*, the resolution provided by our markers confirms the taxonomic distinction between *B*. *asparagoides* and *B*. *clavata* and supports the idea that both families Bonnemaisoniaceae and Naccariaceae as presently circumscribed should be reviewed. The sequencing of new markers, such as LSU and EF2, together with a large sampling of Bonnemaisoniales species should be necessary to achieve a conclusive taxonomic assessment of their intergeneric relationships.

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Table 1

Collection data, Herbarium accession numbers (BCN-Phyc.) and GenBank accession numbers for *rbc*L and SSU sequences of the species used in this study.

Species	Voucher	Collection site	Collection date	*rbc*L	SSU
Asparagopsis armata	2778	Ondarreta, Guipúzcoa, Spain.	12.v.06	GQ337068	GQ337070
A. taxiformis	2739	La isleta del Moro, Almeria, Spain.	13.iv.06	GQ337069	GQ337074
Bonnemaisonia asparagoides	2777	Sant Francesc cove, Girona, Spain.	23.v.06	GQ337065	GQ337071
B. clavata	1660	Sant Francesc cove, Girona, Spain.	16.v.06	GQ337067	GQ337072
B. hamifera	2776	Playa del Camello, Santander, Spain.	30.iv.06	GQ337066	GQ337073

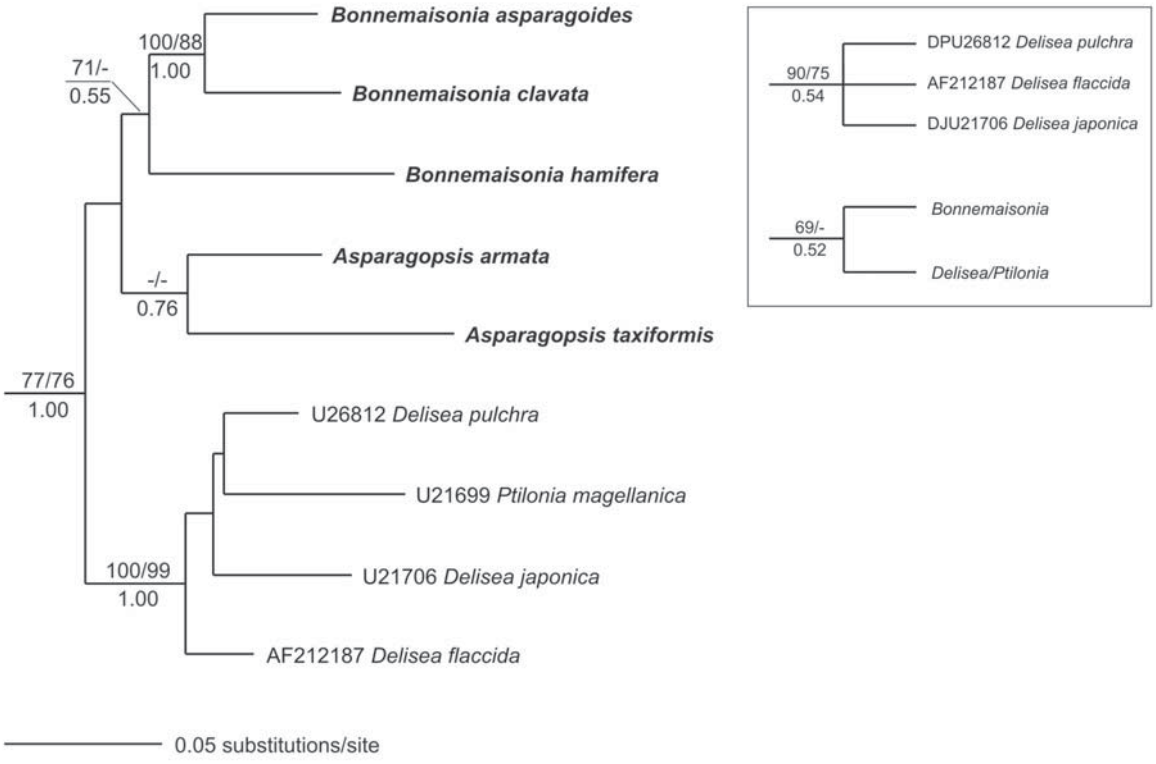

Fig. 1 - ML phylogram (ln L = -9526.62880), with bootstrap proportion values inferred from respectively NJ, MP analyses and obtained Bayesian posterior probabilities superimposed at internal branches. Relationships not resolved in ML but supported by other analyses are reported in the box on the right side of the tree. Outgroup taxa are represented in gray. Sequences generated in the present study are indicated in bold.

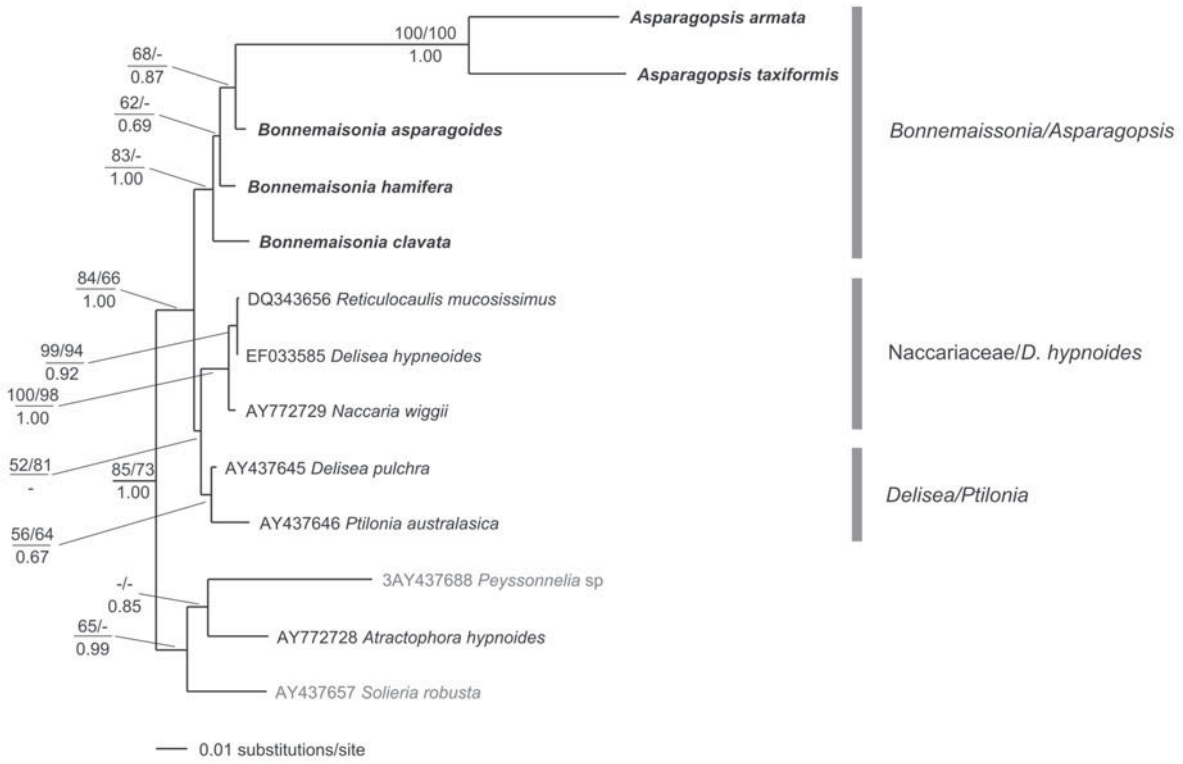

Fig. 2 - ML phylogram (ln L = -6785.41237), with bootstrap proportion values inferred from respectively NJ, MP analyses and obtained Bayesian posterior probabilities superimposed at internal branches. Outgroup taxa are represented in gray. Sequences generated in the present study are indicated in bold.

Mapas de distribución de algas marinas de la Península Ibérica y las Islas Baleares. XXII. Bonnemaisoniales (Bonnemaisoniaceae, Rhodophyta).

Noemi Salvador, Amelia Gómez Garreta & M. Antonia Ribera. 2006. ***Botanica Complutensis*** 30: 161-166.

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Mapas de distribución de algas marinas de la Península Ibérica y las Islas Baleares. XXII. *Bonnemaisonia (Bonnemaisoniaceae, Rhodophyta)*[*]

Noemi Salvador Soler, Amelia Gómez Garreta y Mª Antonia Ribera Siguan[1]

Resumen: Salvador Soler, N.; Gómez Garreta, A. & Ribera Siguan, Mª A. 2006. Mapas de distribución de algas marinas de la Península Ibérica y las Islas Baleares. XXII. *Bonnemaisonia* (*Bonnemaisoniaceae*, *Rhodophyta*). *Bot. Complut.* 30: 161-166.

Se presentan los mapas de distribución de las especies del género *Bonnemaisonia* C. Agardh (*Bonnemaisoniaceae*, *Rhodophyta*) en la Península Ibérica y las Islas Baleares: *B. asparagoides* (Woodward) C. Agardh, *B. clavata* G. Hamel y *B. hamifera* Hariot.

Palabras clave: corología, distribución, mapas, algas marinas, *Bonnemaisoniaceae*, *Bonnemaisonia*, España, Portugal.

Abstract: Salvador Soler, N.; Gómez Garreta, A. & Ribera Siguan, Mª A. 2006. Distribution maps of marine algae from the Iberian Peninsula and the Balearic Islands. XXII. *Bonnemaisonia* (Bonnemaisoniaceae, Rhodophyta). *Bot. Complut.* 30: 161-166.

We publish here the distribution maps along the Iberian Peninsula and the Balearic Islands of the taxa belonging to the genus *Bonnemaisonia* C. Agardh (Bonnemaisoniaceae, Rhodophyta): *B. asparagoides* (Woodward) C. Agardh, *B. clavata* G. Hamel and *B. hamifera* Hariot.

Keywords: corology, distribution, maps, marine algae, Bonnemaisoniaceae, *Bonnemaisonia*, Portugal, Spain.

INTRODUCCIÓN

Bonnemaisonia C. Agardh es el género tipo de la familia *Bonnemaisoniaceae* Schmitz. Esta familia, situada inicialmente en el orden *Rhodymeniales*, fue más tarde incluida en el orden *Nemalionales* por Kylin (1916). En base a las observaciones de Feldmann & Feldmann (1939) sobre la presencia de un ciclo biológico trigenético heteromórfico en algunas de las especies de la familia *Bonnemaisoniaceae*, estos mismos autores propusieron dar a esta familia categoría de orden (Feldmann & Feldmann 1942), incluyendo posteriormente también la familia *Naccariaceae* en el nuevo orden *Bonnemaisoniales* (Feldmann & Feldmann 1952). Posteriores estudios morfo-anatómicos corroboraron la validez taxonómica del orden *Bonnemaisoniales* (Chihara & Yoshizaki 1972, Pueschel & Cole 1982). La familia *Bonnemaisoniaceae* incluye los géneros: *Asparagopsis* Montagne, *Bonnemaisonia* C. Agardh, *Delisea* Lamouroux, *Leptophyllis* J. Agardh, *Pleuroblepharidella* M. J. Wynne y *Ptilonia* J. Agardh. El género *Bonnemaisonia* se encuentra ampliamente distribuido tanto en regiones frías como en templadas y subtropicales. A nivel mundial está representado por siete especies (Guiry *et al*. 2005) de las cuales únicamente *B. asparagoides* (Woodward) C. Agardh, *B. clavata* G. Hamel y *B. hamifera* Hariot están presentes en las costas de la Península Ibérica y las Islas Baleares.

El ciclo biológico del género *Bonnemaisonia* consta de un gametófito monoico o dioico que alterna con un tetrasporófito erecto en el caso de *B. hamifera* (=*Trailliella intricata*) o postrado como ocurre en *B. asparagoides* y *B. clavata* (=*Hymenoclonium*).

MATERIALES Y MÉTODOS

Para la realización de los mapas de distribución del género *Bonnemaisonia* en la Península Ibérica y las Islas Baleares ha sido revisado el material, correspondiente a estos taxones, conservado en los principales herbarios españoles (BCN-Phyc., BIO-Algae, HGI-A,

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MA-Algae, MGC-Phyc., SANT-Algae, VAB-Phyc.). También fueron consultados diferentes herbarios europeos (FI: Herbarium Universitatis Florentinae, Firenze; GB: Botanical Museum, Göteborg; W: Naturhistorisches Museum, Wien), encontrando material procedente de la Península Ibérica e Islas Baleares únicamente en el del Muséum national d'histoire naturelle de París (PC). La ordenación de las citas y la elaboración de los mapas se detalla en Gómez Garreta *et al*. (1994).

RESULTADOS

Bonnemaisonia asparagoides (Woodward) C. Agardh (Mapa 1)

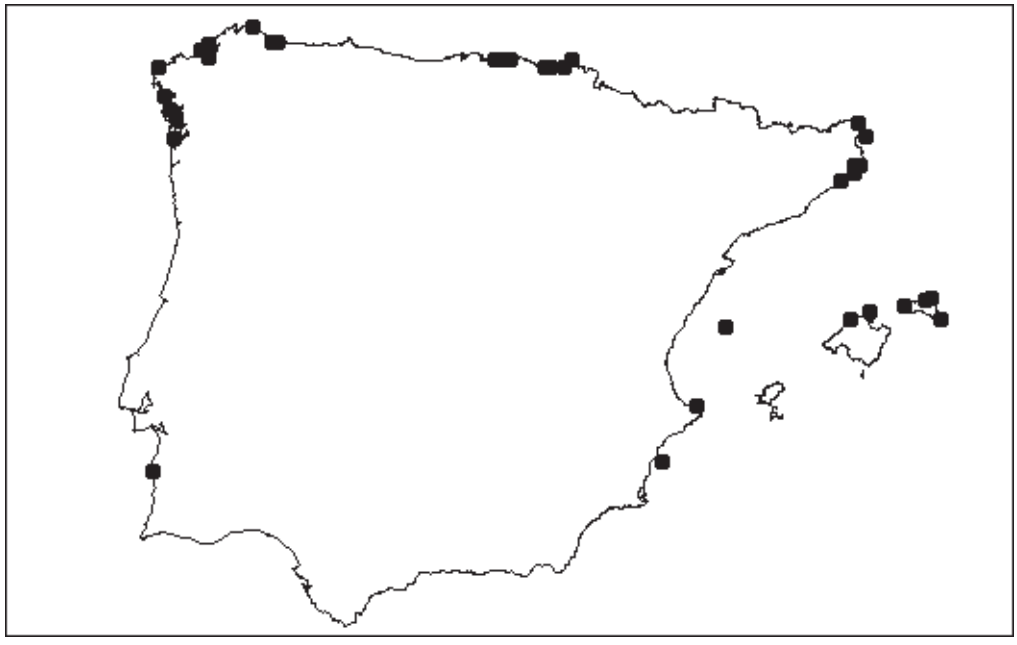
Hymenoclonium serpens (Crouan & Crouan) Batters *stadium*

Guipúzcoa: **30TWP90:** Fuenterrabía, mayo-1979, C. Casares 145; Fuentarrabía, cabo Híguer, 16-04-1988, C. Casares 775. **30TWN89:** San Sebastián, bahía de la Concha, 07-10-1985, cistocarpos, C. Casares 144. **30TWN69:** Ondarreta, 25-05-2005, BCN-Phyc. 1621; Zumaya, 15-05-1987, HGI-A 1273. **30TWN59:** Motrico, junio-1978, cistocarpos, C. Casares 146.

Vizcaya: **30TWP00:** Armintza, 22-07-1986, cistocarpos y ramas anteridiales, BIO-Algae 603; Ibíd., 21-08-1986, BIO-Algae 604; Ibíd., 12-06-1987, cistocarpos y ramas anteridiales, BIO-Algae 668; Ibíd., -4 m, 05-05-1996, BIO-Algae 1757; Gorliz, Errotatxu, -5 m, 28-07-1997, cistocarpos, BIO-Algae 2241. **30TVP90:** La Galea, -6 m, 30-06-1995, cistocarpos, BIO-Algae 1631; Zierbana, 30-05-1983, cistocarpos y ramas anteridiales, BIO-Algae 321; Ibíd., escollera, -6 m, 23-04-1997, cistocarpos y ramas anteridiales, BIO-Algae 2098; Arrigunaga, -4 m, 06-07-1995, cistocarpos y ramas anteridiales, BIO-Algae 1619. **30TVP80:** Kobaron, -9 m, 27-07-1982, cistocarpos, BIO-Algae 288; Ibíd., 12-06-1984, cistocarpos y ramas anteridiales, BIO-Algae 492; Ibíd., sublitoral, 23-08-1997, cistocarpos, BIO-Algae 851.

Lugo: **29TPJ52:** Rinlo, punta Corbeira, -3/-5 m, 21-07-1993, cistocarpos, SANT-Algae 3904. **29TPJ42:** punta del Castro, -1 m, 14-05-2003, SANT-Algae 14740. **29TPJ14:** ría de Viveiro, -10/-15 m, 23-07-1996, cistocarpos, SANT-Algae 8528.

La Coruña: **29TNJ51:** punta Fornelos, -10/-15 m, 07-07-1986, cistocarpos, PC 72722; Ibíd., MA-Algae 2492; Ibíd., SANT-Algae 2768; Ibíd., -3/-5 m, 08-07-1986, cistocarpos, SANT-Algae 594; ría de Ferrol, -10 m, 13-07-1991, cistocarpos y ramas anteridiales, SANT-Algae 381. **29TNJ50:** punta Gaboteira, -7 m, 26-06-2001, SANT-Algae 13382; Canide, -11 m, sin fecha, SANT-Algae 10981; Oleiros, punta Bufadoiro, -15 m, 17-06-2000, cistocarpos, SANT-Algae 11967; ría de La Coruña, San Roque, 16-04-1991, SANT-Algae 4428. **29TNJ40:** La Coruña, playa de Riazor, arrojada, 07-05-1996, cistocarpos, SANT-Algae 8817. **29TNH59:** Bastiagueiro, -8 m, 23-07-1992, cistocarpos, MA-Algae 5079; Ibíd., SANT-Algae 13049; Ibíd., PC 72721; Ibíd., arrojada, 11-07-1985, cistocarpos, SANT-Algae 3311; Ibíd., SANT-Algae 4390. **29TNH10:** A Sistela, -10 m, 27-03-1994, SANT-Algae 6420; Ibíd., SANT-Algae 6453. **29TMH93:** Muros, punta Roncadora, -2 m, 20-05-1989, SANT-Algae 5046. **29TMH87:** Lires, 15-06-1987, cistocarpos, SANT-Algae 3312.



Mapa 1— Distribución de *Bonnemaisonia asparagoides* en la Península Ibérica y las Islas Baleares.

Pontevedra: **29TNH01:** ría de Arosa, -9/-16 m, 09-05-1996, SANT-Algae 8797; Ibíd., -18 m, 16-06-1995, cistocarpos, SANT-Algae 7398. **29TNG17:** Cangas, punta Borneira, -5 m, 15-04-1997, cistocarpos, SANT-Algae 4998.

Baixo Alentejo: **29SNB19:** Sines, 30-06-1961, cistocarpos, PC 72720.

Alicante: **30SYH22:** isla de Tabarca, 04-05-2004, cistocarpos, ABH-Algae 324. **31SBD50:** Penyal d'Ifac, -8 m, 11-07-1984, VAL-Algae 646B.

Castellón de la Plana: **31SCE01:** illes Columbrets, La Ferrera, 0/-5 m, 31-07-2004, BCN-Phyc. 1635.

Girona: **31TDG81:** Blanes, cala Sant Francesc, -5 m, cistocarpos, 04-05-2005, BCN-Phyc. 1619. **31TEG02:** Sant Feliu de Guíxols, -8 m, 21-03-1996, cistocarpos y ramas anteridiales, HGI-A 2545. **31TEG03:** Calonge, roques planes, 17-04-1988, cistocarpos y ramas anteridiales, HGI-A 3201; Ibíd., HGI-A 3200. **31TEG13:** Palamós, roca fosca, 24-05-1987, cistocarpos y ramas anteridiales, HGI-A 3199. **31TEG27:** Roses, cap Norfeu, -5/-8 m, 24-04-1996, cistocarpos, HGI-A 3846. **31TEG19:** Llançà, cala Canyelles, 14-05-2005, cistocarpos, BCN-Phyc. 1615; Llançà, redes, 08-06-1992, cistocarpos, BCN-Phyc. 1627.

Baleares: Mallorca: **31SDE81:** Es Cavalls, -15 m, 02-06-2004, cistocarpos, BCN-Phyc. 1623; Ibíd., -13 m, 02-06-2004, cistocarpos, BCN-Phyc. 230. **31SEE12:** cala Figuera, -15/-20 m, 05-06-2004, cistocarpos, BCN-Phyc. 1620. Menorca: **31SFE10:** illa de l'Aire, -6 m, 20-06-2003, BCN-Phyc. 1636. **31TEE62:** cala Piques, -10/-20 m, 24-06-2003, BCN-Phyc. 1637; Ibíd., BCN-Phyc. 1655, como *Hymenoclonium serpens* sobre *B. asparagoides*. **31TEE93:** cap Cavalleria, -10/-25 m, 23-06-2003, BCN-Phyc. 1654. **31TFE03:** Addaia, cova, -24 m, 21-06-2003, BCN-Phyc. 1652.

Bonnemaisonia clavata G. Hamel (Mapa 2)

Hymenoclonium stadium

Vizcaya: **30TWP00:** Bilbao, Meñakoz, arrojada, 20-07-1984, cistocarpos, BIO-Algae 520; Ibíd., BIO-Algae 521. **30TVN99:** Ereaga, -6 m, 27-06-1995, ramas anteridiales, BIO-Algae 1539; Ibíd., BIO-Algae 1285.

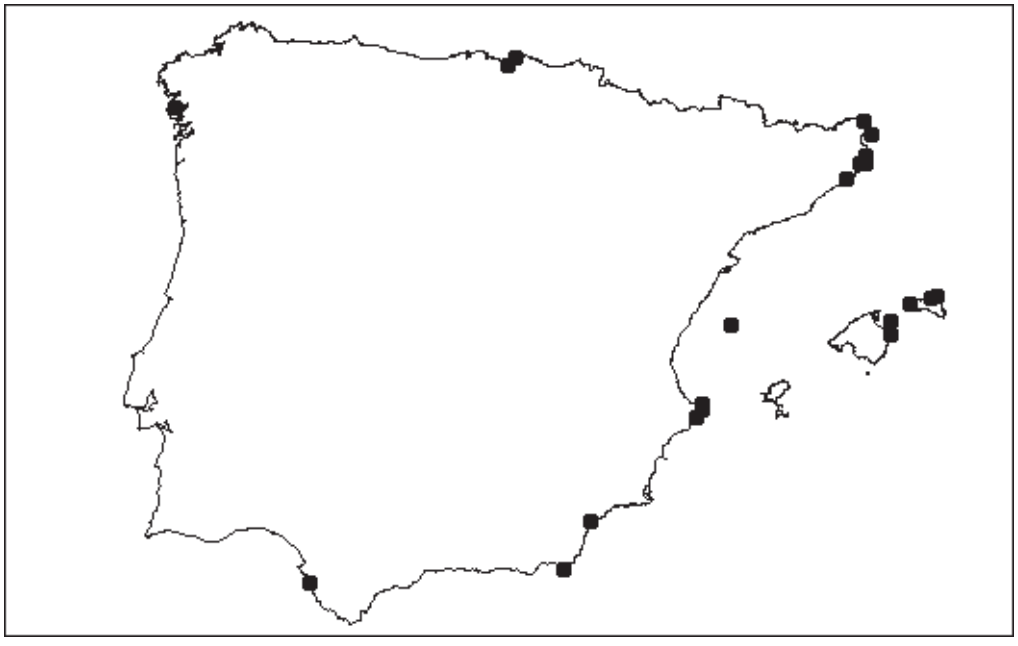
La Coruña: **29TNH01:** isla de Rúa, -18 m, 16-06-1995, SANT-A 7398, como *B. asparagoides*.

Cádiz: **29SQA44:** playa de la Victoria, 03-07-1960, cistocarpos, BCN-Phyc. 1630, como *B. asparagoides*.

Almería: **30SWF76:** Islote de San Pedro, -23 m, 02-05-1992, cistocarpos, MGC-Phyc. 3745, como *B. asparagoides*. **30SXG13:** Los Terreros, isla Negra, junio-1984, cistocarpos, MGC-Phyc. 1478, como *B. asparagoides*.

Alicante: **31SBC48:** Les Rotes, -8 m, 10-05-1993, un ejemplar con cistocarpos y otro con ramas anteridiales, VAL-Algae 1168B. **31SBC59:** Portitxol, flotando, 30-05-1982, BCN-Phyc. 1626, como *B. asparagoides*; Ibíd., -8 m, 16-06-1989, cistocarpos, VAL-Algae 45, como *B. asparagoides*; Xàvia, 22-05-1983, cistocarpos, VAL-Algae 40, como *B. asparagoides*. **31SBD50:** cova Tallada, arrojada, 20-06-2004, cistocarpos, ABH-Algae 335.

Castellón de la Plana: **31SCE01:** illes Columbrets, El Navarrete, -15/-21 m, 31-07-2004, BCN-Phyc. 1657.



Mapa 2— Distribución de *Bonnemaisonia clavata* en la Península Ibérica y las Islas Baleares.

Girona: **31TDG81:** Blanes, cala Sant Francesc, sin fecha, BCN-Phyc. 1633; Ibíd., -5 m, 04-05-2005, BCN-Phyc. 1631; Ibíd., 23-05-2005, cistocarpos, BCN-Phyc. 1616; Ibíd., -2/-7 m, BCN-Phyc. 1632. **31TEG03:** Calonge, cala Gogó, arrojada, 07-04-1985, cistocarpos, HGI-A 3198, como *B. asparagoides*; Ibíd., HGI-A 3197, como *B. asparagoides*. **31TEG13:** Palamós, roca d'en Grau, -17 m, 03-05-1993, cistocarpos, HGI-A 3202, como *B. asparagoides*; Ibíd., cistocarpos, HGI-A 3203, como *B. asparagoides*; Ibíd., cistocarpos, HGI-A 3204, como *B. asparagoides*; Ibíd., cistocarpos, HGI-A 3205, como *B. asparagoides*; Palamós, roca del Mero, -25 m, 03-05-1993, un ejemplar con cistocarpos y otro con ramas anteridiales, HGI-A 3206, como *B. asparagoides*. **31TEG14:** Begur, Aiguafreda, -5 m, 14-04-2005, BCN-Phyc. 1634; Ibíd., cistocarpos, BCN-Phyc. 1617; Begur, Es Furió de Fitó, -15 m, 14-05-2000, cistocarpos, BCN-Phyc. 1628, como *B. asparagoides*. **31TEG27:** Roses, cap Norfeu, -5/-8 m, 24-04-1996, cistocarpos, HGI-A 2541, como *B. asparagoides*. **31TEG19:** Llançà, cala Canyelles, -3 m, 14-05-2005, cistocarpos, BCN-Phyc. 1618.

Baleares: Mallorca: **31SEE30:** faralló d'Aubarca, 06-06-2004, cistocarpos, BCN-Phyc. 1622. **31SED38:** cala Bona, 03-06-2004, un ejemplar con cistocarpos y otro con ramas anteridiales, BCN-Phyc. 229. Menorca: **31TEE62:** cala Piques, -10/-20 m, 24-06-2003, BCN-Phyc. 1656. **31TEE93:** cap Cavalleria, -10/-25 m, 23-06-2003, BCN-Phyc. 1653. **31TFE03:** Addaia, cova, -24 m, 21-06-2003, BCN-Phyc. 1652.

Bonnemaisonia hamifera Hariot (Mapa 3)
Trailliella intricata Batters stadium

Guipúzcoa: **30TWP90:** Fuentarrabía, 25-04-1986, C. Casares 147; Ibíd., náutico, 19-03-1988, C. Casares 710; Ibíd., 16-04-1988, C. Casares 755. **30TWN89:** San Sebastián, náutico, arrojada, 14-05-1984, C. Casares 148; Ibíd., C. Casares 149. **30TWN69:** Guetaria, 27-03-1986, BCN-Phyc. 1658, como *T. intricata*; Ibíd., 24-04-2005, BCN-Phyc. 1649, como *T. intricata*; Ibíd., BCN-Phyc. 1650; Ibíd., cistocarpos, BCN-Phyc. 1651. **30TWN59:** Deba, punta Endata, 06-05-1993, BIO-Algae 914.

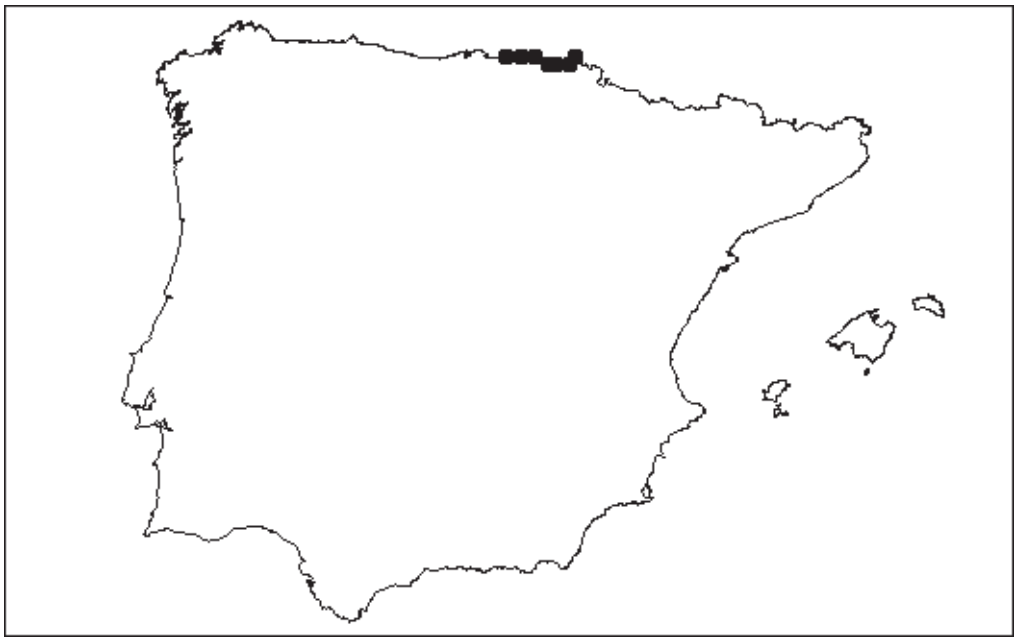
Vizcaya: **30TWP30:** Ea, 08-10-1994, tetrásporas, BCN-Phyc. 1659, como *T. intricata*. **30TWP10:** Arrastraculos, sin fecha, BIO-Algae 3679. **30TVP90:** La Galea, -2 m, 28-03-1995, BIO-Algae 2019; Ibíd., -6 m, 30-06-1996, cistocarpos, BIO-Algae 1644; Arrigunaga, 18-03-1996, BIO-Algae 1839; punta Lucero, -2 m, 22-01-1996, tetrásporas, BIO-Algae 1434, como *T. intricata*; Ibíd., -6 m, 27-10-1997, tetrásporas, BIO-Algae 727, como *T. intricata*.

DISCUSIÓN

Bonnemaisonia asparagoides y *B. clavata* son dos especies de gran parecido morfológico, tanto sus gametófitos (=*Bonnemaisonia*) como sus tetrasporófitos (=*Hymenoclonium*), mostrando además una distribución mundial muy similar. Por este motivo y teniendo en cuenta la escasa información descriptiva sobre la estructura vegetativa de *B. clavata* presente en la bibliografía, ambas especies han sido frecuentemente confundidas en ausencia de estructuras reproductoras. El estudio morfoanatómico que estamos llevando a cabo sobre estos taxones ha puesto de manifiesto nuevos caracteres taxonómicos tanto para el gametófito como para el tetrasporófito que permiten separar con exactitud ambas especies. Siguiendo este criterio hemos enmendado algunas determinaciones del material de herbario revisado. Si bien hemos podido determinar con exactitud la distribución de los gametófitos, la escasez de citas y de material de herbario referentes a ambos tetrasporófitos en la Península Ibérica, no permite determinar su verdadera distribución.

B. asparagoides se encuentra en el Atlántico a lo largo de las costas europeas (Noruega, Suecia, Irlanda, Gran Bretaña, Francia, Portugal, España) y en Marruecos, así como en el mar Mediterráneo (Francia, Grecia, Italia, España, Argelia, Túnez) (Guiry *et al*. 2005). En la Península Ibérica, *B. asparagoides* se encuentra ampliamente distribuida en las costas atlánticas (Guipúzcoa, Vizcaya, Lugo, La Coruña, Pontevedra, Baixo Alentejo) y mediterráneas (Alicante, islas Columbrets, Girona, islas Baleares). Aunque *B. asparagoides* estaba citada en diversas provincias de la costa de Andalucía, después de la revisión de los ejemplares de herbario de dicha costa hemos podido determinar que todos los pliegos de *B. asparagoides* corresponden a *B. clavata*. Por lo tanto, de momento excluimos las citas de *B. asparagoides* de las costas andaluzas en espera de nuevas prospecciones. Tanto los pliegos de herbario de *B. asparagoides* anteriores al año 1950 (Menorca, 28-09-1891, cistocarpos, J. Rodríguez, PC 72714; Ibíd., 18-09-1891, cistocarpos, J. Rodríguez, PC 72715; Gijón, 16-09-1985/05-10-1895, cistocarpos, PC 72712) como aquellos que por su estado no han podido ser comprobados (Menorca, hacía Canutells, 28-09-1989, cistocarpos, J. Rodríguez, MA-Algae 4132) no han sido utilizados para la elaboración de los mapas de distribución.

B. clavata se encuentra en el océano Atlántico (Gran Bretaña, Francia, España, Marruecos) y en el mar Mediterráneo (Francia, Grecia, Italia, España, Argelia, Túnez) (Hamel 1930, South & Tittley 1986, Furnari *et al*. 2003, Guiry *et al*. 2005). En la Península Ibérica, *B. clavata* está presente tanto en las costas atlánticas como en las mediterráneas. Al contrario que *B. asparagoides*, está escasamente distribuida en el litoral atlántico (Vizcaya, La Coruña, Cádiz) y bien representada a lo largo del litoral mediterráneo (Almería, Alicante, islas Columbrets, Girona, islas Baleares). La corrección de



Mapa 3— Distribución de *Bonnemaisonia hamifera* en la Península Ibérica y las Islas Baleares.

los ejemplares de herbario de las costas andaluzas, comentada anteriormente, nos ha permitido aportar las primeras citas de esta especie para la flora de Andalucía. Existe un pliego de herbario citado como *B. asparagoides* (VAL-Algae 1242), procedente de las islas Chafarinas -no incluidas en estos mapas- que también corresponde a *B. clavata*. Así mismo, aportamos la primera cita para Baleares.

Bonnemaisonia hamifera es una especie de amplia distribución. Se encuentra en el Pacífico oriental y occidental (Rusia, Japón, Corea, California, Méjico), en el Atlántico oriental a lo largo de las costas de Europa (Islandia, islas Faroes, Noruega, Países Bajos, Gran Bretaña, Irlanda, Francia, España), Marruecos, islas Canarias y Sudáfrica, en el Atlántico occidental (Québec, Maine, New Hampshire, Massachusetts, Connecticut, Long Island, Virginia) y en el Mediterráneo en Francia, Marruecos (como *Trailliella intricata*), Argelia (como *T. intricata*), Túnez (como *T. intricata*), Italia y Sicilia (Gil-Rodríguez & Alfonso-Carrillo 1980, Meñez & Mathieson 1981, Coppejans 1983, Noda 1987, González 1992, Curiel *et al*. 1997, Furnari *et al*. 2003, Guiry *et al*. 2005). Esta amplia distribución contrasta con su escasa presencia en la Península Ibérica, restringida a las costas de Guipúzcoa y Vizcaya.

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Somatic meiosis in the life history of Bonnemaisonia asparagoides and Bonnemaisonia clavata (Bonnemaisoniales, Rhodophyta) from the Iberian Peninsula.

Noemi Salvador, Amelia Gómez Garreta & M. Antonia Ribera. 2009*a*.

European Journal of Phycology 44 (3): 381-393.

Meiosi somàtica en el cicle vital de Bonnemaisonia asparagoides i Bonnemaisonia clavata (Bonnemaisoniales, Rhodophyta) de la península Ibèrica

Bonnemaisonia asparagoides i *Bonnemaisonia clavata* presenten un cicle vital en el qual mai no s'ha demostrat l'existència de tetrasporangis. El cultiu de les carpòspores d'ambdues espècies, fet en cambres de germinació sota condicions controlades, va donar lloc al seus corresponents "Hymenoclonium", sobre els quals es van formar per desenvolupament directe nous gametòfits. L'observació en detall dels primers estadis de desenvolupament ens va permetre detectar que els gametòfits s'originen a partir d'unes protuberàncies de l' "Hymenoclonium" amb aparença de tetrasporangis.

La comparació de l'àrea de fluorescència relativa (rfa) del DNA nuclear en els diferents estadis del cicle vital de les dues espècies (gametòfits, carpòspores i generacions prostrades) va indicar que els mínims nivells de ploïdia registrats en els gametòfits van ser d'1-2C, mentre que els mínims nivells registrats en la generació "Hymenoclonium" van ser de 2-4C, amb l'excepció de les protuberàncies (1C) que donen lloc als gametòfits.

Les observacions d'aquest estudi demostren que *B. asparagoides* i *B. clavata* presenten un cicle vital diplohaploide heteromòrfic, en què la generació gametofítica és haploide i s'origina directament, mitjançant una meiosi somàtica, sobre la generació prostrada "Hymenoclonium" que és diploide.

Aquest treball ens permet concloure que el cicle vital de *B. asparagoides* i *B. clavata* correspon al tipus "Lemanea", observat fins ara únicament en algues vermelles d'aigua dolça de l'ordre *Batrachospermales sensu lato*. Aquest fet suggereix que s'haurien d'estudiar els cicles vitals d'altres bonnemaisonials que presentin una generació tipus "Hymenoclonium" sense formació de tetrasporangis.

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Somatic meiosis in the life history of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata* (Bonnemaisoniales, Rhodophyta) from the Iberian peninsula

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Carpospores isolated from *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata* (Bonnemaisoniaceae, Rhodophyta) and grown in culture developed into their respective 'Hymenoclonium' prostrate phases. In both species, young gametophytes were initiated directly on the prostrate phase from tetrasporangia-like protuberances. Comparison of the relative fluorescence area (rfa) of nuclear DNA over the sequence of life-history stages indicated that the lowest ploidy levels (1–2C) occurred in the gametophytes, whereas the lowest ploidy levels in the prostrate phases were 2–4C. Rfa data demonstrated that meiosis occurred in the tetrasporangia-like protuberances where 1C values were recorded. The present observations establish that *B. asparagoides* and *B. clavata* have a heteromorphic diplohaplontic life history, which involves a haploid gametophyte produced directly on a diploid prostate phase after somatic meiosis. We conclude that the life history of these taxa corresponds to the *Lemanea*-type. This indicates that the life history of several Bonnemaisoniales with a 'Hymenoclonium' phase but lacking tetrasporangia requires re-investigation.

Key words: *Bonnemaisonia*, Bonnemaisoniales, culture, DNA content, 'Hymenoclonium', image analysis, *Lemanea*-type, life history, meiosis, nuclear patterns

Introduction

Bonnemaisonia C. Agardh, the type genus of the family Bonnemaisoniaceae Schmitz, is represented by seven species (Guiry & Guiry, 2008) of which only *Bonnemaisonia asparagoides* (Woodward) C. Agardh, *Bonnemaisonia clavata* G. Hamel and *Bonnemaisonia hamifera* Hariot occur on the Iberian Peninsula. Initially, the Bonnemaisoniaceae was included in the Rhodymeniales, but was later transferred to the Nemalionales on the basis of a presumed haplobiontic life history (Kylin, 1916). Subsequent reports of an alternation of heteromorphic generations in some species prompted the creation of the order Bonnemaisoniales (Feldmann & Feldmann, 1939, 1942). Recognition of the Bonnemaisoniales as currently circumscribed (Saunders & Hommersand, 2004) is supported by a suite of morphological characters, the ultrastructure of its pit-plugs and cap layers (Pueschel, 1989), and plastids (Chihara & Yoshizaki, 1972), as well as molecular studies (Freshwater *et al.*, 1994; Saunders & Hommersand, 2004).

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Species of the Bonnemaisoniaceae have a triphasic isomorphic or heteromorphic life history. In the genus *Bonnemaisonia*, this alternation is always heteromorphic (Womersley, 1996), with the gametangial thallus (=*Bonnemaisonia*) being erect, conspicuous and much branched, whereas the tetrasporophyte thallus is small, filamentous and tufted (='Trailliella') or prostrate (='Hymenoclonium'). Of the Iberian taxa, *B. hamifera* is characterized by a tufted tetrasporophyte with tetrasporangia, while in *B. asparagoides* and *B. clavata* this phase is prostrate. The life history of *B. hamifera* has been described in detail by several researchers (Koch, 1949; Chihara, 1961; Breeman *et al.*, 1988). Harder & Koch (1949) were the first to culture tetraspores of *Trailliella intricata* Batters from European isolates, while Chihara (1961) completed the life history for Japanese isolates from cultured carpospores and tetraspores. Contrastingly, the life history of *B. clavata* remains unknown (Salvador *et al.*, 2008) and although the life history of *B. asparagoides* has been the subject of numerous investigations, it remains unclear and the presence of tetrasporangia has never been clearly demonstrated despite extensive studies of material from its entire range

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(Rueness & Åsen, 1982). In addition, the original description and illustration of the tetrasporangia of *Hymenoclonium serpens* (Crouan et Crouan) Batters are unclear (Crouan & Crouan, 1859) and the described 'Hymenoclonium' could be confused with prostrate phases of other heteromorphic red algae (Rueness & Åsen, 1982).

Cytological studies, including chromosome numbers for the carpogonium, spermatium and zygote (Kylin, 1916; Svedelius, 1933), did little to clarify the life history of *B. asparagoides*. Svedelius (1933) incorrectly reported that meiosis occurred in the zygote, generating the assumption that the three phases (gametophyte, carposporophyte and tetrasporophyte) were haploid. Feldmann & Feldmann (1942) accepted Svedelius' observations and reported that the alternation of heteromorphic phases in the Bonnemaisoniaceae was independent of an alternation of nuclear phases. Magne (1960, 1964) strongly refuted this proposal after determining a haploid chromosome number in the gametophyte ($n = $ c.18) and a diploid one in the carposporophyte ($2n = 36$) of *B. asparagoides*, consistent with a diplobiontic life history involving heteromorphic phases as in *B. hamifera* and *Asparagopsis armata* Harvey (Feldmann, 1965; Desikachary *et al.*, 1990). Magne's model of alternating haploid and diploid phases was subsequently supported by diploid chromosome counts ($2n = 30$) in the 'Hymenoclonium' phase of *B. asparagoides* (Rueness & Åsen, 1982). These authors observed both generations in the field for a year and cultured carpospores and field-collected 'Hymenoclonium' phases under different conditions (daylength and temperature), but never saw sporangia or other reproductive structures. However, in their cultures, young gametophytes also grew directly on the 'Hymenoclonium' phase, which was recorded around the base of field-collected *B. asparagoides* (Rueness & Åsen, 1982). Consequently, Rueness & Åsen (1982) suggested that meiosis could occur inside the buds that give rise to the gametophytic thalli, but they were unable to confirm their assumption.

In red algae, karyological data are a prerequisite to a thorough understanding of the life history (Dixon, 1982) and of the sequence of species-specific somatic and nuclear phases (Drew, 1949; Lee *et al.*, 1995). Unfortunately, karyological studies on red algae are difficult and results are often imprecise (Godward, 1966; Cole, 1990; Goff & Coleman 1990; Kapraun, 1993). Microspectrophotometry has been widely used to demonstrate nuclear DNA content variation associated with haploid and diploid phases (Goff & Coleman, 1984; Deshmukhe & Tatewaki, 1993; Bennett & Leitch, 2005; Kapraun, 2007), but the required equipment is expensive and increasingly unavailable (Choi *et al.*, 1994). In contrast, image analysis systems which provide accurate relative nuclear DNA content estimates (Vilhar *et al.*, 2001; Hardie *et al.*, 2002) are more readily available and have been successfully used for algal investigations (Choi *et al.*, 1994; Kapraun & Nguyen, 1994; Lee *et al.*, 1995; Masuda *et al.*, 1996; Schnetter *et al.*, 2000; Bleckwenn *et al.*, 2003).

Initial developmental stages of *B. asparagoides* and *B. clavata* have been previously described in cultures originating from carpospores (Salvador *et al.*, 2008). In the present investigation, extended culture studies were undertaken to complete the life history of *B. clavata*, and to determine the origin and development of gametophytes from the 'Hymenoclonium' phase of both taxa. In addition, the nuclear DNA content of different life history stages was measured to locate the site of meiosis and to confirm the alternation of haploid and diploid phases.

Materials and methods

Culture studies

The 'Hymenoclonium' phases of *B. asparagoides* and *B. clavata* were obtained from carpospores. For three consecutive years (2005–2007), cystocarpic specimens of both species were collected in spring from Sant Francesc cove, Girona (41°40′N; 02°48′E), and transported to the laboratory at c. 5°C. Voucher specimens and slides are deposited in the BCN-Phyc. Herbarium (Documentation Center of Plant Biodiversity, University of Barcelona, Spain). Carpospore release and collection was achieved by submerging isolated thalli for 24 h in spring conditions (15°C, 12-h:12-h light–dark photoperiod) in small seawater aquaria with coverslips lining the bottom. Coverslips with attached carpospores were placed in vessels with 200 ml of culture medium prepared from filtered seawater (0.22 µm filter pore size), sterilized in a microwave oven (900 W, 10 min) and enriched using a quarter-strength modified von Stosch's medium (Guiry & Cunningham, 1984). Penicillin-G (4 mg l^{-1}) and GeO_2 (5 mg l^{-1}) were added to the medium to inhibit diatom and bacterial growth (Vergés *et al.*, 2004). Cultures were maintained in a growth chamber (Radiber, Barcelona, Spain) under controlled conditions (15°C, 15 µmol photons m^{-2} s^{-1}, 12-h:12-h light–dark photoperiod). Every week, the cultures were examined, the medium was changed and the algae were carefully cleaned with a paintbrush to eliminate epiphytes. Photomicrographs were made using a Coolpix 4500 camera (Nikon, Tokyo, Japan) connected to an Optiphot-2 light microscope (Nikon, Tokyo, Japan).

Nuclear DNA analysis

In spring 2007, Iberian specimens of *B. asparagoides* and *B. clavata* were collected from Aiguafreda cove, Girona

(41°57′N; 03°13′E). *Bonnemaisonia clavata* was also collected at Cape Cruz, A Coruña (42°35′N; 08°60′W). Voucher specimens are deposited in the herbaria BCN-Phyc. and SANT-Algae (Pharmacy Faculty, University of Santiago, Spain). Carpospores of *B. asparagoides* and *B. clavata* were obtained and cultured as described above. Carnoy's solution was used to fix samples of vegetative cells, spermatia and carpospores inside the cystocarp of freshly collected plants. Different life history stages were also fixed as follows: carpospores at the first divisions after settling, 1-week-old 'Hymenoclonium' germlings and 3-week-old 'Hymenoclonium' phases. After 24 h, material was transferred to 70% ethanol for storage at 4°C (Kapraun, 2005). Preserved material was rehydrated in water and treated with 5% w/v EDTA for 12–24 h (Goff & Coleman, 1990). Algal material was stained with DAPI (0.5 µg/ml 4′, 6-diamidino-2-phenylindole, Sigma-Aldrich Química, Madrid, Spain) according to Goff & Coleman (1990) and Kapraun (2005). Nuclear DNA content estimates were derived from an image analysis system, following the procedure of Choi *et al*. (1994) and Lee *et al*. (1995). Images were acquired with a Cooled CCD Micromax RTE 782-Y high performance digital camera (Princeton, Evry, France) placed in a DMRB fluorescent microscope (Leica, Wetzlar, Germany) and analysed using MetaMorph software (Molecular Devices, Toronto, Canada) and measurements were logged directly to a Microsoft® Excel file.

Nuclear DNA content, referred to as C-values (Gregory, 2005), represents multiples of the minimum amount of DNA corresponding to the non-replicated haploid chromosome complement (Gall, 1981; Goff & Coleman, 1984). In this study, the determination of the nuclear ploidy level was based on the positive correlation between DNA content and nuclear size (Price, 1976; Whittick, 1986; Kapraun, 1994; Kapraun & Nguyen, 1994). By measuring the relative fluorescence area (rfa) of DAPI-stained nuclear DNA, the cell-cycle stage and the C-value of the cells were inferred. The numerical relationship between the rfa and C-value was established by measuring spermatia and gametophytic apical cells (not polyploid), which have a replicated haploid (2C) genome (Goff & Coleman, 1990).

Results

Culture studies

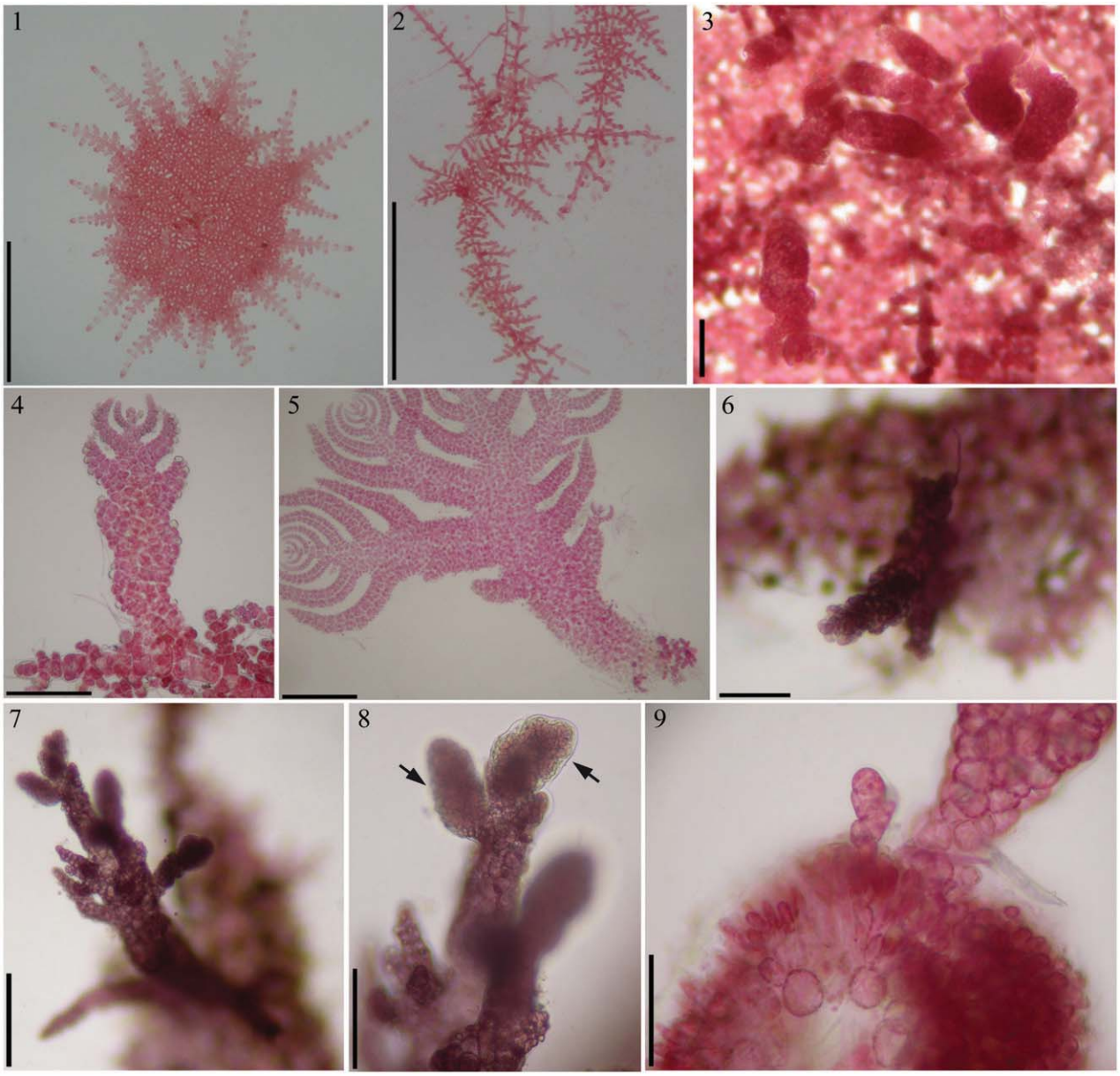
Carpospores isolated from *B. asparagoides* and *B. clavata* grew into well-developed 'Hymenoclonium' phases after 2 months in culture. In some cases, vegetative propagation of 'Hymenoclonium' by fragmentation was observed. The 'Hymenoclonium' phase of *B. asparagoides* is disc-shaped whereas in *B. clavata* it is elongated (Figs 1, 2). Young gametophytes developed directly on the 'Hymenoclonium' phase (Fig. 3). In *B. clavata* the gametophytes appeared after 2 months in culture whereas in *B. asparagoides* they typically required 4 months for development (Figs 4–6). Gametophyte development was not restricted to specific areas of the thallus, but occurred over the entire 'Hymenoclonium'. Young gametophytes of *B. clavata* formed exclusively male reproductive structures after three months in culture (Figs 7, 8). In one culture, gametophyte development was observed from a spermatangial branch (Fig. 9).

In *B. asparagoides*, initial stages of young gametophyte development were clearly observed in the 2007 carpospore cultures (Figs 10–19). Gametophytes developed from distinctive apical cells of the filaments (Fig. 10). These apical cells are pink and covered by a thick mucilaginous layer while the other 'Hymenoclonium' cells are red and covered by a thin mucilaginous layer. The cells that initiate gametophytes divided rapidly forming tetrasporangia-like protuberances with four uninucleate cells (Figs 11, 12). One cell of the protuberance initiated a young gametophyte, and the other three cells remained undivided (Figs 13, 14). After a few divisions, a little-differentiated gametophyte originated with a well-defined apical cell (Fig. 15). Young gametophytes quickly formed secretory or vesicular cells (Figs 16, 17), its characteristic distichous branching (Fig. 18) and the first basal rhizoids (Fig. 19). In both taxa, thalli continued branching (Fig. 4), growing to approximately 1.5 cm (Fig. 5).

Nuclear DNA analysis

C-values inferred from the nuclear rfa at different stages of the life history of *B. asparagoides* and *B. clavata* are given in Tables 1–3. Similar ploidy levels were observed in equivalent cell types for both species. The lowest ploidy level (1C) was observed in spermatia, gametophyte vegetative cells and tetrasporangia-like protuberance cells. The highest ploidy levels were observed in carpospores and gametophyte axial cells. In the carpospores, the maximum ploidy level (32C) was observed in *B. asparagoides*. In both species, values greater than 32C were observed in gametophyte axial cells, approaching 96C (270 rfa) in *B. asparagoides*. However, since all measurements were taken from the apical parts of the thallus, it is possible that even higher values occur in basal zones.

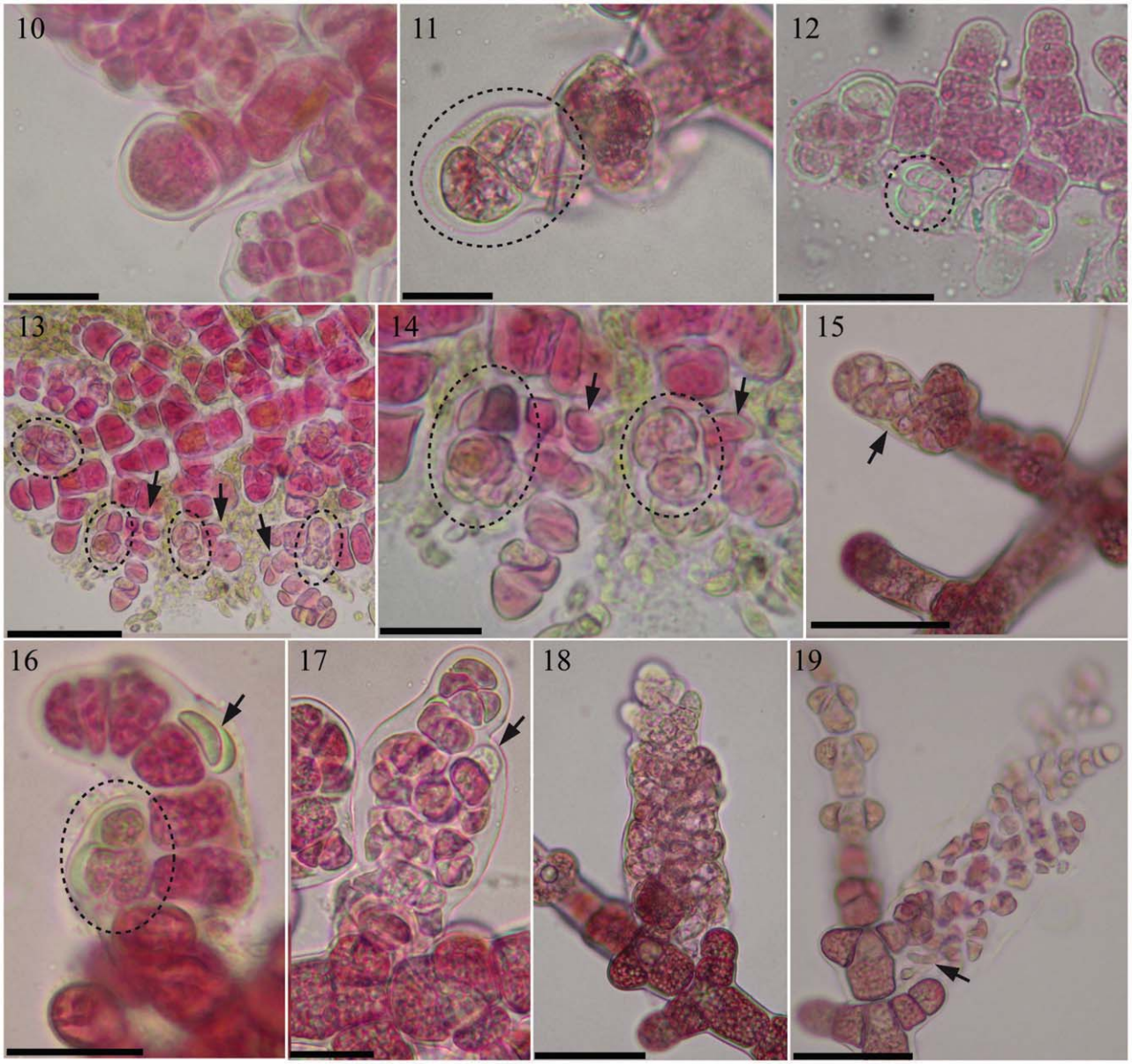
Some ploidy levels overlapped between different cell types in the gametophyte and prostrate phases, these were the 2C and 4C levels (Tables 1, 2). However, the spermatia of both species had the lowest value (1C), together with the cortical cells of *B. asparagoides* and apical cells of *B. clavata*. In contrast, the lowest ploidy level in a mature 'Hymenoclonium' phase was 2C, in vegetative cells of both taxa, and in the secondary apical cells of *B. clavata*.



Figs 1–9. Different stages in the life history of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*. Living material, unless otherwise indicated. Fig. 1. 'Hymenoclonium' phase of *B. asparagoides*. Fig. 2. 'Hymenoclonium' phase of *B. clavata*. Figs 3, 4. Direct development of young gametophytes on 'Hymenoclonium' phases of *B. asparagoides*, slide BCN-Phyc. 3213. Fig. 5. Well-developed gametophyte of *B. asparagoides*, slide BCN-Phyc. 3211. Fig. 6. Direct development of a young gametophyte on the 'Hymenoclonium' phase of *B. clavata*. Figs 7, 8. Spermatangial branches (arrows) of a young gametophyte of *B. clavata*, slide BCN-Phyc. 3214. Fig. 9. Gametophytes of *B. clavata* originated from a spermatangial branch, slide BCN-Phyc. 3210. Scale bars: 0.5 mm (Figs 1, 2), 100 µm (Figs 3, 4, 6, 8), 200 µm (Figs 5, 7), 50 µm (Fig. 9).

Nuclear DNA content of carpospores increases during maturation and elongation, and then decreases during the first divisions after attachment (Tables 1, 2; Figs 20–27). Although the carpospores of both *B. asparagoides* and *B. clavata* exhibited the same pattern, carpospores of the former always had higher ploidy levels than those of the latter. The lower ploidy levels in immature carpospores of *B. asparagoides* (4C) and *B. clavata* (3C) represent diploid values and confirm the presence of fertilization in a sexual life cycle. Attached uninucleate carpospores had the highest ploidy levels of all carpospore stages (32C in *B. asparagoides* and 12C in *B. clavata*). After the first carpospore division, a gradual reduction of ploidy level was observed in both species, with values of 8–16C in *B. asparagoides* and 8C in *B. clavata*. Reduction in ploidy level to 2C occurred in carpospores of both taxa with more than four nuclei.

Comparison of the rfa of nuclei from different vegetative cell types suggests similar patterns of nuclear DNA content variation for both *B. asparagoides* and *B. clavata* (Tables 1, 2). All cell types were uninucleate. In gametophytes of both species, growth results from the division



Figs 10–19. Initial developmental stages of the gametophyte of *Bonnemaisonia asparagoides* from the 'Hymenoclonium' phase. Living material, unless otherwise indicated. Fig. 10. Apical cell before gametophytic thallus is formed. Figs 11, 12. Tetrasporangia-like protuberances (dotted oval). Figs 13, 14. Several young gametophytes (arrows) growing on the 'Hymenoclonium' phase from tetrasporangia-like protuberances (dotted oval). Fig. 15. First cells of the gametophyte (arrow). Fig. 16. Gametophyte initiated from a tetrasporangia-like protuberance (dotted oval) with its first vesicular cell (arrow). Fig. 17. Young gametophyte with the first vesicular cell (arrow). Fig. 18. Gametophyte showing distichous branching. Fig. 19. First rhizoids (arrow) on the basal part of the gametophyte, slide BCN-Phyc. 3212. Scale bars: 20 μm (Figs 10, 11, 14, 17), 50 μm (Figs 12, 13, 15, 16, 18, 19).

of an apical cell with a non-polyploid nucleus (1–2C). These cells initiate uninucleate axial cells with a polyploid nucleus, which progressively increases in size and ploidy level from the apical to the basal part of the thallus. Consequently, rfa values of axial cells were recorded (Fig. 28) but mean values were not calculated. Even though the 'Hymenoclonium' phase grows from apical cells (as do gametophytes), in this study these apical cells were polyploid (4C–8C) and produced derivative cells with lower ploidy levels (Fig. 29). The decrease in the nuclear area of cells in the main filaments was also progressive, resulting in vegetative cells of 2–4C in *B. asparagoides* and 2–3C in *B. clavata*. No differences were observed between the ploidy levels of vegetative cells from 1-week and 3-week-old 'Hymenoclonium' phases. However, a different nuclear pattern was observed between the prostrate phases of the two species. In *B. asparagoides* values of 8C were found in apical cells of 1- and 3-week-old specimens as well as in secondary apical cells. As a result all

Table 1. Rfa and C-values ranges of nuclei in the different cell types of *Bonnemaisonia asparagoides*.

B.asparagoides							
Rfa (μm^2)	3	6	12	24	48	96	
C-Value[a]	1C	2C	4C	8C	16C	32C	n[b]
Gametophyte							
Spermatia	*	*					91
Cortical cells	*	*	*				78
Apical cells		*					14
Carpospores							
Immature carpospores			*	*	*		37
Carpospores (one nucleus)					*	*	26
Carpospores (two nuclei)				*	*		11
Carpospores (four nuclei)				*	*		10
Carpospores (> four nuclei)		*	*	*			87
Hymenoclonium							
Vegetative cells (3-week-old)		*	*	*			98
Vegetative cells		*	*	*			55
Apical cells (1-week-old)			*	*			31
Apical cells (3-week-old)				*			34
Secondary apical cells (3-week-old)				*			18
Protuberance cells	*						15

[a]C-value inferred from rfa values. [b]Number of nuclei examined.

Table 2. Rfa and C-values ranges of nuclei in different cell types of *Bonnemaisonia clavata*.

B.clavata						
Rfa (μm^2)	3	6	12	24	48	
C-Value[a]	1C	2C	4C	8C	16C	n[b]
Gametophyte						
Spermatia	*	*				142
Cortical cells		*	*			42
Apical cells	*	*				32
Carpospores						
Immature carpospores			*	*		12
Carpospores (one nucleus)				*	*	77
Carpospores (two nuclei)				*		138
Carpospores (four nuclei)			*	*		17
Carpospores (> four nuclei)		*	*	*		60
Hymenoclonium						
Vegetative cells (3-week-old)		*	*			114
Vegetative cells		*				57
Apical cells (1-week-old)		*	*			19
Apical cells (3-week-old)			*	*		23
Secondary apical cells (3-week-old)		*	*			16

[a]C-value inferred from rfa values. [b]Number of nuclei examined.

the apical cells had higher ploidy levels than the vegetative cells (Fig. 30). By contrast, in the 'Hymenoclonium' phase of *B. clavata*, both apical cells from 1-week-old specimens (2–3C) and secondary apical cells (2–4C) had nuclear areas similar to those of vegetative cells. The exception was the apical cells of the main filaments analysed in 3-week-old thalli, which had higher values of 4–8C (Table 2, Fig. 31).

Table 3. Summary of C-levels and rfa values in nuclei in cell types of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*.

	B. asparagoides			*B. clavata*		
	rfa ± SD	C-value[a]	n[b]	rfa ± SD	C-value[a]	n[b]
Gametophyte						
Spermatia	3.0 ± 0.8	1	91	2.5 ± 0.3	1	142
	5.6 ± 0.6	2		5.0 ± 1.2	2	
Cortical cells	3.3 ± 0.9	1	78	5.3 ± 1.1	2	42
	6.8 ± 1.0	2		11.8 ± 0.3	4	
	15.3 ± 4.0	4				
Apical cells	5.0 ± 1.0	2	14	2.8 ± 0.6	1	32
				5.9 ± 0.5	2	
Carpospores						
Immature carpospores	14.9 ± 3.0	4	37	9.5 ± 2.8	3	12
	25.5 ± 1.2	8		19.8 ± 2.7	6	
	51.8 ± 2.8	16				
Carpospores (one nucleus)	49.0 ± 10.3	16	26	22.3 ± 4.8	8	77
	94.8 ± 11.7	32		39.4 ± 5.3	12	
Carpospores (two nuclei)	24.2 ± 7.0	8	11	26.8 ± 10.4	8	138
	46.9 ± 6.4	16				
Carpospores (four nuclei)	26 ± 7.0	8	10	19.3 ± 2.0	3	17
	44.4 ± 2.3	16			6	
Carpospores (> four nuclei)	6.2 ± 1.3	2	87	6.2 ± 0.5	2	60
	11.5 ± 2.4	4		8.1 ± 0.5	3	
	22.6 ± 2.6	8		11.8 ± 2.0	4	
Hymenoclonium						
Vegetative cells (3-week-old)	6.2 ± 1.0	2	98	6.0 ± 1.1	2	114
	8.9 ± 0.6	3		9.4 ± 0.6	3	
	11.8 ± 0.7	4				
Vegetative cells	5.7 ± 0.8	2	55	5.8 ± 0.9	2	57
	9.1 ± 0.9	3				
	12.1 ± 1.0	4				
Apical cells (1-week-old)	12.6 ± 2.9	4	31	6.0 ± 1.3	2	19
	20.4 ± 2.9	8		10.2 ± 2.0	3	
Apical cells (3-week-old)	24.2 ± 3.6	8	34	13.3 ± 3.6	4	23
				24.1 ± 3.8	8	
Secondary apical cells (3-week-old)	22.7 ± 3.3	8	18	7.7 ± 1.0	2	16
				12.7 ± 1.9	4	
Protuberance cells	2.6 ± 0.6	1	15			

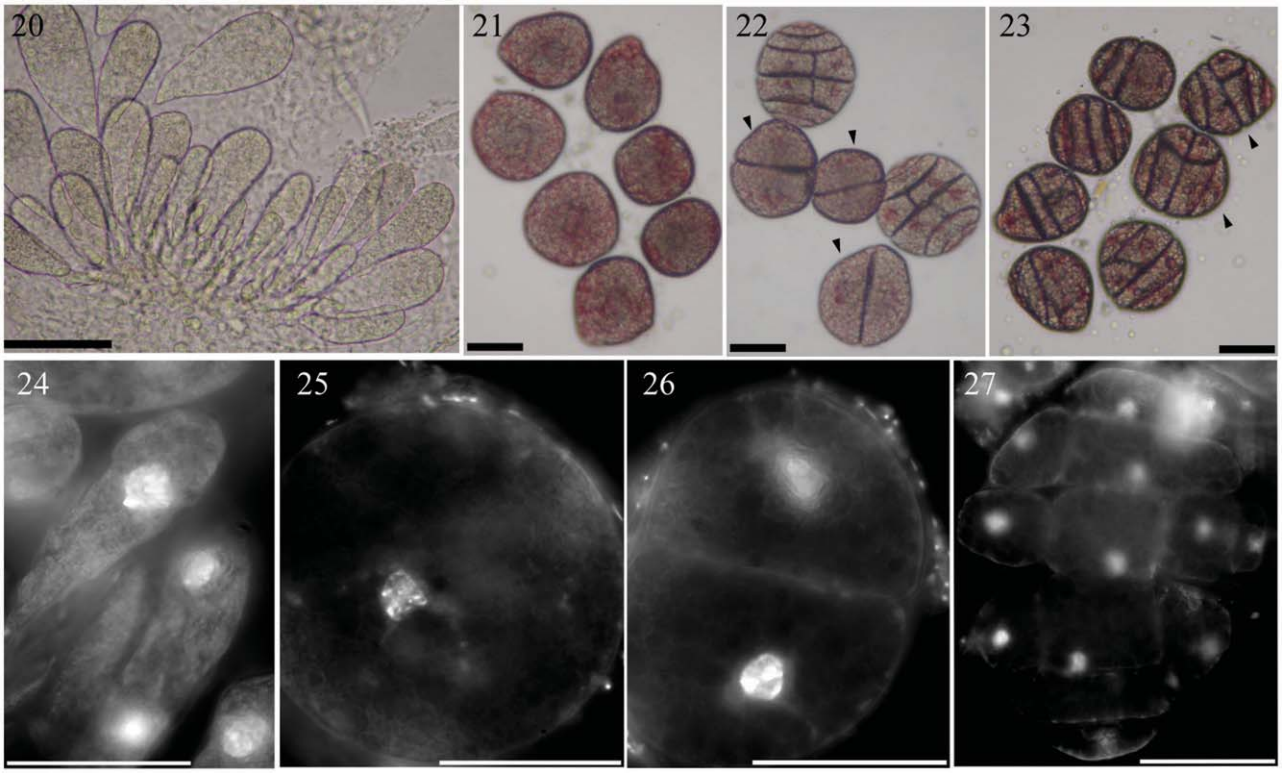
[a]C-value inferred from rfa values. [b]Number of nuclei examined.

Discussion

Life history in culture

Early culture studies of *B. asparagoides* initiated from carpospores consistently resulted in the production of the 'Hymenoclonium' phase, which was initially considered a protonema (Chemin, 1937; Feldmann & Mazoyer, 1937). After structures described as tetrasporangia were reported on the 'Hymenoclonium' phase, it was re-interpreted as a tetrasporophyte (Feldmann & Feldmann, 1942). Later, Kylin (1945) cultured carpospores of *B. asparagoides* and obtained the same structures, but noted inter-cellular pit-connections within them as well as the direct development of young gametophytes from these structures. As a result, Kylin concluded that these structures were unlikely to be tetrasporangia and proposed a life history model involving a protonema rather than a tetrasporophyte. Kylin's interpretation was not totally accepted. Some authors continued to reaffirm the presence of tetrasporangia on the 'Hymenoclonium' phase (Feldmann & Feldmann, 1946; Magne, 1960) while others, citing direct development of gametophytes on the 'Hymenoclonium' phase, supported Kylin's interpretation (Chemin, 1937; Feldmann & Mazoyer, 1937; Feldmann, 1966).

Later, Cortel-Breeman (1975) suggested the existence of two pathways for gametophyte development, one by spores and the other by direct development. In fact, direct development of the gametophyte from the prostrate phase has been described in several species of the Bonnemaisoniales including *Atractophora hypnoides* Crouan et Crouan, *Delisea compressa* Levring, *Delisea pulchra* (Greville) Montagne (as *Delisea fimbriata* (Lamouroux) Montagne), *Naccaria wiggii* (Turner) Endlicher, *Ptilonia magellanica* (Montagne) Agardh (as *Ptilonia okadai* Yamada) and *Ptilonia mooreana* Levring (Chihara, 1962; Boillot, 1967; Bonin & Hawkes, 1988*a*, *b*). Some authors also suggested that the life history of these taxa corresponds to the



Figs 20–27. Carposporangia and carpospore segmentation stages of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*. Fig. 20. Carposporangia of a field-collected specimen of *B. asparagoides*. Fig. 21. Undivided carpospores of *B. asparagoides*. Fig. 22. Carpospores of *B. asparagoides* with two nuclei (arrowheads). Fig. 23. Carpospores of *B. asparagoides* after more than four divisions (arrowheads). Fig. 24. DAPI-stained carposporangia of *B. asparagoides*. Fig. 25. DAPI-stained carpospore of *B. clavata* with one nucleus. Fig. 26. DAPI-stained carpospore of *B. clavata* with two nuclei. Fig. 27. DAPI-stained carpospore of *B. asparagoides* after more than four divisions. Scale bars: 50 μm (Figs 20–23, 24, 27), 25 μm (Figs 25, 26).

Nemalion-type (Chihara, 1962) or the *Lemanea*-type (Bonin & Hawkes, 1988*a*, *b*).

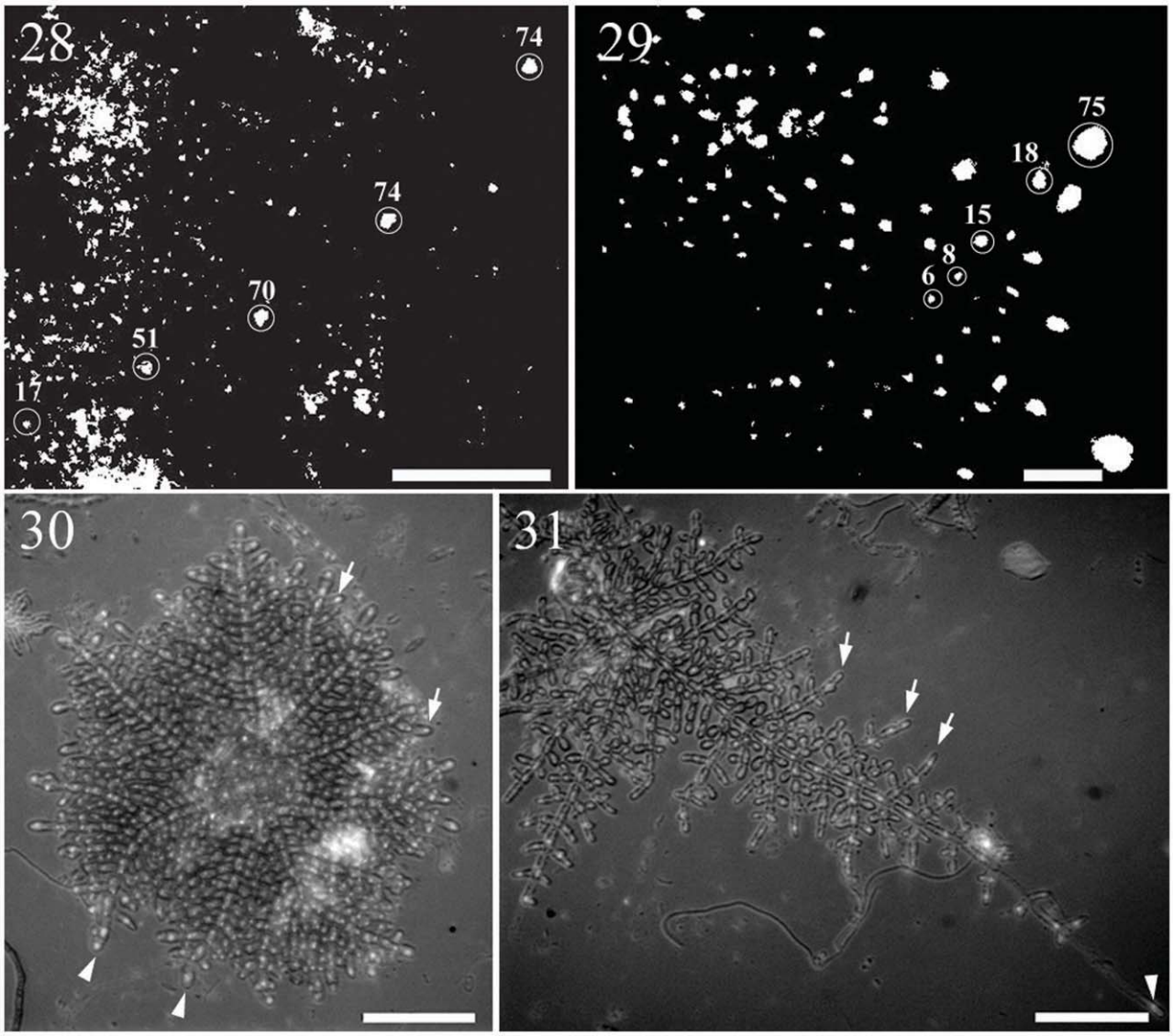
More recently, Rueness & Åsen (1982) suggested that a somatic meiosis could occur in the buds, which gave rise to young gametophytes on the 'Hymenoclonium' phase of *B. asparagoides*.

In the present study, functional tetrasporangia were never observed in either *Bonnemaisonia* species, although the cultures were repeated for three consecutive years. In contrast, all the new gametophytes grew on the 'Hymenoclonium' phase, both in *B. asparagoides* and *B. clavata*. For the first time, the structures that initiated the young gametophytes, here called tetrasporangia-like protuberances, were isolated and their development recorded. As both taxa showed the same gametophyte development, it can be deduced that the gametophytes were initiated by tetrasporangia-like protuberances in both species, although these structures were only observed in *B. asparagoides*. Comparing the life history of *B. asparagoides* and *B. clavata*, young gametophytes on the 'Hymenoclonium' phase appeared earlier in *B. clavata* than in *B. asparagoides* and only developed reproductive structures in the former. These observations are in agreement with differences in growth rates, carpospore germination times and developmental patterns of these taxa observed by Salvador *et al.* (2008).

The *Bonnemaisonia*-type life history, initially named *Asparagopsis*-type (Segawa & Chihara, 1954), was described for *Asparagopsis hamifera* (Hariot) Okamura. Later, Dixon (1973) renamed this life history the *Bonnemaisonia*-type, reflecting the re-assignment of *A. hamifera* to the genus *Bonnemaisonia* (Feldmann & Feldmann, 1942). However, neither *B. clavata* nor *B. asparagoides* (type species of the genus) has a *Bonnemaisonia*-type life history. This supports the necessity to re-instate the original term (*Asparagopsis*-type) to describe a life history that involves a sequence of morphologically distinct gametophyte, carposporophyte and tetrasporophyte phases.

Nuclear DNA content analysis and development

The homologous gametophyte cells show similar ploidy levels in both species. Spermatia and apical cells have rfa values (3–6 μm^2) that correspond to 1–2C levels and G_1–G_2 stages. However, a 1C-value was observed in the outer cortical cells of *B. asparagoides*, probably due to the measurement of an apical part of the thallus. In both *B. asparagoides* and *B. clavata*, the ploidy



Figs 28–31. Nuclear patterning of *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*, material fixed in Carnoy's and stained with DAPI. The numbers indicate the relative fluorescent area (μm^2) of the nuclei examined (circles). Fig. 28. Axial cells of the apical zone of the *B. asparagoides* gametophyte. Fig. 29. Apical zone of a main filament of the *B. asparagoides* prostrate phase. Fig. 30. Major nuclear pattern of *B. asparagoides* prostrate phase after 3 weeks of culture, main (arrowheads) and secondary (arrows) apical cells indicated. Fig. 31. Major nuclear pattern of *B. clavata* prostrate phase after 3 weeks in culture, main (arrowheads) and secondary (arrows) apical cells indicated. Scale bars: 100 μm (Figs 28, 30, 31), 50 μm (Fig. 29).

level of axial cells increases from the apical cell to the basal part of the thallus, in accordance with their apical growth pattern. The gametophytes grow from a uninucleate apical cell, initiating a uninucleate derived axial cell, which increases its size proportionally with its ploidy level (Fig. 28). A similar nuclear pattern characterized by progressive increase in DNA content was described for *Wrangelia plumosa* Harvey (Goff & Coleman, 1990).

In the carpospores of *B. asparagoides* and *B. clavata*, 4–16C and 3–6C values were observed, respectively, and DNA content appears to be correlated with their carpospore size differences (Salvador *et al.*, 2008). The relationship between nuclear DNA content and cell dimensions in red algae has been described previously (Goff & Coleman, 1990; Kapraun & Dunwoody, 2002). During carpospore development ploidy levels of 3C (*B. clavata*) or 4C (*B. asparagoides*) were typical, with higher ploidy level is of 6C (*B. clavata*), and 8–16C (*B. asparagoides*) observed. Similar ploidy levels were reported in carpospores of *Polysiphonia mollis* Hood et Harvey (4C; Goff & Coleman, 1986), *Wrangelia plumosa* (2C–8C; Goff & Coleman, 1990) and *Dasysiphonia chejuensis* Lee et West (8C; Choi *et al.*, 1994).

Although mature carpospores were obtained before attachment, it was not possible to determine their ploidy levels as nuclear dimensions

could not be clearly measured. This could be due to interference from auto-fluorescence associated with cell walls and intracellular granules (Goff & Coleman, 1986). After settlement, the carpospores increased their nuclear sizes considerably, from 3 to 12C in *B. clavata* and from 4 to 32C in *B. asparagoides*. Goff & Coleman (1984) described a similar increase in carpospore size in *Polysiphonia mollis* after attachment. In our study, the ploidy levels in the first divisions of the attached carpospores from both species exhibited a sequential reduction of DNA contents due to the absence of DNA replication during consecutive mitoses (Goff & Coleman, 1986, 1990; Garbary & Clarke, 2002). Lower ploidy levels (<8C) were observed in *B. clavata* nuclei after the second division, whereas in *B. asparagoides* this was seen after the third division. In both taxa, DNA content reduction was complete after the third carpospore division, when the nuclei reached their lowest ploidy level (2C). This corresponds to the G_1 stage of the diploid phase. It should be noted that G_1 and G_2 cell-cycle stages were observed in carpospores with one, two and four nuclei. However, carpospores with more than four nuclei showed three distinct ploidy levels: 2C, 4C and 8C in *B. asparagoides*, and 2C, 3C and 4C in *B. clavata*. Elevated ploidy levels in the carpospores of *B. asparagoides* could be related to the polyploidy observed in the apical cells of its 'Hymenoclonium' phase.

In the 'Hymenoclonium' phase of both species, some homologous cells showed different C-values (Tables 1 and 2), perhaps reflecting differences in their growth patterns (Salvador *et al.*, 2008). In *B. asparagoides*, similar values were found for the main and secondary apical cells, reflecting the uniform growth of all filaments, which produces their characteristic stellate developmental pattern. In contrast, in *B. clavata* the main apical cells had higher ploidy levels than the secondary, perhaps because only its two main filaments grow continuously, resulting in a characteristic bipolar development pattern. Cells from 1- and 3-week-old filaments had dissimilar values. Both the initial cells from the carpospores and the main apical cells of the filaments from 1-week-old thalli had lower values than the apical cells from 3-week-old thalli. This could be because the main filaments are not well-defined during the first week, and their apical cells have similar C-values to other vegetative cells. During the third week of culture, the apical cells of the main filaments became highly endopolyploid with increased C-values.

It is interesting to note that the nuclear pattern in the 'Hymenoclonium' phase in both taxa is distinct from that of the gametophyte. Whereas gametophyte growth is associated with a progressive increase in DNA content, the 'Hymenoclonium' phase growth involves a cascade of DNA values until the nuclei reach 2–4C levels.

Nuclear DNA content analysis and life history

The ploidy levels obtained in the different stages (vegetative and reproductive gametophyte cells, carpospores, 'Hymenoclonium' cells and tetrasporangia-like protuberances) of the life histories corroborated the culture observations. Firstly, the 1C-level was only observed in the gametangial phase and in the tetrasporangia-like protuberances, whereas the 2C-level was the minimum observed in the 'Hymenoclonium' phase (Tables 1, 2). This supports a life-history model characterized by an erect haploid gametophyte, diploid carpospores and a prostrate diploid 'Hymenoclonium' phase (Fig. 32) consistent with a sexual life cycle as reported in other red algae (Goff & Coleman, 1990; Sheath *et al.*, 1996; Kapraun *et al.*, 2007). Secondly, for the first time meiosis is confirmed cytologically in the tetrasporangia-like protuberances. This somatic meiosis produces four cells with haploid nuclei, one of which initiates a new gametophyte, while the other three remain at the base of the gametophyte and take no part in its development. As none of the four meiotic cells are released, these structures are inconsistent with the concept of rhodophyte tetrasporangia (Guiry, 1990).

In this study, the life history of *B. clavata* has been completed for the first time and the occurrence of meiosis in the life histories of *B. asparagoides* and *B. clavata* has been demonstrated cytologically and in culture. The life histories of these taxa are characterized by the following: the presence of only one spore type (carpospore), the development by somatic meiosis of the gametophyte on apical cells of the diploid prostate phase, and the production of a young gametophyte from only one of the four meiotic nuclei. As a result, we affirm that this life history corresponds to the *Lemanea*-type (Magne, 1967; Necchi & Carmona Jiménez, 2002) and is therefore demonstrated for the first time in marine red algae. This suggests that somatic meiosis is not unique to the freshwater Batrachospermales. Further studies will be required to confirm this life history in several Bonnemaisoniales species within the genera *Atractophora*, *Delisea*, *Naccaria* and *Ptilonia*, as well as other marine algae with direct development (Dixon, 1982; Hawkes, 1990). We strongly recommend the use of our image analysis system to estimate nuclear DNA content for the study of complex life histories because only small quantities

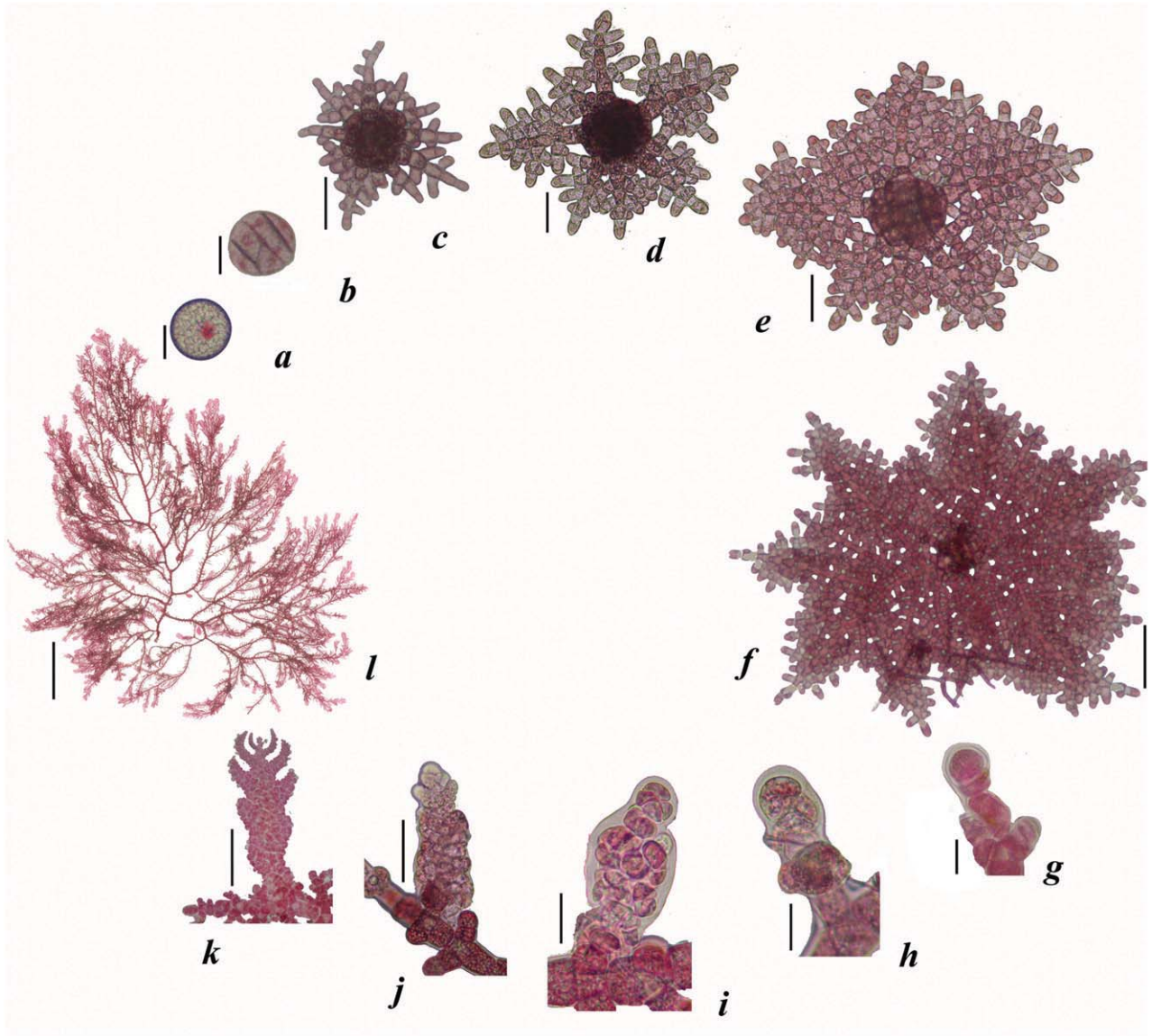


Fig 32. Diagrammatically arranged images to show the life history of *B. asparagoides*. (*a*) Attached carpospore at 12 h. (*b*) Divided carpospore at 48 h. (*c*) 'Hymenoclonium' germling after 6 days. (*d*) 'Hymenoclonium' after 8 days. (*e*) 'Hymenoclonium' after 13 days. (*f*) 1-month-old 'Hymenoclonium'. (*g*) Initial cell of a tetrasporangia-like protuberance. (*h*) Tetrasporangia-like protuberance. (*i–j*) Upright gametophyte thalli. (*k*) Young gametophyte. (*l*) Gametophyte. Scale bars: 20 µm (*g–i*), 50 µm (*a–e*, *j*), 100 µm (*f*, *k*), 2 cm (l).

of material are needed, which is important when working with cultured material.

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Nuclear DNA content variation in life history phases of the Iberian Bonnemaisoniaceae (Rhodophyta).

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Variació del contingut de DNA nuclear en el cicle vital de les Bonnemaisoniaceae (Bonnemaisoniales, Rhodophyta) de la península Ibèrica

Es van examinar les cinc espècies de la família *Bonnemaisoniaceae* presents a la península Ibèrica (*Asparagopsis armata*, *Asparagopsis taxiformis*, *Bonnemaisonia clavata*, *Bonnemaisonia asparagoides* i *Bonnemaisonia hamifera*) i dues espècies de Nova Zelanda (*Ptilonia willana* i *Delisea plumosa*).

L'espectrofluorimetria i l'anàlisi d'imatges, mitjançant l'ús del fluorocrom DAPI (4', 6-diamidino-2-fenilindol, dilactate) com a localitzador de DNA, van ser les tècniques emprades per estimar el contingut del DNA nuclear en el gametòfit i en la generació prostrada o esporòfit corresponent.

El contingut mínim de DNA corresponent als valors 2C dels gametòfits (espermacis o cèl·lules vegetatives) va oscil·lar entre 0,5-0,8 pg, rang similar a l'obtingut en extrapolar els valors 2C (0,6-0,85 pg) a partir dels 4C obtinguts en les generacions esporofítiques o prostrades dels mateixos espècimens. Aquests resultats confirmen l'alternança de fases nuclears haploides i diploides prèviament suggerida en algunes espècies de l'ordre *Bonnemaisoniales*.

Les observacions presentades en aquest estudi concorden amb la presència d'una fecundació i d'una meiosi en el cicle vital d'aquestes algues diplobiòntiques, tant en els tàxons que presenten tetrasporogènesi (*A. armata* i *A. taxiformis*), com en els que s'ha descrit un desenvolupament directe del gametòfit (*B. asparagoides* i *B. clavata*).

Es va observar una àmplia variació en el contingut del DNA dins una mateixa planta en alguns gametòfits, en què es van registrar valors de fins a 8C. Els recomptes cromosòmics coneguts d'algunes espècies i la superposició del contingut de DNA de les espècies examinades sobre un arbre filogenètic hipotètic, basat en les filogènies en què s'inclouen les *Bonnemaisoniales*, ha permès determinar que la tendència evolutiva de la quantitat de DNA en aquest ordre es caracteritza per la conservació d'un genoma ancestral.

Les mesures obtingudes en aquest estudi van permetre ampliar la base de dades del contingut de DNA en les algues (http://people.uncw.edu/kapraund/DNA/), amb la incorporació de set noves espècies de quatre gèneres diferents.

Nuclear DNA content variation in life history phases of the Iberian Bonnemaisoniaceae (Rhodophyta).

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Abstract

The DNA-localizing fluorochrome DAPI (4', 6-diamidino-2-phenylindole, dilactate) and RBC (chicken erythrocytes) standard were used with image analysis and static microspectrophotometry to estimate nuclear DNA contents in gametophyte and sporophyte phases of 5 species of Bonnemaisoniales from the Atlantic and Mediterranean coasts of Spain. Estimated nuclear genome sizes for the Bonnemaisoniales expand our data base to include 7 species representing 4 genera. DNA content estimates from mean values for 2C nuclei in the haploid gametophytes range from 0.5 – 0.8 pg. A similar range was observed by extrapolating from the 4C mean values of the prostrate/sporophytic phases. In *Asparagopsis armata, Asparagopsis taxiformis, Bonnemaisonia asparagoides* and *Bonnemaisonia clavata,* I_f values in mature 2C male gamete (spermatia) nuclei closely approximate 50% of 4C values in vegetative cells of their respective prostrate/sporophytic phase, consistent with meiosis and a sexual life history in these diplobiontic algae. A wide intraplant variation (endopolyploidy) of DNA contents to 8C was demonstrated in the gametophyte phase. Availability of a consensus higher-level phylogenetic tree for Bonnemaisoniales has opened the way for determining evolutionary trends in DNA amounts. Both estimated genome sizes and published chromosome numbers for Bonnemaisoniales suggest a narrow range of values consistent with conservation of an ancestral genome.

Key words: *Asparagopsis*, *Bonnemaisonia*, Bonnemaisoniales, DAPI, DNA content, image analysis, life history, microspectrophotometry, polyploidy.

Introduction

The marine red algal genera *Asparagopsis* and *Bonnemaisonia* (Bonnemaisoniales) have been the subject of numerous studies concerning life history (Feldmann & Feldmann, 1942; Chihara, 1961; Shevlin & Polanshek, 1978; Rueness & Åsen, 1982; Bonin & Hawkes, 1987; Salvador *et al*., 2009), ecology as invasive species (Farnham, 1994; Ni Chualáin *et al*., 2004; Altamirano *et al*., 2009), phylogeography (Andreakis *et al*., 2004, 2007; Sherwood, 2008) and potential applications of their bioactive metabolites (Haslin *et al*., 2001; Salvador *et al*., 2007). Despite continuing interest in members of this order, modern molecular techniques are only now beginning to overcome a history of pervasive taxonomic and nomenclatural confusion (Ní Chualáin *et al*., 2004; Salvador *et al*., 2008, 2009). Although Bonnemaisoniales was separated from Nemaliales on the basis of their then known alternation of generations (Feldmann & Feldmann, 1942), it is now understood that this life history pattern lacks taxonomic significance and many orders of red algae are heterogeneous with regard to life history (Garbary & Gabrielson, 1990). The distinction of these two orders is now generally recognized on the basis of ultrastructural details of pit plugs and caps (Pueschel, 1989) and plastids (Chihara & Yoshizaki, 1972) as well as molecular studies (Freshwater *et al*., 1994; Le Gall & Saunders, 2007).

Bonnemaisoniales, as originally proposed, is characterized by a heteromorphic life history (Feldmann & Feldmann, 1942). According to Dixon (1982), the information available for members of these taxa indicates both a 'Bonnemaisonia'-type life history as well as a direct development of gametophytes from vegetative branches of the assumed diploid sporophyte with an absence of tetrasporogenesis (Chihara, 1962; Feldmann, 1966; Boillot, 1967; Chen *et al*., 1970). In addition, in *Bonnemaisonia asparagoides* and *Bonnemaisonia clavata*, somatic meiosis has been described (Rueness & Åsen, 1982; Salvador *et al*., 2009) as reported in the 'Lemanea'-type life history (Necchi & Carmona, 2002). Despite the numerous studies carried out on the life history of the Bonnemaisoniales, the sequence of nuclear phases has been demonstrated only in *B*. *asparagoides* and *B*. *clavata* (Salvador *et al*., 2009).

Microspectrophotometry with the DNA-localizing fluorochrome DAPI (4', 6-diamidino-2-phenylindole) has been employed successfully to demonstrate an

alternation of ploidy levels associated with meiosis and sexual reproduction in other red algae (for a review, see Kapraun, 2005 and Kapraun *et al*., 2007), including members of Batrachospermales and Thoreales which share a 'Lemanea'-type life history (Huth, 1981; Necchi & Carmona, 2002). The present investigation of nuclear DNA contents in Bonnemaisoniales from the Atlantic and Mediterranean coasts of Spain was initiated to determine the extent of nuclear DNA content variation, to identify any correlation between genome size and phylogeny, to determine if DNA contents are diagnostic and represent synapomorphies at either genus or species level and to corroborate an alternation of haploid and diploid nuclear DNA contents in gametophyte and prostrate/sporophytic phases, respectively.

Materials and methods

Source of specimens

Five species of Bonnemaisoniales were collected from the Mediterranean [Aiguafreda and Llançà (Girona), Porto Colom (Majorca)] and Atlantic [Cabo Cruz (A Coruña), Zumaya (Guipúzcoa)] coasts of Spain. Specific information for collection locations is available in the Table 1. Due to the difficulty in obtaining 'Hymenoclonium' phases of *B. clavata* and *B. asparagoides*, these phases were cultured in the laboratory from carpospores which produced young gametophytes (Salvador *et al*., 2008).

Identification of collected material

The taxonomic validity of *A. armata* and *A. taxiformis* as distinct species as well as morphological criteria useful in distinguishing them (Bonin & Hawkes, 1987) have been confirmed (Andreakis *et al*., 2004). However, their respective sporophytic phases, *Falkenbergia rufolanosa* and *Falkenbergia hillebrandii*, have been described as 'indistinguishable' (Womersley, 1996). According to Ni Chualáin *et al*. (2004), "The only morphological differences apparent among the *Falkenbergia* isolates are the sizes of the cells" at three different distances from the apex. For these distances, the cells of *F. rufolanosa* are considerably shorter and narrower than those in *F. hillebrandii*.

However, these observations were made from cultured specimens and may not be representative of field collected plants (Andreakis *et al*., 2004). Recent morphological and anatomical studies of *B. asparagoides* and *B. clavata*, including their respective prostrate phases, provide additional taxonomic characters to identify and distinguish these algae (Salvador *et al*., 2008).

Nuclear DNA content estimates

Algal material was fixed in Carnoy's solution and stored in 70% ethanol at 4 ºC (Kapraun, 2005). Preserved material was rehydrated in water and softened in 5% w/v EDTA (Goff & Coleman, 1990) for 12 h. Algal specimens were transferred to cover slips treated with subbing solution, air dried and stained with DAPI (0.5 μg mL^{-1}) (Sigma Chemical Co., St. Louis, MO 63178) as previously described (Goff & Coleman, 1990; Kapraun & Nguyen, 1994). Nuclear DNA content estimates based on microspectrophotometry with DAPI followed procedures specified previously (Kapraun & Nguyen, 1994; Kapraun, 1994) using a protocol modified after Goff & Coleman (1990). Nuclear DNA content estimates based on image analysis of DAPI-stained specimens followed a procedure modified from Kapraun & Dunwoody (2002) and Choi *et al*. (1994) using a Cooled CCD Miramax RTE 782-Y high performance digital camera placed on a Leica DMRB fluorescence microscope and subsequently analyzed using MetaMorph software (Molecular Devices, Toronto, Canada). For a recent, comprehensive review of theory and practice of DNA quantification by densitometry, see Hardie *et al*. (2002).

Chicken erythrocytes (RBC) with a DNA content of 2.4 pg (Clowes *et al*., 1983; Riechmann *et al*., 2000) were used to quantify mean fluorescence intensity (I_f) values obtained from image analysis and microspectrophotometry for algal specimens (Kapraun, 1994; Kapraun & Dunwoody, 2002). DAPI binds by a non-intercalative mechanism to adenine and thymine rich regions of DNA which contain at least four A-T base pairs (Portugal & Waring, 1988). Consequently, chicken erythrocytes (RBC) can be used directly as standards for determining amounts of DNA only when the A-T contents of both standard and experimental DNA are equivalent (Coleman *et al*., 1981). *Gallus* has a nuclear DNA base composition of 42-43 mol % G + C (Marmur & Doty,

1962). Published data indicate similar mean mol % values for the Rhodophyta (Dutcher *et al*., 1990; Kapraun *et al*., 1992; Kapraun *et al*., 1993*a*, 1993*b*; Le Gall *et al*., 1993; Lopez-Bautista & Kapraun, 1995). Algae investigated in this study are assumed to have a similar range of base pair compositions, and linearity is accepted between DAPI-DNA binding in both RBC and algal samples (Le Gall *et al*., 1993). Nuclear DNA contents were estimated by comparing the I_f values of the RBC standard and algal samples (Kapraun & Nguyen, 1994; Kapraun, 2007).

Supplementary materials and methods, information for collection locations, and data for number of algal nuclei examined in each sample and estimates of nuclear genome size (pg) ± SD are available at http://people.uncw.edu/kapraund/dna.htm (see Table III Rhodophyta, Appendix III Rhodophyta and Supplementary Table 1). Nuclear DNA content data for these and other red algae are incorporated into a database of plant genome sizes (Kapraun, 2005; Gregory *et al*., 2007) compiled and hosted by the Royal Botanic Gardens (RBG) Kew web page (http://www.rbgKew.org.uk/cval/homepage.html).

Assignment of ploidy level

Assignment of estimated nuclear DNA contents to specific C-values is presumptive in that no karyological investigations were conducted on the algal samples used for nuclear DNA content estimates. In the present study, the smallest genome sizes in mature spermatia were assumed to represent the single 2C (G_2) content (Table 1) of a prophase nucleus according to Goff & Coleman (1990).

Results and discussion

Estimated nuclear genome sizes of Bonnemaisoniales examined here expand our data base to include 7 species representing 4 genera. Table 1 summarizes nuclear genome size estimates (pg) ± SD obtained for Bonnemaisoniales and Table 2 includes the mean estimates for 2C and 4C values.

Nuclear DNA content estimates

DAPI staining with the protocol modified from Goff & Coleman (1990) yielded reproducible, stable nuclear fluorescence with little apparent interference from autofluorescence, non-specific binding, or other cellular material. Nuclear DNA content estimates for replicated (2C) haploid (1N) genomes should be considered accurate only to ± 0.1 pg (Kapraun & Shipley, 1990; Kapraun & Dutcher, 1991; Hinson & Kapraun, 1992; Kapraun & Bailey, 1992).

Measurement of mean I_f values in mature spermatia from Spanish Bonnemaisoniales resulted in 2C nuclear genome size estimates of 0.5 – 0.8 pg (Table 1). A similar range (0.6 – 0.85 pg) was observed by extrapolating from the 4C mean values (Table 2) found in prostrate/sporophytic phases (Kapraun & Dutcher, 1991; Hinson & Kapraun, 1992). Some vegetative cells from the *B. asparagoides* and *B. clavata* gametophytes showed comparable 2C values as well (Table 1). In most gametophyte samples (vegetative cells and spermatia) as well as in carpospores, a cluster of DNA content values was observed approximating twice the estimated 2C values and, presumably, represent 4C nuclei with a range of 0.9 – 1.8 pg (Table 1). In addition, populations of synthesis (S) phase nuclei (Goff & Coleman, 1990) were recorded in most samples.

Bonnemaisonia clavata gametophytes collected from Girona (Mediterranean coast) and A Coruña (Atlantic coast) showed the same nuclear genome sizes. Nuclear DNA content levels obtained in this study from the I_f were in agreement with those obtained from nuclear areas of *B. asparagoides* and *B. clavata* by Salvador *et al*. (2009).

Present 2C genome sizes of 0.5 – 0.8 pg are best appreciated when compared with the minimum amount of DNA estimated for specifying the mRNA sequences required for angiosperm development. Specifically, the genome of *Arabidopsis thaliana* (L.) Heynhold, with 0.16 pg = 157 Mb (Bennett *et al*., 2003) is one of the smallest found in angiosperms (Bennett & Smith, 1976) but still has 30,000 or twice the estimated 15,000 genes per haploid genome required for development (Flavell, 1980). Single copy (1C) values of 245 – 392 Mbp (0.25 – 0.4 pg) can be derived for species of

Bonnemaisoniales using the expression 1 pg = 980 Mbp (Cavalier-Smith, 1985; Bennett *et al*., 2000).

Comparison of static microspectrophotometry and image analysis

Nuclear genome size estimates for algae obtained with image analysis measurements have been found to be highly correlated with the estimates obtained with microspectrophotometry (Kapraun & Nguyen, 1994; Kapraun & Buratti, 1998; Kapraun & Dunwoody, 2002). In the present study, a regression analysis revealed a high correlation between estimates using these techniques ($r = 0.93$, $p < 0.01$).

Presence of polyploid nuclei

Intraplant variation (polyploidy) of DNA contents was observed in both gametophyte and prostrate/sporophytic phases of Bonnemaisoniales investigated, and 4C – 8C nuclei were quantified in axial cells of gametophytes, carpospores and some vegetative cells of the prostrate/sporophytic phase of *A*. *armata, B*. *asparagoides, B*. *clavata* and *B*. *hamifera* (Table 1). A similar intraplant variation was also observed from nuclear areas in a parallel investigation of *B. asparagoides* and *B. clavata* (Salvador *et al*., 2009). According to these authors, the uninucleate apical cells of the gametophytes produce axial cells by a sequential increase in ploidy levels which was also observed in the released carpospores before their germination (Salvador *et al*., 2009). Our observations suggest that the gametophytes of the Bonnemaisoniales studied here show similar nuclear pattern. Nuclear DNA content variation from 2C – 16C has been documented previously in vegetative cells of other red algae (Goff & Coleman, 1990; Kapraun, 2005). Higher nuclear DNA levels typically correlate with increased cell size in both green (Kapraun & Nguyen, 1994) and red algae (Kapraun, 2005), with nuclear DNA variation of over two orders of magnitude within the same thallus reported in the latter (Goff & Coleman, 1990). For contemporary reviews of polyploidy effects on genomic plasticity and phenotypic variation in plant systems see Chen (2007) and Leitch & Leitch (2008).

Nuclear DNA content variation associated with a diplobiontic life history

Considerable life history variation has been reported in species of Bonnemaisoniales (Dixon, 1982; Rueness & Åsen, 1982; Hawkes, 1990) which typically involves a sexual gametophyte, a carposporophyte and a tetrasporophyte (Chihara, 1962). Culture studies suggest intraspecific variability in development of *Delisea pulchra* (Greville) Montagne (Bonin & Hawkes, 1988). In *Atractophora* and *Naccaria*, gametophytes are reported to develop directly from the prostrate protonemal stage produced from carpospores (Boillot, 1967). *Bonnemaisonia asparagoides* (monoecious) and *B. clavata* (dioecious) have an alternation of heteromorphic generations with 'Hymenoclonium' phases (Rueness & Åsen, 1982; Salvador *et al*. 2008, 2009). Direct development of *B. asparagoides* on the 'Hymenoclonium' phase was observed (Feldmann, 1966; Rueness & Åsen, 1982). Recent investigations of *B. asparagoides* and *B. clavata* from Spain confirm direct development of gametophytes from prostrate protonema following vegetative meiosis (Salvador *et al*., 2009). In contrast, *Bonnemaisonia hamifera* is dioecious and alternates with a 'Trailliella' tetrasporophyte (Chen *et al*., 1970) and *Bonnemaisonia geniculata* Gardner is reported to have another type of tetrasporophyte (Shevlin & Polanshek, 1978). *Asparagopsis taxiformis* and *A. armata* exhibit an alternation of generations with *F. hillebrandii* and *F. rufolanosa* representing their respective tetrasporophyte phases (Feldmann & Feldmann, 1942; Dixon, 1964; Andreakis *et al*., 2004; Ní Chualáin *et al*., 2004). Tetrasporogenesis has been reported in both *A. taxiformis* (Chihara, 1961, 1962) and *A. armata* (Feldmann & Feldmann, 1942; Bonin & Hawkes, 1987), and precise environmental conditions required for tetrasporogenesis have been described (Lüning, 1990; Guiry & Dawes, 1992; Andreakis *et al*., 2004).

The DNA-localizing fluorochrome DAPI and microspectrophotometry have been used to demonstrate variations in nuclear DNA levels consistent with an alternation of haploid (2C) and diploid (4C) phases in red algae associated with a sexual life cycle (Kapraun, 1994; Kapraun *et al*., 1992, 2007). Despite numerous life history investigations on Bonnemaisoniales, the sequence of nuclear phases has been demonstrated only in *B. asparagoides* and *B. clavata* (Salvador *et al*., 2009). In the present study, no evidence of tetrasporogenesis was observed in either collected or cultured material. However, in comparing the mean values obtained between phases, the

gametophytes showed a 2C range of 0.6 – 0.8 pg whereas their prostrate/sporophytic phases ('Falkenbergia', 'Hymenoclonium' and 'Trailliella') had a 4C range of 1.2 – 1.7 pg (Table 2). In addition, the mean nuclear genome sizes in gametophytes with presumed 2C replicated haploid nuclei approximate 50% of those in prostrate/sporophytic phases with presumed 4C replicated diploid nuclei (Table 2).

These results confirm the alternation of haploid and diploid phases previously suggested by previous cytological investigations (Magne, 1960, 1964; Rueness & Åsen 1982) and culture studies (Chihara, 1961, 1962; Chen *et al*., 1970; Rueness & Åsen, 1982; Salvador *et al*., 2009) in the Bonnemaisoniales. Assignment of ploidy levels and reproductive phases for some isolates and cultures investigated remains problematic (Table 1) as gametophytes can develop on the tissue of the prostrate phase making it difficult to distinguish the phases (Chen *et al*., 1970; Salvador *et al*., 2009).

In Batrachospermales, reports of a unique pattern of somatic meiosis (Necchi & Carmona, 2002) in the development of haploid gametophytes from vegetative branches of the microscopic diploid sporophyte, 'Chantransia' stage, was confirmed with microspectrophotometry (Kapraun *et al*., 2007). In the Bonnemaisoniales studied here, 2C levels in mature spermatium nuclei closely approximate 50% of 4C values in vegetative cells of mature sporophyte/prostrate phases (Table 1). These results are consistent with a sexual life history and the presence of meiosis in these diplobiontic algae, both in taxa exhibiting tetrasporogenesis (*A. armata* and *A. taxiformis*) as well as in taxa with gametophyte development (*B. asparagoides* and *B. clavata*) from vegetative branches of the microscopic diploid sporophyte/prostrate phase (Rueness & Åsen, 1982; Salvador *et al*., 2009).

Molecular phylogeny and patterns of genome size variation

New availability of both a DNA C-values database (Kapraun, 2005) and trees derived from a consensus of several different studies has opened the way for determining evolutionary trends in DNA amounts for red algae (Kapraun, 2005). A phylogenetic hypothesis for Bonnemaisoniales (Freshwater *et al*., 1994; Ní Chualáin *et al*., 2004; Le Gall & Saunders, 2007) provides a picture of nuclear genome size evolution among

these taxa. Southern hemisphere genera *Delisea* and *Ptilonia* are basal or sister group to a *Bonnemaisonia-Asparagopsis* clade according to the phylogenetic tree resulting from SSU alignment (Ní Chualáin *et al*., 2004). Nuclear genome size data for these taxa (Kapraun, 2005) and from the present study indicate 2C nuclear genome sizes in Bonnemaisoniales of about 0.5 – 0.8 ± 0.1 – 0.2 pg, approximating the ranges observed in other members of the florideophycidae (Kapraun, 2005). It is perhaps noteworthy that nuclear DNA contents are similar for dioecious and monoecious (only *B*. *asparagoides*) species in the Bonnemaisoniales (Fig. 1).

Asparagopsis armata has become widely distributed in Europe as an alien introduction (Farnham, 1994) and fits the definition of a marine invader (Cronk & Fuller, 1995). In flowering plants (Stebbins, 1971; Jackson, 1976) and green algae (Kapraun & Martin, 1987), competitive and aggressive species or 'weeds' are often characterized by elevated nuclear DNA contents or polyploidy. Although the 2C genome size of *A. armata* is unremarkable and similar to that of *A. taxiformis*, their respective tetrasporophytes have the highest 4C levels observed in Bonnemaisoniales (Fig. 1). Consequently, it is interesting to note that the tetrasporophyte is the most resilient phase in *Asparagopsis* (Andreakis *et al.,* 2004) and the primary means of dispersal (Ní Chualáin *et al*., 2004).

Published karyological studies, limited to three species of *Asparagopsis* and *Bonnemaisonia*, suggest that 1n chromosome number range of 18 – 25 (Cole, 1990). Reported chromosome complements of 1n = 10 in *A. armata* (Svedelius, 1933) should be reinvestigated. The chromosome complement of 1n = ca. 30 in *B. asparagoides* from Scandinavia may represent an aneuploid population. In red algae, the postulated basal (ancestral) nucleotype is characterized both by small genome sizes and small chromosome complements (Kapraun, 2005). Chromosome complements greater than 1n = 10 probably reflect ancestral polyploidy events (Cole, 1990; Kapraun *et al*., 2007).

In Bonnemaisoniales, published chromosome number variation and nuclear DNA content estimates in the present study are consistent with one or more instances of aneuploidy following an ancestral (fossil) polyploidy event (Kapraun & Bailey, 1992; Kapraun & Buratti, 1998). A similar sequence of karyological events was postulated in Gelidiales (Kapraun *et al*., 1993*a*) which are characterized by a small range of nuclear genome sizes (2C = 0.35 – 0.45 pg). In Gelidiales and Bonnemaisoniales, speciation

probably was not accompanied exclusively by loss or gain of chromosomes (non-disjunction), but rather, has involved translocations, and fusion and/or fission processes which produce changes in chromosome numbers independent of nuclear DNA contents (Kapraun, 1993). The many complex causal factors behind these mechanisms have been discussed elsewhere (Wenzel & Hemleben, 1982; Pichersky, 1990; Lynch & Conery, 2000; Bennetzen, 2002).

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Table 1. Nuclear DNA content of Bonnemaisoniales. Data standardized to the DNA level of chicken erythrocytes (RBC = 2.4 pg).

					Nuclear Genome Size (pg)			
Species	**Origin**	**Phase**	**Cell type**	**Nº of nuclei**	**2C**	**4C**	**8C**	**Method**
Asparagopsis armata Harvey	Girona	G	C	11			3.2 ± 0.6	IA
	(Spain)	"	V	48		1.7 ± 0.1		IA
		"	V	89		1.7 ± 0.2		IA
		"	Sp	51		1.8 ± 0.3		IA
		"	Sp	61	0.6 ± 0.1			M
		"	Sp	62	0.7 ± 0.1			M
		S	V	157		1.6 ± 0.1		M
		"	V	14			2.7 ± 0.4	M
Asparagopsis taxiformis (Delile) Trevisan	Majorca	G	V	63		1.8 ± 0.2		IA
	(Spain)	"	Sp	67		1.7 ± 0.1		IA
		"	Sp	4		1.6 ± 0.1		M
		"	Sp	69	0.6 ± 0.1			M
		"	Sp	42	0.7 ± 0.1			M
		"	Sp	49	0.8 ± 0.2			M
		"	Sp	7	0.6 ± 0.1			IA
		"	Sp	22	0.7 ± 0.1			IA
		"	V	17			2.4 ± 0.4	IA
		"	V	10			2.9 ± 0.4	M
		S	V	102		1.7 ± 0.3		M
Bonnemaisonia asparagoides (Woodward) C. Agardh	Girona	G	V	51	0.6 ± 0.1			M
	(Spain)	"	V	46	0.6 ± 0.2			IA
		"	Sp	12	0.6 ± 0.2			IA
		"	Sp	21	0.5 ± 0.1			M
		"	Sp	39		0.9 ± 0.1		IA
		"	V	13		0.9 ± 0.1		M
		"	V	30		1.1 ± 0.2		M
		"	V	3		1.3 ± 0.2		IA
		"	V	31		1.6 ± 0.1		IA
		"	V	46		1.8 ± 0.2		IA
		"	C	5			2.3 ± 0.2	IA
	Culture	G	C	61		1.6 ± 0.1		IA
		"	C	26		1.6 ± 0.2		IA
		"	C	12		1.7 ± 0.3		IA
		"	V	29			2.2 ± 0.1	IA
	Culture	S	V	104		1.1 ± 0.1		M
		"	V	64		1.2 ± 0.2		M
		"	V	49			2.3 ± 0.1	IA
		"	V	134			2.2 ± 0.0	IA
Bonnemaisonia clavata Hamel	A Coruña	G	Sp	20	0.6 ± 0.1			M
	(Spain)	"	Sp	31	0.8 ± 0.1			M
		"	Sp	46		0.9 ± 0.1		M
		"	Sp	13		1.1 ± 0.1		M
	Girona	G	V	25		1.6 ± 0.1		IA
	(Spain)	"	Sp	83	0.6 ± 0.2			IA
		"	V	8	0.6 ± 0.2			IA
		"	C	71			2.2 ± 0.2	IA
	Culture	G	C	8		1.4 ± 0.1		IA
		"	C	24		1.5 ± 0.2		IA
		"	C	25		1.6 ± 0.1		IA
		"	C	27		1.7 ± 0.1		IA
	Culture	S	V	87		1.2 ± 0.2		M
		"	V	69			1.9 ± 0.2	IA
		"	V	44			2.3 ± 0.0	IA
		"	V	70			2.4 ± 0.0	IA
Bonnemaisonia hamifera Hariot	Guipúzcoa	G	V	104	0.8 ± 0.2			M
	(Spain)	"	V	89		1.5 ± 0.3		M
		"	V	16			2.7 ± 0.7	M
		S	V	49		1.3 ± 0.2		IA
		"	V	29		1.3 ± 0.1		IA
		"	V	47		1.4 ± 0.3		IA
		"	V	47		1.4 ± 0.2		IA
		"	V	110			3.2 ± 0.6	M
[1] *Delisea plumosa* Levring	New Zealand	G	V	47		1.0 ± 0.2		M
[1] *Ptilonia willana* Lindauer	New Zealand	G	M	165	0.6 ± 0.1			M

[1] Data from Kapraun (2005)

Abbreviations: G= Gametophyte, S= Sporophyte/prostrate phase, C= Carpospore, V= Vegetative cell, Sp= Spermatium, IA= Image analysis, M= Microspectrophotometry.

Table 2. Nuclear genome size mean estimates from 2C and 4C values for gametophytes and sporophytic/prostrate phases, respectively.

Species	Reproductive Phase	Nuclear Genome Size (pg) 2C	2C (50% of 4C)	4C
Asparagopsis armata Harvey	G	0.7	0.85	1.7
(='Falkenbergia' phase)	S		0.8	1.6
Asparagopsis taxiformis (Delile) Trevisan	G	0.7	0.85	1.7
(='Falkenbergia' phase)	S		0.85	1.7
Bonnemaisonia hamifera Hariot	G	0.8	0.75	1.5
(='Trailliella' phase)	S		0.7	1.4
Bonnemaisonia asparagoides (Woodward) C. Agardh	G	0.6	0.6	1.2
(='Hymenoclonium' phase)	S		0.6	1.2
Bonnemaisonia clavata Hamel	G	0.7	0.6	1.2
(='Hymenoclonium' phase)	S		0.6	1.2

Abbreviations: G=gametophyte, S=sporophytic/prostrate phase.

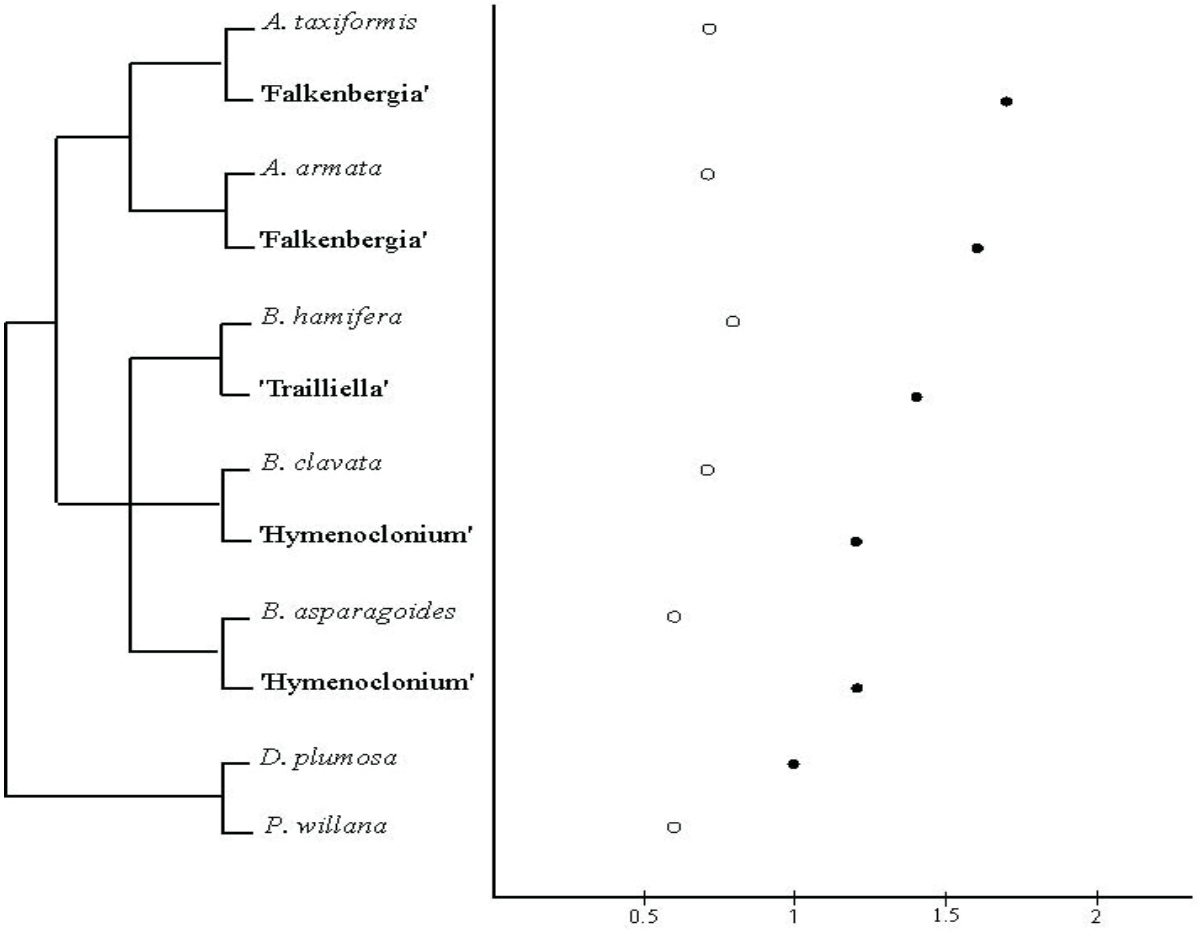

Figure 1. Estimated nuclear DNA contents (○= 2C, ●= 4C) superimposed on a phylogenetic tree of Bonnemaisoniales inferred from cpDNA RFLPs and Bayesian analyses of the SSU alignment (Ní Chualáin *et al*., 2004), plastid *rbc*L gene sequence analysis (Freshwater *et al*., 1994) and combined analysis of EF2, SSU and LSU (Le Gall & Saunders, 2007).