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THE INVASIVE GENUS *ASPARAGOPSIS* (BONNEMAISONIACEAE, RHODOPHYTA): MOLECULAR SYSTEMATICS, MORPHOLOGY, AND ECOPHYSIOLOGY OF *FALKENBERGIA* ISOLATES[1]

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The genus *Asparagopsis* was studied using 25 *Falkenbergia* tetrasporophyte strains collected worldwide. Plastid (cp) DNA RFLP revealed three groups of isolates, which differed in their small subunit rRNA gene sequences, temperature responses, and tetrasporophytic morphology (cell sizes). Strains from Australia, Chile, San Diego, and Atlantic and Mediterranean Europe were identifiable as *A. armata* Harvey, the gametophyte of which has distinctive barbed spines. This species is believed to be endemic to cold-temperate waters of Australia and New Zealand and was introduced into Europe in the 1920s. All isolates showed identical cpDNA RFLPs, consistent with a recent introduction from Australia. *Asparagopsis taxiformis* (Delile) Trevisan, the type and only other recognized species, which lacks spines, is cosmopolitan in warm-temperate to tropical waters. Two clades differed morphologically and ecophysiologically and in the future could be recognized as sibling species or subspecies. A Pacific/Italian clade had 4–8° C lower survival minima and included a genetically distinct apomictic isolate from Western Australia that corresponded to the form of *A. taxiformis* originally described as *A. sanfordiana* Harvey. The second clade, from the Caribbean and the Canaries, is stenothermal (subtropical to tropical) with some ecotypic variation. The genus *Asparagopsis* consists of two or possibly three species, but a definitive taxonomic treatment of the two *A. taxiformis* clades requires study of field-collected gametophytes.

Key index words: ***Asparagopsis*; chloroplast RFLPs; ecophysiology; *Falkenbergia*; introductions; molecular systematics; phylogeography**

Abbreviations: **cpDNA, chloroplast DNA; SSU, small subunit**

The marine red algal genus *Asparagopsis* (Bonnemaisoniales, Rhodophyta) has been well studied with respect to its morphology (Bonin and Hawkes 1987), life history (Chihara 1962), cytology (Svedelius 1933), physiology (Oza 1977, Guiry and Dawes 1992), secondary metabolites (Sauvageau 1925, Marshall et al. 1999), and potential applications (e.g. as an antiviral agent; Haslin et al. 2001). *Asparagopsis* is of particular interest because it was the first red alga shown to have a heteromorphic life history (Feldmann and Feldmann 1939a, b) and because *A. armata* has become widely distributed in Europe as an alien introduction (Dixon 1964, Farnham 1994). *Asparagopsis armata* is regarded here as invasive according to Cronk and Fuller's (1995) definition because it spreads naturally in natural habitats and produces a significant change in terms of community composition.

Since its proposal as a genus, *Asparagopsis* has suffered extensive taxonomic and nomenclatural confusion. Montagne (1841) described the genus for *Dasya delilei* Montagne (1841), *nom. illeg.*, based on *Fucus taxiformis* Delile (1813) from Egypt (Fig. 1a). The combination *Asparagopsis taxiformis* was made by Trevisan (1845). Two new *Asparagopsis* species, *A. sanfordiana* (Fig. 1b) and *A. armata* (Fig. 1c), were described from Western Australia by Harvey (1855). The conspicuous harpoon-like barbed spines of *A. armata* (Fig. 1c, arrow) are very distinctive. *Asparagopsis sanfordiana* was said to differ from *A. taxiformis* (as *A. delilei*) only by the

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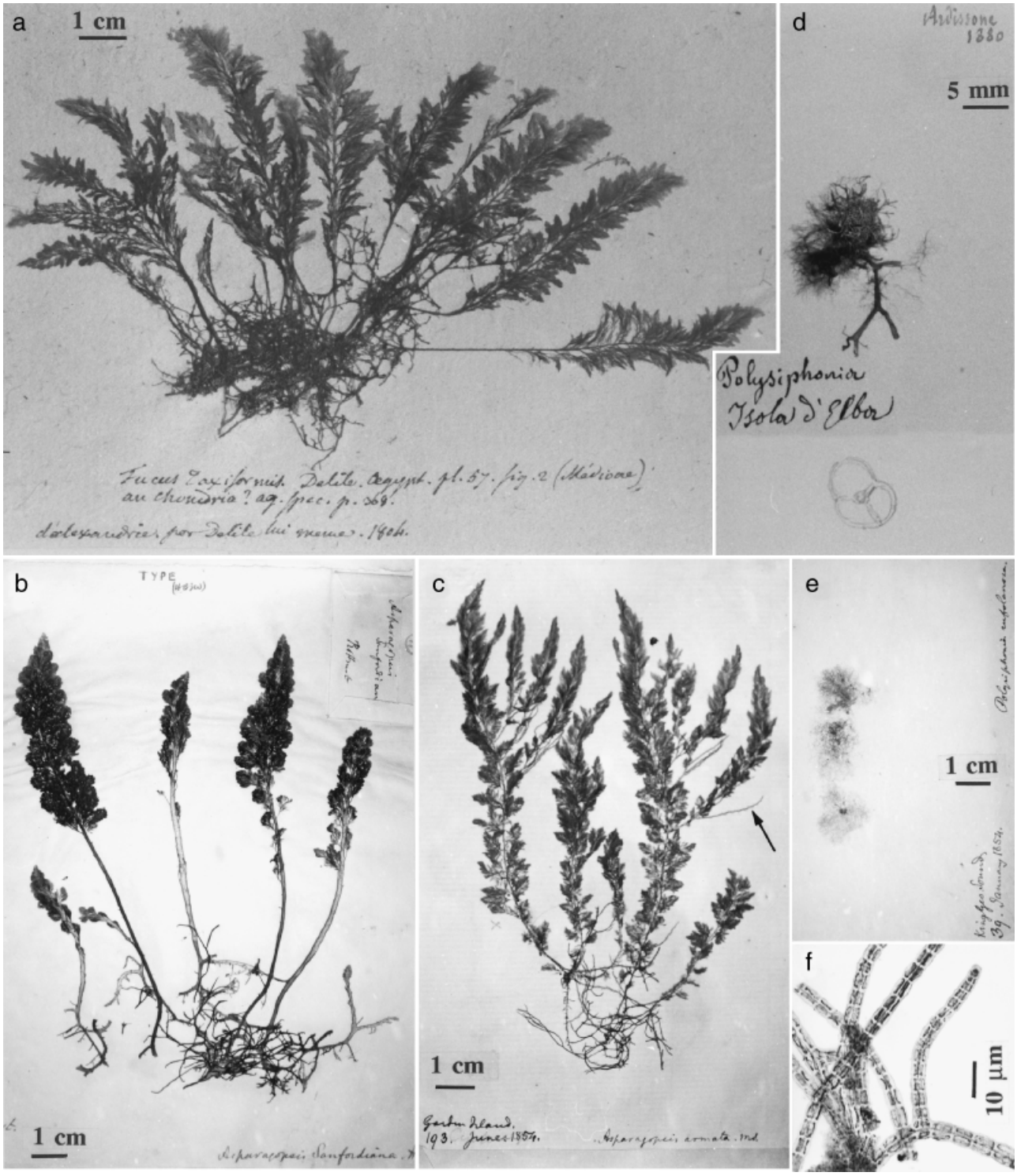

FIG. 1. Morphology of *Asparagopsis* species and their tetrasporophytic *Falkenbergia* phases, illustrated with type material. (a) *Asparagopsis taxiformis*. Isotype of *Fucus taxiformis* Delile from Alexandria, Egypt (PC). (Holotype is in CN; Dixon 1964.) (b) *Asparagopsis sanfordiana* Harvey from Rottnest Island, Western Australia (holotype, TCD). (c) *Asparagopsis armata* Harvey from Garden Island, Western Australia (lectotype, TCD), showing conspicuous harpoon-like spines (arrow). (d–f) Falkenbergia phases. (d) Lectotype of *Polysiphonia hillebrandii* Bornet in Ardissone, the basionym of *Falkenbergia hillebrandii* (Bornet) Falkenberg, from Elba (PC), with original sketch of transverse section. (e and f) Holotype of *Polysiphonia rufolanosa* Harvey, the basionym of *Falkenbergia rufolanosa* (Harvey) Schmitz, from King George Sound, Western Australia (TCD). (e) Habit. (f) Detail of branching of polysiphonous axes. (Photographs of specimens in PC were kindly taken by M. Dumont, Muséum National d'Histoire Naturelle, Paris.)

long naked bases of the main axes (Harvey 1855), and it has generally been treated as conspecific with *A. taxiformis* (Feldmann and Feldmann 1942). No further members of the genus have been accepted, so *A. armata* and *A. taxiformis* are the only species currently recognized (Bonin and Hawkes 1987), distinguishable by the presence or absence of spines. Spineless populations of *A. armata* in England provoked queries as to whether *A. armata* and *A. taxiformis* really represent separate species (Dixon 1964, 1965, Dixon and Irvine 1977). Nevertheless, sympatric populations of the two species at Rottnest Island in Western Australia can be distinguished by the more open feathery branching of *A. armata* (Fig. 1c) compared with the closer spaced laterals of *A. taxiformis* (Fig. 1a) (Bonin and Hawkes 1987).

The life history of *Asparagopsis* includes the filamentous "genus" *Falkenbergia* J. and G. Feldmann (1939a, 1942). *Asparagopsis* carpospores from Algeria germinated into a tetrasporophytic phase identified as *Falkenbergia rufolanosa* (Harvey) Schmitz (based on *Polysiphonia rufolanosa* Harvey [1855] from Western Australia [Fig. 1, e and f] [Feldmann and Feldmann 1939a, 1942]). Tetrasporogenesis in *A. armata* requires precise environmental conditions (Lüning 1981, Guiry and Dawes 1992). Chihara (1961, 1962) established that *A. taxiformis* from Japan has the same life history as *A. armata* and identified its tetrasporophyte as *Falkenbergia hillebrandii* (Bornet) Falkenberg (basionym: *Polysiphonia hillebrandii* Bornet in Ardissone [1883] from Elba, near Italy [Fig. 1d]). However, the *Falkenbergia* phases of the two species are thought to be indistinguishable (Feldmann and Feldmann 1942, Dixon 1964, Dixon and Irvine 1977).

Asparagopsis taxiformis and *A. armata* have contrasting geographical distributions. *Asparagopsis taxiformis* is widely distributed in the tropics and subtropics (Bonin and Hawkes 1987, Huisman and Walker 1990). It was originally described from Alexandria in Egypt, so its Mediterranean distribution is particularly critical and is the focus of a recent molecular taxonomic study (Andreakis et al. 2004). *Asparagopsis armata* apparently is endemic to the southern hemisphere (Southern and Western Australia [Womersley 1996], New Zealand and the Chatham Islands [Bonin and Hawkes 1987], and perhaps Chile [Santelices 1988]). It was introduced into the Atlantic Ocean and Mediterranean Sea in the 1920s (Feldmann and Feldmann 1942), presumably from southern Australia where it was and is abundant (Sauvageau 1925, Womersley 1996). Tetrasporophytes and gametophytes were discovered almost simultaneously at four European sites, interpreted at the time as four separate introductions (Westbrook 1930, Svedelius 1933). Some evidence supporting this hypothesis was obtained in preliminary ecophysiological studies (Guiry and Dawes 1992). The *Falkenbergia* phase spread rapidly in Ireland and Britain (De Valéra 1942, Drew 1950), and *A. armata* is now widely distributed in the North Atlantic southward to Sénégal (Neto 2000) and in the western basin of the Mediterranean (Guiry and Dawes 1992).

The aim of the present study was to confirm that *A. armata* and *A. taxiformis* represent separate species, to determine whether the tetrasporophytic phases of the two species can be distinguished, and to evaluate relationships between conspecific isolates with a view to developing a better understanding of the

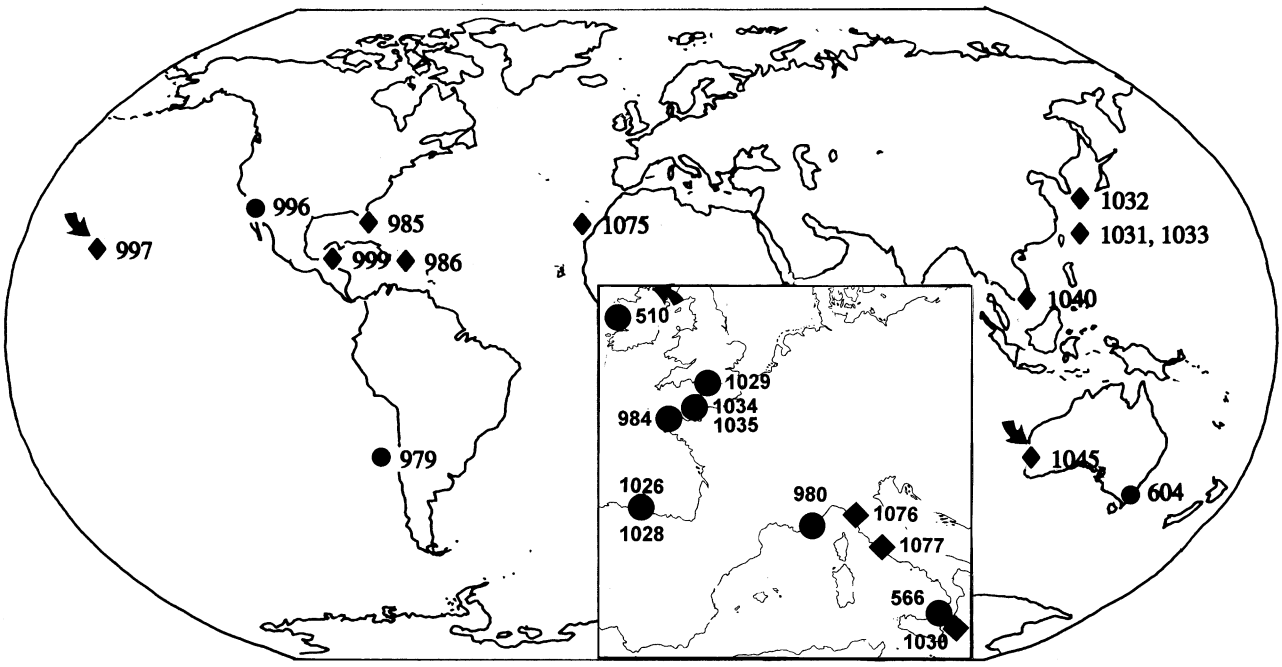

FIG. 2. Geographical origins of *Falkenbergia* phase isolates of *Asparagopsis* species, as detailed in Table 1. Most isolates were identified by their plastid DNA RFLP patterns with reference to those obtained from morphologically identifiable gametophytes. Filled circles, *Asparagopsis armata*; diamonds, *Asparagopsis taxiformis*. Arrows indicate sites where additional samples were collected for SSU sequencing.

biogeography of *Asparagopsis*. This required the development of various approaches to the identification of tetrasporophytes and a taxonomic and nomenclatural study of both tetrasporophytes and gametophytes. Comparative data were obtained on the life histories, morphology, and ecophysiology of the same isolates, and two different classes of molecular marker were used. First, we used RFLP analysis of the plastid genome of numerous *Falkenbergia* phase isolates of *Asparagopsis* collected worldwide (Fig. 2). The technique simultaneously samples conserved and variable regions of the plastid genome, providing information relevant to phylogenetic reconstruction at different levels in red algae (Maggs and Ward 1996, Wattier et al. 2001). It was previously used to identify the source of an introduced red seaweed, *Pikea californica* Harvey (Maggs and Ward 1996). The small subunit (SSU) rRNA gene was sequenced for exemplar isolates chosen from the results of the RFLP analysis, and other collections, to compare the results from plastid and nuclear genomes and to link our RFLP data to this widely used marker (Harper and Saunders 2001).

MATERIALS AND METHODS

Cultures. Stock cultures were initiated from field-collected plants of the *Falkenbergia* phase or (for three isolates: 604, 1040, and 1045) carpospores of *Asparagopsis* (Table 1, Fig. 2). A single thallus of each isolate was selected for a clonal stock culture. Cultures were maintained at 15, 20, or 25° C (Table 1) at a 16:8-h light:dark cycle under 25 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Seawater was filtered, heat sterilized to 89° C on 2 successive days, and enriched using Guiry and Cunningham's (1984) quarter-strength (VS5) modification of von Stosch's medium.

Plastid DNA RFLP analysis. DNA was extracted from about 200 mg fresh weight of blotted fresh algal material by a modified phenol-chloroform method (Maggs and Ward 1996). Organellar DNA from field-collected 5-g samples of the *Trailliella* phase of *Bonnemaisonia hamifera* Hariot was cesium chloride purified as described by Maggs and Ward (1996) and labeled with digoxigenin (Roche Diagnostics, Basel, Switzerland) as described by Maggs and Ward (1996). Restriction digests of *Falkenbergia* total genomic DNA were set up using the six-base cutting endonucleases *Pst* I, *Eco*RI, *Bgl* II, and *Kpn* I. One microliter of 100 mM spermidine was added to each 20-μL reaction mixture to aid digestion. Restriction fragments were separated on 0.5% agarose gels beside Dig III digoxigenin-labeled molecular weight markers (Roche Diagnostics) and a 1-kb marker (Invitrogen Corp., Carlsbad, CA, USA). After electrophoresis, the gels were photographed and Southern blotted onto Hybond-N hybridization transfer membrane (Amersham Biosciences UK Ltd, Chalfont St. Giles, Bucks, UK) as detailed in the manufacturer's instructions. The filters were hybridized with the *Trailliella* organellar DNA probe and immunologically detected according to the manufacturer's instructions, except that they were prehybridized for 3 h and then hybridized overnight. Although mitochondrial DNA was a contaminant of both the restriction digests and the labeled probe, its low abundance relative to the plastid DNA rendered it effectively undetectable.

Sizes of resulting fragments were determined from the marker lanes. Banding patterns were analyzed visually. Restriction fragments were scored as present or absent to generate a data matrix. An unrooted phylogenetic tree was constructed using Dollo parsimony in PAUP 3.1.1 (Swofford 1993). Dollo is particularly suitable for RFLP data because it weights gains of restriction sites relative to losses. The algorithm explains the presence of the state 1 by allowing up to one forward change $0 \rightarrow 1$ and as many reversions $1 \rightarrow 0$ as are necessary to explain the pattern of states seen, minimizing the number of $1 \rightarrow 0$ reversions necessary (Farris 1977). Analyses used heuristic searches, bisection-reconnection branch swapping, branch-and-bound search option, and no designated outgroup and were bootstrapped 1000 times. The most parsimonious tree was printed with midpoint rooting.

SSU rDNA sequence analyses. DNA was extracted (Saunders 1993) from eight isolates of the Bonnemaisoniales (Table 2). The SSU rDNA was PCR amplified using the oligonucleotide primers listed in Saunders and Kraft (1994), according to the protocols as modified by Saunders and Kraft (1996). The PCR products were agarose gel purified following the procedure of the Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA). Sequencing of DNA was completed with the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosytems [ABI], Foster City, CA, USA), and data were collected with the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

All new SSU sequences were added to an alignment of 18 other sequences representing orders of the florideophyte lineage 4 (Saunders and Bailey 1997, Saunders and Kraft 1997). The taxa included representatives of the following orders: Bonnemaisoniales: *Bonnemaisonia hamifera* Hariot (L26182), *Delisea pulchra* (Greville) Montagne (AY437645), *Ptilonia australasica* Harvey (AY437646); Gelidiales: *Gelidium pusillum* (Stackhouse) Le Jolis (U32564), *Gelidium vittatum* (Linnaeus) Kützing (AF515300), *Ptilophora pinnatifida* (J. Agardh) R. E. Norris (U60345); Gigartinales: *Dasyphloea insignis* Montagne (U09614), *Eucheuma denticulatum* (N. L. Burman) Collins et Hervey (U25439), *Mychodea carnosa* Hooker f. et Harvey (U33135), *Schottera nicaeensis* (Lamouroux ex Duby) Guiry et Hollenberg (U33137), *Sphaerococcus coronopifolius* Stackhouse (U09622); Halymeniales: *Grateloupia filicina* (Lamouroux) C. Agardh (U33132), *Sebdenia flabellata* (J. Agardh) P. G. Parkinson (U33138); Plocamiales: *Plocamiocolax pulvinata* Setchell (U09618), *Plocamium angustum* (J. Agardh) Hooker f. et Harvey (U09620); Rhodymeniales: *Dictyothamnion saltatum* A. Millar (AF085264), *Hymenocladiopsis crustigena* R. L. Moe (AF085274), *Rhodymenia leptophylla* J. Agardh (U09621). The alignment is available in GenBank under the above listed accession numbers.

The resulting alignment contained 23 taxa and 1813 sites, of which 104 were considered ambiguously aligned or were complementary to external PCR primer sequence (removed before analysis). Modeltest (v 3.06, Posada and Crandall 1998) was used to estimate an appropriate model for these data. A general-time-reversible model was selected with a gamma distribution and invariant sites. The program Mr. Bayes (v. 2.01, Huelsenbeck and Ronquist 2001) was used to complete Bayesian inference of phylogeny under a GTR model with invariant sites and a gamma distribution. Four Markov chains were used, the temperature was set to 0.2, and 10^6 generations were run with sampling every 100 generations. Log-likelihood values stabilized around 70,000 generations, and we used the final 6000 trees (4000 burnin) to calculate the posterior probabilities. Parsimony and distance analyses were completed in PAUP 4.0b4a (Swofford 2001). Unweighted parsimony and minimum evolution distance (parameters as identified in Modeltest) analyses were completed under a heuristic search using 100 random additions with the tree-bisection-reconnection branch swapping option in effect. The robustness of the resulting phylogenies was assessed with 2000 bootstrap replicates (100 random additions) under the respective analyses (Felsenstein 1985). The final topologies were rooted along the

TABLE 1. *Falkenbergia* phases of *Asparagopsis* species collected worldwide and isolated from tetrasporophytes except where indicated (see Fig. 2 for locations).

Culture no.	Origin	Latitude/longitude	Collector	Collection date	Habitat	Optimal growth temperature
Asparagopsis armata						
0510	New Quay, Co. Clare, Ireland	53.15°N, 9.08°W	E. M. Cunningham	6 February 1985	Epiphytic, low intertidal	20° C
0566	Straits of Messina, Sicily, Italy	38.13°N, 15.33°E	M. D. Guiry	4 June 1987	On intertidal blocks	20° C
0604[a]	Sorrento Back Beach, Victoria, Australia	38.22°S, 144.19°E	M. D. Guiry	28 October 1987	Lower eulittoral	15° C
0979	Isla Robinson Crusoe, Chile	c. 33.67 °S, c. 79.00°W	D. G. Müller	Unknown	Unknown	15° C
0980	Villefranche-sur-mer, France	43.42°N, 7.18°E	D. G. Müller	1 September 1980	Subtidal, at 20 m	20° C
0984	Roscoff, France	48.43°N, 3.59°E	Probably H. A. von Stosch	Unknown	Unknown	20° C
0996	San Diego, California, USA	32.45°N, 117.10°W	J. A. West	1972	Unknown	20° C
1026	Puerto di Porticella, Oviñana, Asturias, Spain	43.50°N, 6.10°W	W. F. Farnham	30 August 1992	Unknown	20° C
1028	Artedo, Spain	43.50°N, 6.10°W	J. M. Rico	October 1992	Unknown	20° C
1029	Horse Ledges, Shanklin, Isle of Wight, UK	50.38°N, 1.10°W	W. F. Farnham	24 September 1992	Unknown	20° C
1034	Portelet Bay, Jersey, Channel Is, UK	50.38°N, 1.10°W	W. F. Farnham	October 1992	Epiphytic in mid-shore pools	20° C
1035	L'Archirondel, Jersey, Channel Is, UK	49.13°N, 2.07°W	W. F. Farnham	26 October 1992	Free-floating in sea	20° C
Asparagopsis taxiformis						
0985	Key Largo, Florida, USA	25.07°N, 80.28°W	C. J. Dawes	1 August 1991	Unknown	25° C
0986	La Paugain, Puerto Rico	c. 18.00°N, 67.00°W	C. J. Dawes	17 March 1991	Unknown	25° C
0997	Hawaii, USA	c. 20.00°N, 156.00°W	R. E. Norris	2 December 1991	Unknown	25° C
0999	Puerto Morales, Quintana Roo, Yucatan, Mexico	25.07°N, 80.28°W	J. A. West	27 July 1980	Unknown	25° C
1030	Villa St. Giovanni, Messina, Sicily, Italy	38.13°N, 15.33°E	M. Gargiulo	16 November 1992	Subtidal	25° C
1031	Komesu, Itoman, Okinawa I., Okinawa Pref., Japan	26.05°N, 127.40°E	M. Masuda	22 March 1992	Tide pools 100 m from 1033	25° C
1032	Hinomi-saki, Taisha, Shimane Pref., Japan	35.28°N, 132.36°E	M. Masuda	1 May 1992	Tide pools	25° C
1033	Komesu, Itoman, Okinawa I., Okinawa Pref., Japan	26.05°N, 127.40°E	M. Masuda	22 March 1992	Tide pools 100 m from 1031	25° C
1040a	Hon Tre, Nha Thrang, Khan Hoa Province, Vietnam	12.15°N, 109.10°E	M. Masuda	6 March 1992	Unknown	25° C
1045a	Little Armstrong Bay, Rottnest I., Western Australia	132.1°S, 115.28°E	J. H. Huisman	26 November 1992	3 m depth on rock	20° C
1075	Las Palmas, Gran Canaria, Canary Is, Spain	28.08°N, 15.27°W	M. D. Guiry	5 October 1993	Epiphytic on algae in pools	25° C
1076	Lerici, La Spezia, Italy	44.04°N, 9.55°E	C. A. Maggs	16 July 1993	Surge zone	25° C
1077	Civitavecchia, Italy	42.05°N, 11.47°E	C. A. Maggs	30 June 1993	3 m depth on *Posidonia*	25° C

[a]Culture isolated from carpospores.

TABLE 2. Samples or cultured isolates (details in Table 1) of members of the Bonnemaisoniales from which SSU rDNA sequences were obtained.

Name	Isolate and/or voucher no.	Collection information (position in cpDNA RFLP analyses)	GenBank accession no.
Asparagopsis armata	GWS000294	*Falkenbergia* phase, Fanad, Co. Donegal, Ireland, intertidal; C. A. Maggs, 18 August 1997	AY772722
Asparagopsis taxiformis	no. 986 GWS000290	Puerto Rico (Caribbean clade)	AY772723
Asparagopsis taxiformis	no. 1045 GWS000292	Rottnest Island (apomictic strain resembling *Asparagopsis sanfordiana*)	AY772724
Asparagopsis taxiformis	G0380	Port Denison, WA, Australia, 6 m depth on breakwater; G. W. Saunders, 9 November 1995	AY772725
Asparagopsis taxiformis	G0435	Kahala, Oahu, Hawaii, USA; G. T. Kraft, 22 March 1996	AY772726
Asparagopsis taxiformis	no. 1030 GWS000291	Messina, Italy (Pacific clade)	AY772727
Atractophora hypnoides P. & H. Crouan	GWS000398	Sarn Badrig, Caernavon, North Wales, UK; subtidal; C. A. Maggs, 17 August 1998	AY772728
Naccaria wiggii (Turner) Endlicher	GWS000397	Near Porth Calmon, North Lleyn, North Wales, UK; subtidal, F. Bunker, 1 January 1998	AY772729

branch separating the Halymeniales, Plocamiales, and Rhodymeniales versus the Bonnemaisoniales, Gelidiales, and Gigartinales, based on our current understanding of ordinal relationships within lineage 4 (cf. Saunders et al. 2004).

Morphology and life history. For each *Falkenbergia* strain, 30 tips were cut six to seven cells from the apex and grown at 20° C at a 16:8-h light:dark cycle to ensure rapid growth without reproduction (which requires short days) so that all strains would be comparable. After 2 weeks, 10 thalli were taken haphazardly, fixed in 4% formalin–seawater solution, and mounted. Permanent slides were made using 60% Lactophenol Blue (Sigma-Aldrich, Inc., Poole, Dorset, UK) in 60% Karo® syrup (Bestfoods, Englewood Cliffs, NJ, USA). Length and width of one of the three pericentral cells was measured at cells 10, 20, 30, 40, and 50 from the apex of one branch of each thallus. Isolates were assigned to taxonomic/biogeographical groups on the basis of the RFLP analyses. Data were analyzed using one-way analysis of variance. In the case of significant factor effects, differences amongst individual means were analyzed using the Tukey multiple-comparison procedure (Zar 1996).

For life history studies, tetrasporogenesis was induced by the following procedure. *Falkenbergia* thalli were chopped in a drop of seawater until there was a relatively homogeneous mass of cuttings 1–2 mm in length that grew into roughly equal-sized plantlets suitable for experiments. Five axes of uniform size of each strain were placed in sterilized conical flasks containing 250 mL of enriched seawater, with 4 ng of KI and 25 ng of As_2O_3 according to Oza (1977). Twelve flasks were incubated in each water bath (model RMT 6, Brinkmann/Lauda, Lauda, Germany) with temperature control accurate to ± 0.2° C. Germinated tetraspores were grown in glass culture dishes or in conical flasks on a mechanical shaker (Rotatest shaker, model R100, AQS Manufacturing Ltd., Horsham, West Sussex, UK) under long-day (16:8-h) conditions at a suitable temperature and microscopically examined every day for reproduction.

Effects of temperature on growth and survival were investigated using isolates placed in water baths at 2° C intervals from 3 to 33° C. For investigations of tetrasporogenesis, an 8:16-h photoperiod was used for all isolates because only temperature was being investigated in relation to reproduction. The 8:16-h photoperiod was permissive for tetrasporogenesis in all isolates of *A. armata* and *A. taxiformis* previously studied (Rojas et al. 1982, Guiry and Dawes 1992). Under a photon irradiance of 14–20 μmol photons $\cdot m^{-2} \cdot s^{-1}$ from cool-white fluorescent light sources, each strain was incubated at all temperatures ranging from 13° C to 31° C at 2° C intervals. When necessary, temperature was monitored every 15 min with a Squirrel data logger fitted with temperature probes accurate to ± 0.1° C (Grant Instruments, Cambridge, UK). Flasks were rotated at random daily to eliminate any positional variation in temperature and irradiance. The culture medium in each flask was replaced after 3 weeks, and total incubation time was 5 weeks, after which tetrasporangia were scored as present or absent.

RESULTS

Molecular systematics. Evaluation of chloroplast DNA (cpDNA) Southern-RFLPs of all *Falkenbergia* phases generated with the restriction enzymes *Pst* I, *Eco*RI, *Bgl* II, and *Kpn* I (Fig. 3) resulted in a data matrix of 180 characters. Three groups of samples were apparent (Fig. 3). The first group (Fig. 3, group on right consisting of samples 1035–510) was unequivocally identifiable as *Asparagopsis armata* because 604 Australia was obtained from carpospores of this species (Table 1). They included all four isolates from Britain and Ireland, all three from Atlantic Europe (France and Spain), two from the Mediterranean (France and Italy), and three from the Pacific Ocean (Chile, California, and southeast Australia).

Falkenbergia phase isolates belonging to the second and third groups of samples were well separated from those of *A. armata* by 57–123 polymorphisms (losses or gains of restriction sites or large indels resulting in length differences; Table 3). These isolates were identified as the *Falkenbergia* phase of *A. taxiformis* by the morphology of parent gametophytes (of 1040 Vietnam and 1045 Australia) and were clearly subdivided into two groups. The first subgroup (Fig. 3, group on left, designated "Pacific/Italy") was composed largely of several isolates with identical RFLPs from the Mediterranean (south and west Italy) and the Pacific Ocean (all three Japanese isolates and Hawaii) and a divergent isolate, 1045 from Rottnest Island, Australia, which showed 23 changes from the rest of this group (Table 3). The second subgroup (Fig. 3, center group, designated "Caribbean") showed divergences of 49–94 changes from the Pacific/Italian isolates. It consisted of

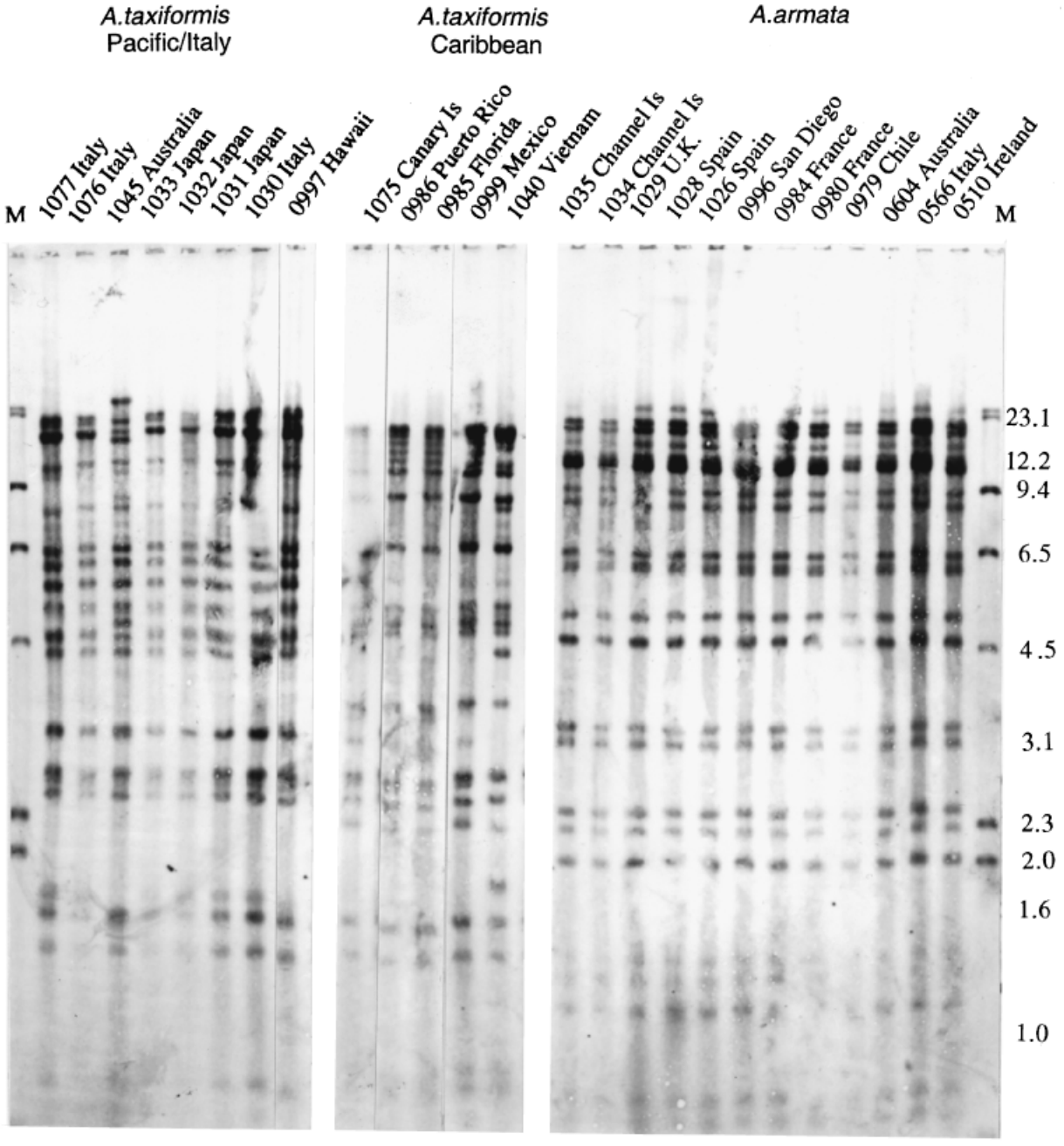

FIG. 3. Southern blot of *Pst* I restriction digests of total DNA of *Falkenbergia* phases of *Asparagopsis* probed with nonradioactively labeled organellar (predominantly plastid) DNA of *Trailliella* phase of *Bonnemaisonia hamifera*. All digests shown here were run on a single gel, but lanes have been regrouped to aid interpretation; all were repeated several times on different gels to overcome minor problems of band resolution. Groups of related samples are labeled with their identifications. Vietnam 1040 isolate was excluded from phylogenetic analyses because high-quality DNA was obtained too late to evaluate bands carefully with respect to other isolates. However, it appears to be related to the *A. taxiformis* Caribbean clade. Sizes in kb are derived from the marker lanes (M) shown and a 1-kb marker lane (not shown). Bands were scored down to sizes of less than 1 kb.

three tropical western Atlantic/Caribbean isolates with identical RFLPs (Florida, Puerto Rico, Mexico), plus Canary Islands (1075), which differed from them by 10 changes (Table 3). Although 1040 Vietnam appeared to be a member of this group, it differed from the other samples by a large number of bands. Unfortunately, DNA extractions from this isolate repeatedly resulted in degraded DNA. Eventually, high molecular weight DNA was obtained (Fig. 3), but this was insufficient to fully evaluate this sample relative to other DNA samples, and it was therefore excluded from analyses of genetic and morphometric data.

Dollo parsimony analysis of the RFLP data matrix produced a single most parsimonious tree (Fig. 4) with length 183 steps, consistency index 0.907, and retention index 0.788, which separated *A. armata* from

TABLE 3. cpDNA RFLPs of *Falkenbergia* phases of *Asparagopsis* spp. generated with *Pst* I, *Eco*RI, *Bgl* II, and *Kpn* I, showing numbers of bands for each enzyme for each group of samples (columns 2–5) and pair-wise comparisons of numbers of bands that differ between the groups (columns 6–9).

	Pst I	*Eco*RI	*Bgl* II	*Kpn* I	*A. armata*	*A. taxiformis* Caribbean	*A. taxiformis* Canary Is	*A. taxiformis* Pacific/Italy
A. armata	27	28	34	8				
A. taxiformis Caribbean	24	21	26	7	102			
A. taxiformis Canary Is	24	24	26	7	57	10		
A. taxiformis Pacific/Italy	22	27	29	10	123	93	52	
A. taxiformis Rottnest I.	23	26	30	10	123	94	49	23

The differences are relatively large because the data matrix consists of all bands found in any of the samples.

A. taxiformis. The *A. taxiformis* clade was subdivided into two well-supported (100%) clades, the Caribbean + Canaries clade and the Pacific/Italian clade (Fig. 4).

The SSU sequences for *A. taxiformis* from Western Australia (no. 1045 Rottnest culture and a field-collected gametophyte), Hawaii (field-collected gametophyte), and Italy (no. 1030) were identical, and thus only one sequence represented the Pacific/Italian clade in the SSU analyses (Fig. 5). The Puerto Rico (Caribbean clade) *A. taxiformis* SSU sequence differed from the previous clade by three nucleotide substitutions. Trees from Bayesian (Fig. 5), parsimony (six trees, length = 740, consistency index = 0.596, retention index = 0.704), and distance analyses yielded similar topologies. In all analyses the Gelidiales was moderately resolved as sister to a variously supported monophyletic Bonnemaisoniales (Bonnemaisoniaceae plus Naccariaceae). *Naccaria* was weakly resolved as sister to the included Bonnemaisoniaceae, whereas *Atractophora*, also included in the Naccariaceae in most current classifications, failed to join *Naccaria* and was basal to all ncluded genera of the order. This aspect of our analyses needs further investigation and will be discussed in detail elsewhere. Within the Bonnemaisoniaceae the relationships among genera were poorly resolved and equivocal among analyses with the exception of a variously supported (strong to moderate for Bayes and distance, weak under parsimony) association between *Bonnemaisonia* and a strongly monophyletic *Asparagopsis*. The three species of *Asparagopsis* had uncharacteristically long branches in the SSU trees, and a second series of analyses was completed after removing these taxa to assess whether these branches were having an impact on other aspects of bonnemaisonialean relationships. No major differences were observed (data not shown).

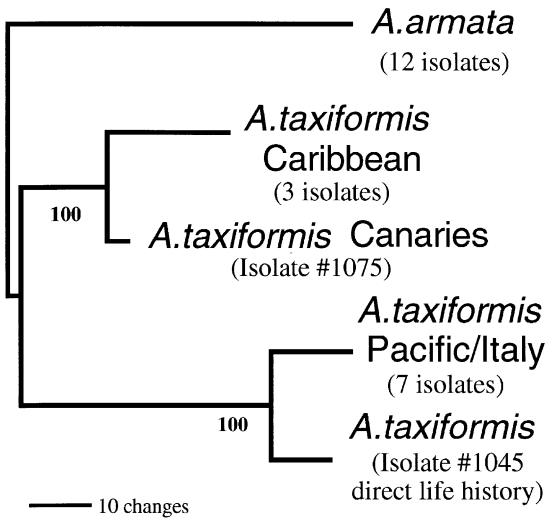

FIG. 4. Phylogenetic tree of cpDNA RFLPs generated with *Kpn* I, *Eco*RI, *Bgl* II, and *Pst* I, constructed using Dollo parsimony, indicating bootstrap values for 1000 replications.

Life history. A heteromorphic life history was demonstrated in nearly all the isolates. Under inductive conditions, tetrasporangia (Fig. 6, a–d) were formed by all *Falkenbergia* phases except for San Diego (1996), which was sterile under all conditions, and 1045 Rottnest (see below). The Vietnam culture grew poorly and showed limited reproduction.

In 9 of 10 *A. armata* isolates, released tetraspores (Fig. 6c) grown under long-day conditions (16:8-h) gave rise to the gametophytic phase, but germination failed in 566 (Messina). After 2 months, spermatangia but no carpogonia or cystocarps were formed on some branches of the Irish, Chilean, and Villefranche isolates. Larger gametophytes did not grow well in culture; after about 6 months, they began to lose color and stopped growing.

Asparagopsis taxiformis Caribbean clade isolates from Florida and Mexico gave rise to gametophytes, but Puerto Rico failed to grow; gametangia were not formed in any culture. Five isolates of the *A. taxiformis* Pacific/Italian clade gave rise to gametophytes (Fig. 6e) that developed spermatangia (Fig. 6f) but not carpogonia. Uniquely, in the Rottnest I (Western Australia) 1045 strain, gametophytes developed directly from the *Falkenbergia* phase at 20 and 21°C, in the absence of tetrasporangia.

Morphology of tetrasporophytes. The only morphological differences apparent among the *Falkenbergia* isolates were the sizes of the cells measured at 30, 40, and 50 cells from the apex. At all three distances from the apex, cells of the *Falkenbergia* phase of *A. armata* were significantly shorter and narrower than those in *Falkenbergia* phases of the *A. taxiformis* Caribbean

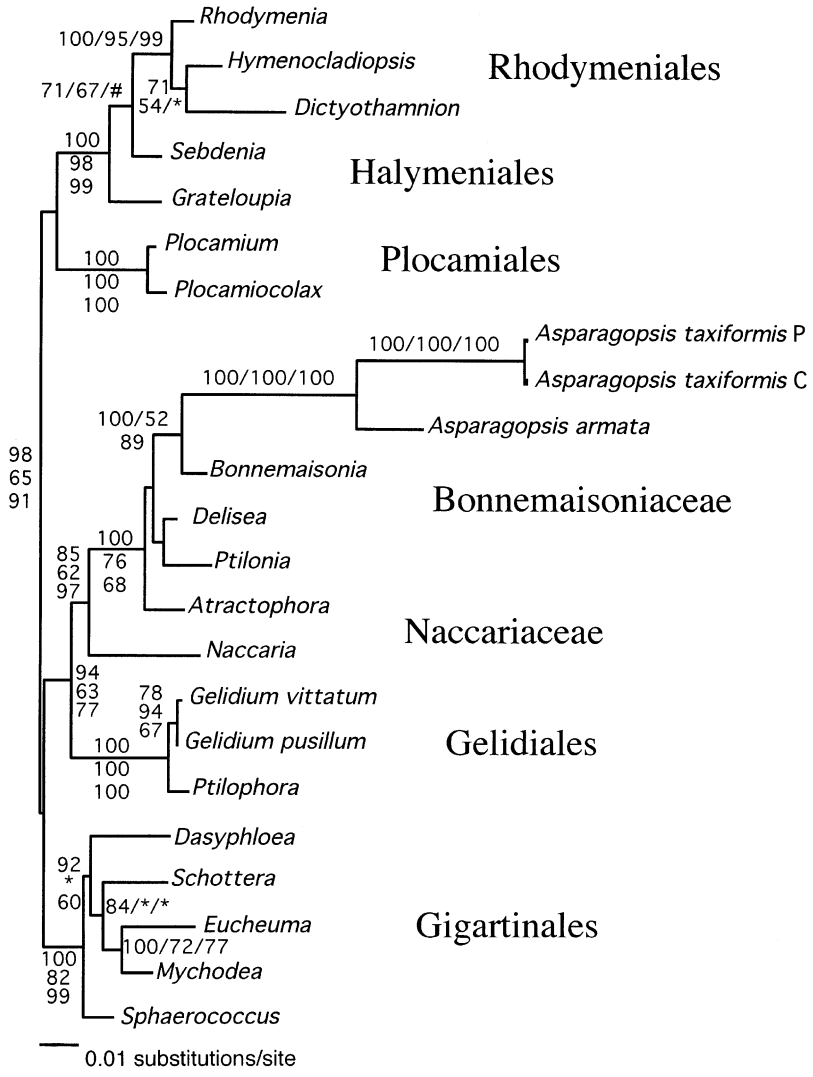

FIG. 5. Phylogenetic tree resulting from Bayesian analyses of the SSU alignment. Values at internal branches represent Bayesian posterior probabilities, and parsimony and distance bootstrap replicates, respectively. Asterisk indicates <70% (posterior probabilities) or <50% (in maximum parsimony, neighbor-joining analyses) support for that node; # *Grateloupia* and *Sebdenia* formed a monophyletic group with 63% support under distance. P and C indicate Pacific/Italian and Caribbean clades, respectively, for *Asparagopsis taxiformis*. Sequences of four samples of the Pacific/Italian clade (no. 1045 Rottnest; field-collected gametophytes from Western Australia and Hawaii and Italy no. 1030) were identical. The Bonnemaisoniaceae as currently circumscribed fails to form a monophyletic group, but a clade of the Bonnemaisoniaceae plus *Atractophora* is fairly well supported.

clade (Fig. 7). Cells of the *Falkenbergia* phase of the *A. taxiformis* Pacific/Italian clade were intermediate in size between the other two groups but differed significantly from both groups at cells 30 and 40 when both length and width were compared (Fig. 7). The Vietnam 1040 isolate was excluded from the analysis, as noted above, due to uncertainty about its attribution to a group.

Morphology of gametophytes. Gametophytes did not grow well in culture, so it was not possible to make meaningful morphological comparisons of cultured *Asparagopsis* strains. However, some cultures were isolated from female gametophytes or collected with them. The gametophyte of *A. armata* from Victoria, Australia, from which isolate 604 was obtained, had typical harpoon-like spines and no holdfasts. This corresponded with type material from Garden Island, Western Australia (Fig. 1c). None of the *A. armata* isolates formed spines in culture. The *A. taxiformis* gametophytic parent of isolate 1040 from Vietnam (Fig. 6 g) had stoloniferous holdfasts, lacked spines, and exhibited markedly conifer-like branching. Gametophytes growing in Japan beside the Pacific/Italian-clade *Falkenbergia* phase (Fig. 6h) were large and robust, similar to the type of *A. taxiformis* (Fig. 1a). The fertilized female from which Rottnest 1045 was obtained was very robust with naked main axes below, resembling the type of *A. sanfordiana* (Fig. 1b).

Effects of temperature on growth and survival of tetrasporophytes. Maximum and minimum temperatures for survival and growth (Fig. 8) broadly divided the *Falkenbergia* phase isolates into the same three groups as the RFLP and SSU analyses, that is, *A. armata* and *A. taxiformis* Pacific/Italian and Caribbean clades. *Asparagopsis armata* isolates were easily recognizable by their low maximum survival temperature of 25° C, 4° C lower than for any other isolates, and low maximum temperatures permitting growth (21–23° C). They also had much lower minimum survival (5–7° C) and growth (9° C) temperatures than other isolates. Chile 979 was remarkable for its survival at 3° C, the lowest temperature tested, and growth at 7° C.

Members of the Caribbean *A. taxiformis* clade were readily identifiable by the high minimum temperature

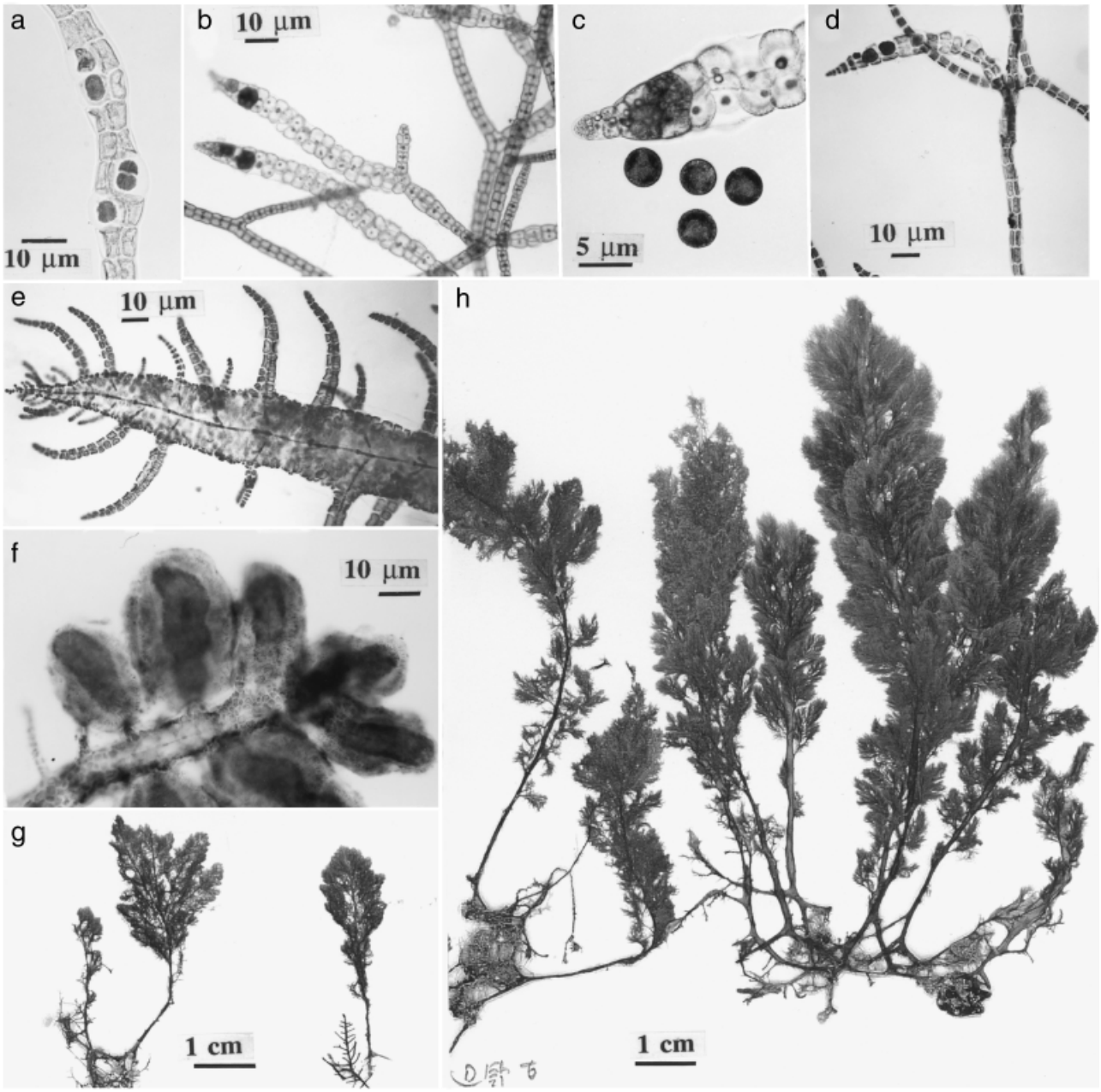

FIG. 6. Life history of *Asparagopsis* isolates. (a–f) stained with 60% Lactophenol Blue. (a) *Falkenbergia* phase tetrasporophyte of *A. armata*, with tetrasporangia; isolate 1029 from Isle of Wight, UK. (b and c) *Falkenbergia* phase tetrasporophyte of *A. taxiformis* (Pacific/Italian clade); isolate 1030 from Messina, Italy. (b) Tetrasporangia near apices of stichidia-like branches that have shed mature tetraspores. (c) Four tetraspores released from tetrasporangium. (d) *Falkenbergia* phase tetrasporophyte of *A. taxiformis* (Caribbean clade) with tetrasporangia; isolate 986 from Puerto Rico. (e and f) *A. taxiformis* (Pacific/Italian clade) gametophyte that grew from tetraspore in culture (isolate 1030). (e) Tip of young gametophyte. (f) Male branchlets bearing spermatangia. (g) *A. taxiformis* herbarium specimens from Hon Tre Island, Nha Thrang, Vietnam, 6 March 1992, leg. & det. M. Masuda (SAP). (h) *A. taxiformis* herbarium specimens from Okinawa, Japan, 9 March 1990, leg. T. Yoshida (SAP).

of 17° C for both survival and growth (Fig. 8). All four isolates examined (the Canaries isolate was not tested) had identical upper survival limits of 31° C. The Vietnam strain was provisionally assigned to this group on the basis of its temperature responses. The Pacific/Italian *A. taxiformis* clade exhibited recognizably lower survival temperatures of 9–13° C, 4–8° C lower than for the Caribbean group but 2–6° C higher than for *A. armata* (Fig. 8). Lower temperature limits were relatively variable within this group, ranging from 9° C for survival and 11° C for growth of Messina 1030 to 17° C for survival and growth of the Hawaiian strain. Rottnest 1045 showed unique temperature responses, growing over a narrow temperature range but surviv-

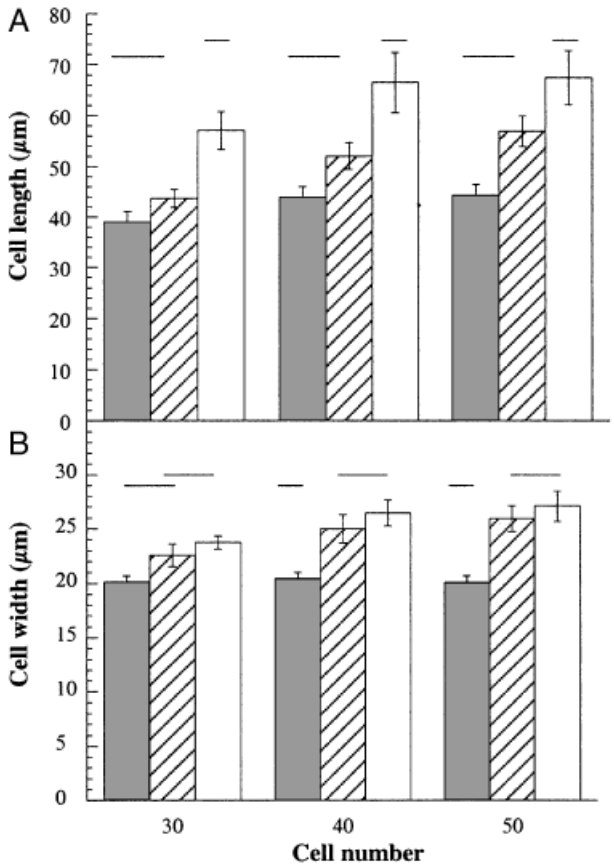

FIG. 7. Mean cell length (A) and width (B) of *Asparagopsis armata* (shaded bars) and the Pacific/Italian (cross-hatched bars) and Caribbean (open bars) clades of *A. taxiformis* at cell numbers 30, 40, and 50 from the apex, with SEs. Lines above histograms are results of Tukey multiple comparison test, indicating whether values for cells at the same distance from the apex are significantly different in the three groups of isolates. The Vietnam 1040 isolate was excluded from the analyses.

ing a much wider range. Upper survival limits of the Pacific/Italian clade, 29–31° C, were similar to those of the Caribbean group.

Effects of temperature on tetrasporogenesis. The optimum day length for induction of tetrasporangia had previously been determined as 8:16 h. At this day length, almost all isolates formed tetrasporangia at one or more temperatures within the range 11 to 29° C (Fig. 9). For *A. armata*, the range of inductive temperatures for an individual strain varied greatly, from 3° C for strains from Chile, Jersey, and Ireland to 11° C for strains from Australia and Jersey. The highest permissive temperature was 21 or 23° C. The Pacific/Italian *A. taxiformis* clade shared the upper part of its inductive temperature range with the Caribbean clade and the lower part with *A. armata*. The temperature range for tetrasporangial production by individual isolates in the Pacific/Italian group was very wide (7–13° C). The minimum temperature for some strains was 17° C and the maximum was 29° C. In the Caribbean clade, tetrasporogenesis occurred over a very limited temperature range between 23 and 29° C. The Vietnamese strain was exceptional in reproducing at only a single temperature (23° C), possibly a result of poor performance in culture.

DISCUSSION

All three classes of evidence (molecular, morphological/life history, and ecophysiological) show that the *Falkenbergia* strains of *Asparagopsis* fall into two well-defined groups, *A. armata* and *A. taxiformis*. We therefore demonstrated using a variety of approaches that *A. armata* is a distinct species from *A. taxiformis*, although spines are not always formed (e.g. in culture). This result has now been corroborated by a recent study using sequences of a plastid marker (RUBISCO spacer), a mitochondrial marker (*cox* 2–3 spacer), and partial large subunit nuclear gene (Andreakis et al. 2004). Based on our results, *A. taxiformis* can be further subdivided into two clades, a Pacific/Italian clade and a Caribbean + Canaries clade. Congruent evidence was obtained from SSU analyses: the sequence of an isolate in the Caribbean clade differed by three bases from that of five members of the Pacific/Italian clade. However, the sole significant morphological difference detected in this study between the *Falkenbergia* phases of the Pacific/Italian and Caribbean clades is slightly larger cells in the Caribbean isolates, and the only conceivable way to separate the clades is to conduct physiological or molecular studies. These two clades may therefore represent cryptic sibling species, like those recently demonstrated in *Caloglossa* and *Spyridia* (Kamiya et al. 1998, Zuccarello et al. 2002) or, alternatively, they could be regarded as geographically isolated subspecies of *A. taxiformis*. Both *A. taxiformis* clades were also recovered by Andreakis et al. (2004) using organellar markers but not in their large subunit analyses, possibly as a result of recombination in the nuclear genome. Further studies based on gametophytes are required to seek diagnostic morphological criteria to distinguish the subspecies or species.

In our study, crossing experiments were impossible because carpogonial branches were not formed in culture, so the biological species concept could not be used. Our lack of success with gametophyte cultures was disappointing and may be a result of the abundant secondary metabolites (Marshall et al. 1999). Field-collected gametophytes decay very rapidly.

The main conclusions for the taxonomy and biogeography of these three groups of isolates are as follows.

Asparagopsis armata. The maximum and minimum temperatures required for reproduction, survival, and growth were much lower than for the two groups of *A. taxiformis* isolates, and cell sizes were significantly smaller. The temperature tolerance data indicate that this species naturally occupies a cold to warm temperate zone. Sorrento, the site from which strain 0604 was collected, is in the native range of *A. armata* in Australia (Womersley 1996). All isolates of *A. armata* from Australia, Europe, Chile, and San Diego were identical using our molecular markers. Identical cpDNA

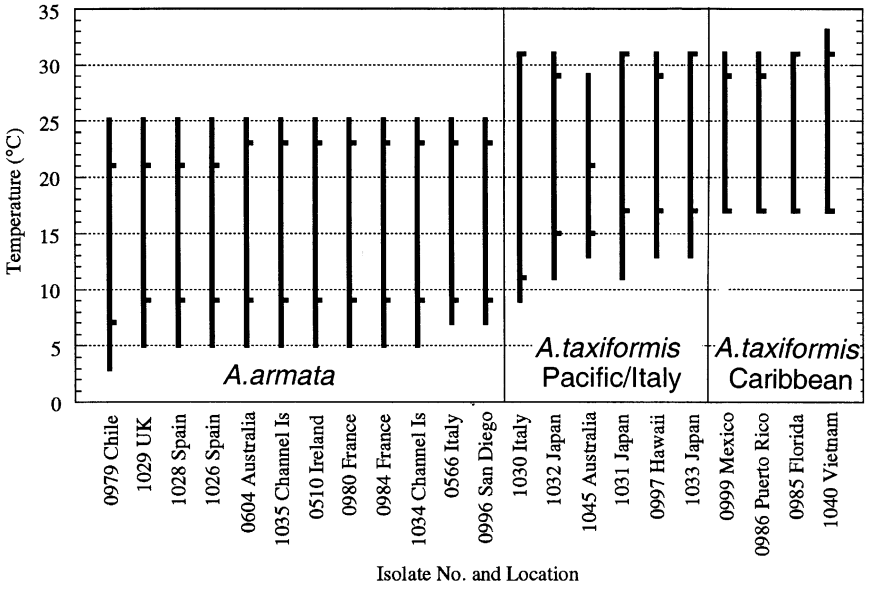

FIG. 8. Temperature effects on survival and growth of three groups of *Falkenbergia* phases of *Asparagopsis* species, as detailed in Table 1 and shown in Figure 4. The Vietnam isolate is provisionally assigned to the Caribbean group on the basis of its ecophysiology. Vertical bar represents temperatures over which isolate survived; marked points on bars are upper and lower temperatures permitting growth.

RFLPs indicate a very close relationship between these geographically disjunct populations, consistent with a recent introduction from Australia. In comparison, considerable intrapopulational variation was detected in European *Ceramium virgatum* Roth (Wattier et al. 2001, Maggs et al. 2002).

The differences between European isolates in growth and survival responses to temperature were minimal (<2° C), providing little evidence for growth and survival temperature ecotypes (Breeman 1988, Breeman and Guiry 1989). There was more variation in the inductive temperatures for tetrasporogenesis (Fig. 9) but no obvious correlation between inductive temperatures and geographical provenance. Two strains from the same site (1034 and 1035 from Jersey) showed marked differences, and although this could be interpreted as evidence for two separate introductions, there are other possible explanations.

There appears to be only one previous record of *A. armata* from Pacific North America (Okamura 1932) where the distribution of this species is very localized in the San Diego area, possibly indicating a relatively recent introduction. The cold south-flowing California Current influences the west coast of North America from the Aleutian Islands to Point Conception, keeping the temperature relatively uniform at 5–14° C in February and 10–17° C in August. Therefore, the Californian coast would be an ideal area for this species to expand, particularly northward, as was the case for the introduced European populations.

Asparagopsis taxiformis, Pacific/Italian clade. The other isolates of *Asparagopsis* must be assigned to

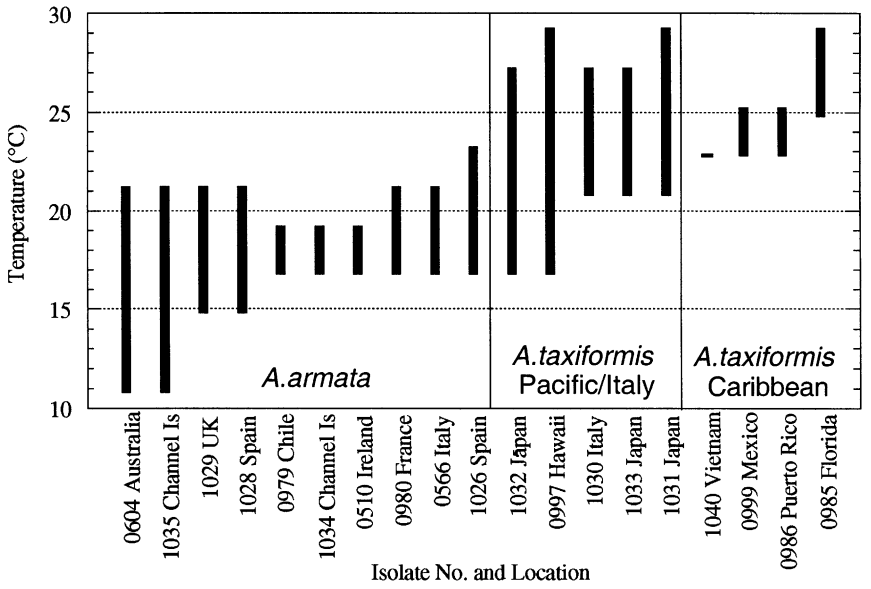

FIG. 9. Temperature ranges over which tetrasporangial formation was induced at a permissive day length in three groups of *Falkenbergia* phases of *Asparagopsis* isolates, as listed in Table 1 and shown in Figure 4.

the only other currently recognized species, *A. taxiformis*, but were divided by molecular, ecophysiological, and morphological data into two groups, the Pacific/Italian isolates and the Caribbean/Canaries isolates. Three isolates from Italy were attributed to the *A. taxiformis* Pacific/Italian clade (the fourth was *A. armata*), although *A. taxiformis* has only been reported rarely in the Mediterranean Sea. The type locality is near Alexandria in Egypt, where it still occurs (Delile 1813, Aleem 1951, 1993), and *A. taxiformis* is also known from Algeria (Dixon 1964). It was reported from Syria (as *A. delilei*; Sauvageau 1925) and Libya (Nizamuddin et al. 1979) and was considered to be rare and localized in the eastern Mediterranean (Feldmann and Feldmann 1939c, Feldmann 1942). *Asparagopsis taxiformis* appears to be the oldest available name for this clade of this species, which has recently become much more abundant on southwestern coasts of Italy (Andreakis et al. 2004).

The type locality of the supposed *Falkenbergia* phase of *A. taxiformis*, *F. hillebrandii* (Bornet) Falkenberg, is Elba off the northwest coast of Italy (Ardissone 1883), quite close to the source of our strains from La Spezia and Civitavecchia. Another early (1908) report of *F. hillebrandii* in the western Mediterranean was cited by Dixon (1965). Because the *Falkenbergia* phases of the three clades can be distinguished from each other in culture only by quantitative morphological features, and possibly not at all in field-grown material, these reports may in fact represent the original date for the arrival of *F. rufolanosa* (= *Asparagopsis armata*) in Europe. However, the rarity of *A. armata* in the Italian Mediterranean, where sea temperatures are probably too high for its survival in summer (Lüning 1990, Andreakis et al. 2004), makes this unlikely. We suggest that our Italian isolates may belong to a species native to warm-temperate to subtropical waters in the Pacific that has been introduced into the Mediterranean. European isolates belonging to this clade died below 9° C, so this entity cannot colonize most of the Atlantic coasts of Europe.

The nonEuropean *A. taxiformis* Pacific/Italian clade members are from Rottnest I, Western Australia (1045), Okinawa, Japan (1031, 1033), and Hinomisaki, Japan (1032). Apart from the Rottnest isolate, these and the Mediterranean strains all had identical plastid DNA RFLPs and clearly belong to the same species. The temperature responses of this group of isolates signify a warm-temperate distribution for this species. The slightly different temperature responses for growth, survival, and tetrasporogenesis of different Pacific isolates may represent temperature ecotypes. Ecotypic differentiation in temperature responses of seaweeds is relatively rare (Breeman 1988), with most reports occurring in seaweed populations that have disjunct distributions, wide latitudinal ranges (Novaczek and Breeman 1990, Novaczek et al. 1990), or both. Temperature ecotypes in species with tropical Atlantic/Mediterranean distributions have been linked to their survival in refugia during the last glacial maximum (Pakker and Breeman 1996).

The Rottnest 1045 isolate, identified as *A. taxiformis* (Pacific/Italian clade), differed genetically from the other members of this clade with our plastid marker but not by its SSU sequence. *Asparagopsis sanfordiana* was described from Garden and Rottnest Islands, Western Australia (Harvey 1855), and the thallus from which this isolate was obtained matched Harvey's protologue. The 1045 isolate is thus closely related to the other Pacific isolates but differs in its life history and by both RFLP and ecophysiological markers. We suggest that *A. sanfordiana* represents an apomictic segregate of *A. taxiformis*, with which it has correctly been placed in synonymy.

Asparagopsis taxiformis, Caribbean clade. The final group identified, the Caribbean clade of *A. taxiformis*, may represent a separate subspecies, or possibly an additional species of *Asparagopsis*, that occupies subtropical to tropical locations. This was the most stenothermal group of isolates, which corresponds with previous findings of very narrow temperature ranges for survival (10–13° C) of various Caribbean seaweeds (Pakker et al. 1995). The lower survival temperature of 17° C was within the range (15–19° C) of those previously observed for Caribbean isolates of red, green, and brown seaweeds (Pakker et al. 1996).

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