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Morphological differentiation of cryptic lineages within the invasive genus Asparagopsis (Bonnemaisoniales, Rhodophyta)

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ABSTRACT: Rapid identification of introduced seaweeds is crucial to support management and conservation decisions, especially when multiple cryptic lineages of high-profile invasive taxa occur sympatrically. The red seaweed genus Asparagopsis (Bonnemaisoniales, Rhodophyta) comprises two recognised morpho-species characterised by heteromorphic life cycles and presumably morphologically identical 'Falkenbergia' tetrasporophyte stages: A. armata and A. taxiformis. Populations of the former were easily identified by the presence of distinctive harpoon-like braches on the gametophyte thalli. Four morphologically cryptic yet genetically distinct mitochondrial lineages of invasive nature were recognised within A. taxiformis. We reported a morphological delineation of tetrasporophytes and gametophytes of Asparagopsis, including cryptic lineages collected from the Mediterranean Sea and the Hawaiian Islands, where multiple Asparagopsis lineages were present. Vegetative anatomical characters of the tetrasporophytes were useful in differentiating A. armata from those of A. taxiformis as well as among tetrasporophyte isolates belonging to the four A. taxiformis lineages. In addition, these characters distinguished lineage 2 native range specimens (Hawaii) from the invasive specimens (Mediterranean Sea), which suggested high levels of morphological plasticity in the invasive taxon. We propose that the taxonomic status of the lineages within A. taxiformis needs to be revised.

KEY WORDS: Falkenbergia, Identification, Management, Morphology, Reproductive, Taxonomy, Vegetative

INTRODUCTION

Since its first description, the red seaweed genus Asparagopsis Montagne (Bonnemaisoniales) has been the subject of many studies related to its complex life cycle (Feldmann 1942; Chihara 1961; Rojas et al. 1982), development (Svedelius 1933; De Valera & Folan 1964; Oza 1977; Guiry & Dawes 1992; Kumar et al. 1999, 2000), allelopathic compounds (Wolk 1968; McConnell & Fenical 1977, 1979; Manilal et al. 2010, 2012), ultrastructure (Jha & Vijayaraghavan 1998) and, most recently, invasive behavior (Dixon 1964; Boudouresque & Verlaque 2002; Flagella et al. 2003, 2005; Kraan & Barrington 2005; Andreakis et al. 2007a; Tsiamis et al. 2007; Altamirano et al. 2008; Bolton et al. 2011). The name Asparagopsis was originally used to describe the

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Table 1. Characters and character states identified from gametophytes, tetrasporophytes and reproductive structures of *Asparagopsis* OTUs.

No.	Character	No. of replicates	Character state 0	1	2	3	4
Gametophyte							
1	Thallus colour	10	reddish	—	—	—	—
2	Habit	10	epilithic	epiphytic	—	—	—
3	Maximum length of thallus (cm)	10	4–6.4	6.4–10.7	10.7–19.9	—	—
4	Maximum width of thallus (cm)	10	0.7–1.2	1.2–5.6	—	—	—
5	Attachment to substrate	10	rhizoids	stolons	—	—	—
6	Shape of thallus	10	ovate	clavate	—	—	—
7	Basal width of main axis (cm)	10	0.10–0.22	—	—	—	—
8	Apical cell – width (μm)	50	3.0–4.5	4.5–10	—	—	—
9	Apical cell – length (μm)	50	7.1–12.3	—	—	—	—
10	Height where branching begins (cm)	10	0.2–1	1–4.1	4.1–7.1	—	—
11	Branchlet positioning	10	radial	irregular	—	—	—
12	Branching	10	opposite	distichous	—	—	—
13	Length of the branchlets (cm)	20	0.6–1.3	1.3–3.9	—	—	—
14	Basal diameter of brachlets (mm)	20	0.2–0.3	0.3–0.6	0.6–0.8	—	—
15	Presence of spines in fixation structure	10	yes	no	—	—	—
16	Ramification of fixation structure	10	yes	no	—	—	—
17	Presence of secondary rhizoids	10	yes	no	—	—	—
Tetrasporophyte							
1	No. of cells between ramifications	20	0–16	—	—	—	—
2	Apical cell division pattern	10	dichotomous	trichotomous	—	—	—
3	Ramification angle (°)	10	>90	90	<90	—	—
4	Vesicular cell – diameter (μm)	30	2.7–4.5	4.5–6.2	6.2–10.2	—	—
5	Vesicular cell – colour	30	blue	—	—	—	—
6	Vesicular cell – position	30	next to the axis	away from the axis	—	—	—
7	Apical cell – width (μm)	40	5.0–6.0	6–12.2	12.2–15.2	—	—
8	Apical cell – length (μm)	40	6.0–6.8	6.8–17.1	17.1–20.7	—	—
9	Apical cell – division pattern	10	parallel	perpendicular	—	—	—
10	Thickness of the cell wall (μm)	20	1.9–3.1	3.1–4.7	4.7–5.3	5.3–8.8	—
11	Axial cell – width (μm)	30	1.5–2.7	2.7–4.2	4.2–5.7	5.7–8.3	—
12	Axial cell – length (μm)	30	17.4–21.9	21.9–33.9	33.9–56.8	56.8–63.7	63.7–92.9
13	Filament width (mm)	20	15.4–34.4	34.4–42	42–63	—	—
14	10° cell from apex – width (μm)	10	9.7–22.1	—	—	—	—
15	10° cell from apex – length (μm)	10	16.3–41.3	—	—	—	—
16	20° cell from apex – width (μm)	10	12–31.3	—	—	—	—
17	20° cell from apex – length (μm)	10	24–43.5	43.5–86.4	—	—	—
18	30° cell from apex –width (μm)	10	12–12.8	12.8–30.7	—	—	—
19	30° cell from apex – length (μm)	10	23.6–45.3	45.3–53.5	53.5–95.7	—	—
20	40° cell from apex – width (μm)	10	11.0–19.0	19–40.5	—	—	—
21	40° cell from apex – length (μm)	10	27.5–32.4	32.4–67.1	67.1–102.4	—	—
22	50° cell from apex – width (μm)	10	14.3–22.0	22.0–25.3	25.3–42.7	—	—
23	50° cell from apex – length (μm)	10	24.2–49.5	49.5–106.0	—	—	—
24	Distance between axial cells (μm)	30	2.2–4.6	4.6–10.1	—	—	—
Gametophyte reproductive traits							
1	Cystocarp – total length (mm)	30	1.0–1.3	1.3–1.5	1.5–2.3	—	—
2	Cystocarp – diameter (mm)	30	0.5–0.9	0.9–1.4	—	—	—
3	Cystocarp – stalk (mm)	30	0.7–1.1	0.4–0.7	—	—	—
4	Spermatangia – width (mm)	30	0.09–0.10	0.10–0.19	0.19–0.20	—	—
5	Spermatangia– length (mm)	30	0.4–0.6	0.6–0.7	0.7–1.1	—	—
6	Carpospores – width (μm)	50	17.8–24.4	39.9–57.9	29.2–37.6	—	—
7	Carpospores – length (μm)	50	39.6–61.8	64.1–84.1	102.6–147.4	—	—
8	Sexuality	10	monoecious	dioecious	—	—	—

gametophyte stage of a triphasic heteromorphic diplohaplontic life cycle. These highly branched pinkish to reddish colored gametophytes can grow up to 40 cm tall, and they commonly occur on rocky substrates or as epiphytes. The tetrasporophytic stage, on the other hand, was described as the genus Falkenbergia Schmitz [F. rufolanosa (Harvey) Schmitz is now known as Asparagopsis armata Harvey, and F. hillebrandii (Bornet) Falkenberg is now known as A. taxiformis (Delile) Trevisan de Saint-Le´on], and the tetrasporophytes are commonly found as microscopic filaments arranged in a pompom-like morphology up to 2 cm in diameter, either free floating or attached to other algae (Feldmann 1942; Chihara 1961). In recent years, morphological, ecophysiological and molecular research performed independently on worldwide collections of Asparagopsis gametophytes and tetrasporophytes has revealed that whereas A. armata is a genetically homogeneous taxon along its temperate distribution range, the pan-tropical A. taxiformis comprises a cryptic species complex (Andreakis et al. 2004, 2007b; Nı´ Chuala´in et al. 2004). The latter is composed of at least four genetically distinct yet morphologically cryptic lineages, each characterised by a distinct geographical distribution

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(Andreakis et al. 2007b). Multiple lineages are known from the Mediterranean Sea and the Indo-Pacific Oceans (lineages 2 and 3 in the Mediterranean Sea; 1, 2 and 4 in Hawaii and the Pacific side of the Isthmus of Panama). The Indo-Pacific Mediterranean lineage 2 is considered native to Australia (Womersley 1996), New Zealand (Horridge 1951) and the Hawaiian Islands (Sherwood 2008), but it is also present in South Africa (Bolton et al. 2011) and Japan (Andreakis et al. 2007b). The aforementioned lineage has recently expanded its distribution range in southern Portugal and the Mediterranean Sea, and it is considered an invasive taxon characterised by high genotypic diversity and polyploidy (Andreakis et al. 2007a, 2009). In addition, lineage 3 shows a distinct Atlantic Ocean and eastern Mediterranean Sea distribution, but it is also found in South Africa, where it is considered invasive (Bolton et al. 2011). Given that A. armata is a genetically homogeneous species whilst four genetically distinct lineages occur in A. taxiformis, for the sake of simplicity in this article we refer to these entities as five Operational Taxonomic Units (OTUs). We acknowledge, however, that the taxonomic status of the A. taxiformis lineages still needs further clarification. Due to their invasive potential, both Asparagopsis armata and A. taxiformis have been included on the lists of the 'worst invasive alien species threatening biodiversity in Europe' (EEA 2007) and the Mediterranean Sea (Streftaris & Zenetos 2006; Andreakis et al. 2007b). In the field, gametophytes of both morpho-species are easily distinguished due to the presence of harpoon-like branches in A. armata (Harvey 1855; Maggs & Stegenga 1999). Their tetrasporophyte stages, however, are apparently morphologically identical. Up to now, their identification has been based only on inference from the presence of the respective gametophytes or by DNA sequencing. A first morphological discrimination of tetrasporophyte isolates showed some marginal differences that were of limited taxonomic use (Nı´ Chuala´in et al. 2004); however, the isolates were grown under the same conditions, suggesting that ecotypes or previously undetected levels of phenetic variation within the morpho-species range may have not been fully captured (Schmidt-Roach et al. 2012). The development of tools for fast identification of introduced seaweeds is crucial to support management and conservation decisions. Herein, we identify diagnostic morphological characters to discriminate among Asparagopsis gametophytes and tetrasporophytes by examining and comparing multiple vegetative and reproductive features. In our approach, we have first sequenced the cox2–3 intergenic spacer (IGS) and established a phylogenetic framework that recognised five OTUs. Next, we measured numerous morphological characters, and using analysis of variance we identified morphological features that provided statistically significant differences for the OTUs. Finally, we prepared a binary matrix of selected characters, and we used character state reconstruction to map the characters on the phylogenetic tree. We then discuss the congruence between genetically distinct lineages and morphological features in discerning Asparagopsis cryptic gametophytes and tetrasporophytes. We finally argue the importance of having accessible tools for rapid in-field Asparagopsis species identifications, especially in regions where multiple native and/or invasive strains of Asparagopsis occur sympatrically.

MATERIAL AND METHODS

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Asparagopsis gametophytes and tetrasporophytes were collected by snorkel and/or SCUBA diving from a depth range of 0–1.5 m (Table S1). Specimens were transported in seawater and darkness to the laboratory, and they were cleaned of epiphytes and separated from other species. Part of the collected material was preserved in silica gel for molecular analyses, and part was maintained as herbarium vouchers that were deposited in the University of Ma´laga Herbarium. Specimens of the Indo-Pacific lineage 2 were sampled from both its invasive Mediterranean range (ML2) and its native Hawaiian distribution range (L2). Gametophytes of A. armata were identified by the presence of characteristic harpoon-like hooks (Bonin & Hawkes 1987); the remaining gametophytes were assigned to A. taxiformis sensu lato. Twenty four vegetative anatomic characters were analysed for tetrasporophytes and 17 for gametophytes (Table 1; Figs 1, 2). Cystocarp measurements were performed by taking into consideration the diameter and the total length of the cystocarp, including the stalk, or only the length of the stalk. Because spermatangia had a clavate form, both total length and width were measured. Carpospores displayed an oval or pyriform shape; thus, both length and width were measured. Tetrasporophyte filaments were stained for 20 min in a solution containing 1 g of aniline blue,

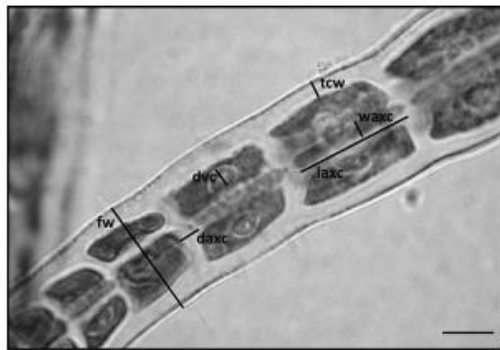

Fig. 1. Tetrasporophyte morphological characters. Scale bar = 10 μm; tcw = thickness of the cellular wall, waxc = axial cell width, laxc = axial cell length, dvc = vesicular cell diameter, daxc = axial cell distance, fw = filament width.

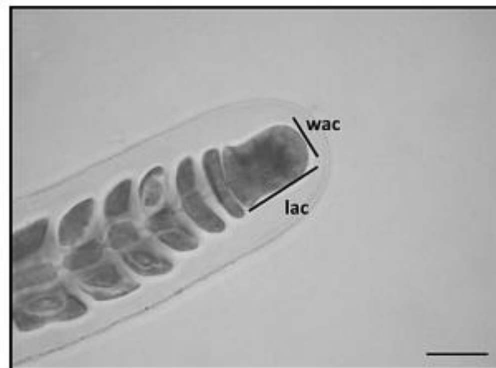

Fig. 2. Tetrasporophyte morphological characters. Scale bar = 10 μm; wac = apical cell width, lac = apical cell length.

100 ml of distilled water and 1 ml of acetic acid. When necessary, the staining was enhanced by heating for 10–15 min or by staining in the aniline solution overnight. Gametophytes were directly analysed without staining or preservation. All material was observed under a dissecting microscope (Leica EZ4, Leica Microsystems, Wetzlar, Germany) and a compound light microscope (Olympus C011, Olympus Corp., Tokyo, Japan). To test the statistical significance of the differences among characters, the homocedasticity of variances was checked by the Fmax test prior to one-way analysis of variance (ANOVA) for all the species and lineages. A Fisher LSD post hoc procedure of multiple comparisons was used when significant differences were found (with a significance value set at a ¼ 0.05). All tests were performed using SigmaPlot (Systat Software Inc., v11.0). DNA extraction and sequencing of the cox2–3 IGS was performed following Andreakis et al. (2004) and Sherwood (2008). New sequences were compared to those in GenBank: EU146211, EU146208 and EU146226 (for lineage 1 of A. taxiformis); EU146198, EU146197 and EU146196 (for lineage 2 of A. taxiformis); AY589524 (for lineage 3 of A. taxiformis); EU146220, EU146221 and EU146222 (for lineage 4 of A. taxiformis); and AY589522 and AY589523 (for A. armata). We used the ClustalW algorithm (Thompson et al. 1994) for sequence alignment as implemented in Bioedit v4.8.5 (Hall 1999); the alignment was refined by eye. Hierarchical likelihood ratio tests were performed using Modeltest v3.06 (Posada & Crandall 1998) to find the bestfitting parameters for maximum likelihood (ML) analysis given the alignment and ML phylogenies, inferred in PAUP* v4.0b10 (Windows version; Swofford 2002).

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Table 2. Tetrasporophyte morphological characters for *A. armata* and *A. taxiformis* cryptic lineages. Values are given as mean ± *sd* ($n = 10$–30). Different lowercase letters show significant differences among lineages and species ($P < 0.001$). All measurements are expressed in µm. See text for *A. taxiformis* lineage definitions.

	A. armata	*A. taxiformis*				
		L1	ML2	L2	L3	L4
Vesicular cell – diameter	6.4 ± 0.9 a	3.4 ± 0.5 b	3.3 ± 0.6 b	7.4 ± 1.5 c	8.2 ± 2.0 c	5.9 ± 1.4 a
Apical cell – width	13.7 ± 1.5 a	5.5 ± 0.5 b	4.6 ± 0.6 b	8.7 ± 0.9 c	12.3 ± 2.0 d	8.1 ± 1.0 c
Apical cell – length	14.8 ± 2.3 a	6.9 ± 0.6 b	6.4 ± 0.4 b	11.7 ± 2.0 c	17.2 ± 3.5 a	13.3 ± 2.1 d
Thickness of the cellular wall	6.8 ± 2.0 a	2.3 ± 0.4 b	2.5 ± 0.4 b	3.9 ± 0.8 c	4.1 ± 1.2 d	3.5 ± 0.6 cd
Axial cell – width	7.0 ± 1.3 a	2.0 ± 0.5 b	2.5 ± 0.6 b	5.0 ± 0.8 c	3.9 ± 1.2 d	2.7 ± 0.7 b
Axial cell – length	78.3 ± 14.6 a	25.3 ± 3.4 b	22.9 ± 5.5 c	39.8 ± 5.9 d	66.3 ± 9.5 e	40.1 ± 11.5 d
Filament width	52.5 ± 10.5 a	19.7 ± 4.3 b	23.2 ± 5.4 b	37.5 ± 3.1 c	46.6 ± 10.0 a	38.3 ± 7.6 c
10th cell from apex – length	32.3 ± 9.7 a	26.9 ± 11.3 ab	24.2 ± 7.9 ab	30.0 ± 9.8 a	35.8 ± 10.1 ca	36.3 ± 5.0 ca
20th cell from apex – length	68.1 ± 18.3 a	33.9 ± 9.9 b	54.2 ± 10.7 a	37.3 ± 10.3 b	58.7 ± 22.0 a	45.8 ± 9.4 b
30th cell from apex – width	21.7 ± 4.5 a	19.4 ± 4.1 a	26.9 ± 7.6 bc	18.8 ± 4.8 a	31.8 ± 5.6 b	23.0 ± 5.2 ac
30th cell from apex – length	79.4 ± 16.3 a	44.1 ± 12.5 b	60.5 ± 15.2 c	41.2 ± 17.6 b	69.0 ± 15.5 ac	46.2 ± 10.3 b
40th cell from apex – width	23.1 ± 7.1 a	21.2 ± 4.5 a	26.0 ± 7.0 b	16.4 ± 5.4 a	31.4 ± 9.1 b	20.4 ± 5.6 a
40th cell from apex – length	85.1 ± 17.3 a	52.4 ± 11.6 bc	55.9 ± 12.0 b	37.5 ± 10.2 d	84.5 ± 17.4 a	40.7 ± 8.3 cd
50th cell from apex – width	26.2 ± 4.2 ab	21.6 ± 3.3 b	26.7 ± 4.8 ca	18.5 ± 4.2 b	34.0 ± 8.7 d	18.3 ± 3.5 b
50th cell from apex – length	7.3 ± 2.8 a	1.6 ± 0.4 b	2.9 ± 0.7 b	3.1 ± 1.0 b	7.1 ± 2.5 a	3.1 ± 0.9 b
Distance between axial cells	7.3 ± 2.8 a	1.6 ± 0.4 b	2.9 ± 0.7 b	3.1 ± 1.0 b	7.1 ± 2.5 a	3.1 ± 0.9 b

Computations were performed using heuristic searches and 10 random sequence additions to find the highest likelihood tree. Bootstrap support for individual clades (Felsenstein 1985) was calculated with 1000 replicates using the same options and constraints as used in the tree inferences. Following identification of statistically supported ranges of values for each of the character states under consideration and given the results of the ANOVA tests (Tables 2–4), a binary matrix for character state mapping was constructed. If no statistical differences were found among average values, they were all given the same character state (Table 1). Finally, morphological characters were scored for each species/lineage and mapped onto the ML phylogram. Their ancestry and evolution across the phylogeny was evaluated individually using a maximum parsimony framework in Mesquite v2.75 (Maddison & Maddison Where lineages 1 (L1), 2 (L2) and 4 (L4) occurred sympatrically (i.e. the Hawaiian Islands), the diameter of the vesicular cell and the length of the apical cell were characters that distinguished the three lineages (Table 2). For the former character, values were up to 20% and 25% larger in the native lineage 2 when compared to the L4 and L1 lineages, respectively; for the latter character, the lowest values were recovered for L1 as well. In the Mediterranean Sea, invasive lineage 2 (ML2) and lineage 3 (L3) coexist. The characters that distinguished them were the diameter of the vesicular cell (higher in L3); the width and length of the apical cell (wider in L3 and longer in ML2); the thickness of the cell wall (thicker in L3); the distance between the axial cells (greater in L3); the length of the 10th, 40th and 50th cells and the width of the 50th cells (always higher in the case of L3). In addition, the filament's width and the dimensions of the axial cells were statistically different in both lineages, with higher values recorded for L3 (Table 2). Interestingly, L2 isolates collected from the lineage's presumed native and introduced ranges (i.e. Hawaii and the Mediterranean Sea, respectively) showed marked differences in all morphological characters except the distance between the axial cells (Table 2). For instance, the diameter of the vesicular cell, the width and length of the apical cell, the width of the cell wall, the width and length of the axial cell and the width of the filament were shown to be larger in Hawaiian populations. In contrast, the height of the 20th, 30th and 40th and the width of the 30th and 40th cells were greater in introduced populations collected from the Mediterranean Sea. All A. taxiformis gametophyte isolates were growing epilithically, attached via rhizoids; they had reddish-coloured thalli with ovate or lanceolate shapes. They were distinguished from A. armata mainly by the absence of harpoonlike lateral branches. The branchlets were opposite and always displayed radially with respect to the main axis. No significant differences were found in the height of the

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Table 4. Morphological characters identified from gametophyte reproductive structures for lineages of *A. taxiformis* (see text for lineage definitions). Values are mean ± *sd* ($n = 30–40$). Different lowercase letters show significant differences among lineages ($P < 0.001$).

	L1	ML2	L2	L3
Cystocarps				
Total length (mm)	1.3 ± 0.2 a	0.5 ± 0.2 b	1.3 ± 0.3 ab	1.9 ± 0.4 c
Diameter (mm)	0.7 ± 0.2 a	0.7 ± 0.2 a	0.8 ± 0.2 a	1.1 ± 0.3 b
Stalk length (μm)	509.2 ± 92.9 a	898.6 ± 185.0 b	513.5 ± 116.2 a	866.6 ± 165.2 b
Spermatangia				
Width (μm)	131.0 ± 17.5 a	233.7 ± 42.3 b	226.0 ± 65.63 b	185.4 ± 21.8 c
Length (μm)	484.9 ± 113.3 a	957.8 ± 152.0 b	517.7 ± 182.3 a	716.6 ± 167.5 c
Carpospores				
Width (μm)	21.1 ± 3.3 a	48.9 ± 9.0 b	23.9 ± 4.3 a	33.4 ± 4.2 c
Length (μm)	50.7 ± 11.1 a	125.0 ± 22.5 b	74.1 ± 10.7 c	75.2 ± 9.9 c

apical cell among the lineages studied. Rhizoids displayed ramifications and bore spine-like structures in all cases. However, statistically significant morphological differences were recovered among sympatric lineages (Table 3). In the Mediterranean Sea, the maximum length and width of the thallus, the height of the thallus where branching began, the length of the branchlets and the basal diameter of the branchlets separated ML2 from L3. All of the aforementioned characters were significantly larger for ML2, in some cases reaching twice the value recovered for L3 (Table 3). In the Hawaiian Islands, useful characters to discriminate sympatric lineages were the length and width of the apical cell and the height of the thallus where branching began. L2 and L4 thalli had similar heights, both being 40% smaller than L1. Furthermore, the width of their apical cells was consistently wider than for L1. Branching in L1 began at a height more than four times higher than in L4 and L2. L4 showed equal width of the main axis to L1 but higher than for L2. The length and the basal diameter of the branchlets were not significantly different among the Hawaiian lineages. Finally, thallus height, basal diameter and length of the branchlets and height where branching begins were greater in ML2 compared to L2; although, L2 had wider apical cells (Table 3). Except for L4, which was not found with reproductive structures, the lineages were monoecious. The diameter of the cystocarps and their total length distinguished the Mediterranean isolates of L2 from L3; these structures were wider and longer in L3 than in ML2. In addition, the width and length of the spermatangia and carpospores had significant diagnostic value with ML2 characterised by considerably wider and longer values (Table 4). In the Hawaiian Islands, the reproductive characters that can be used to distinguish lineages were the width of the spermatangia and the length of the carpospores, which were significantly higher in L2 (Table 4). Interestingly, significant differences were noted among native and introduced isolates of lineage 2 with ML2 exhibiting longer cystocarps and spermatangia as well as wider and longer carpospores (Table 4). Character-state reconstructions of vegetative and reproductive characters over the cox2–3 IGS phylogeny is shown in Figures 3

Table 3. Morphological characters identified from gametophytes of the *A. taxiformis* cryptic lineages (see text for lineage definitions). Values are given as mean ± *sd* ($n = 10$). Different lowercase letters show significant differences among lineages ($P < 0.001$).

	L1	ML2	L2	L3	L4
Thallus					
Length (cm)	8.5 ± 2.2 a	15.7 ± 4.2 b	5.2 ± 1.2 c	7.4 ± 1.4 a	5.1 ± 1.3 c
Maximum width (cm)	2.2 ± 0.8 ab	3.5 ± 2.1 c	2.7 ± 1.5 cb	1.9 ± 0.3 ab	1.2 ± 0.6 a
Main axis					
Basal diameter (mm)	1.4 ± 0.3 a	1.1 ± 0.2 ab	1.1 ± 0.2 ab	1.5 ± 0.3 ac	0.15 ± 0.2 ac
Apical cell – width (μm)	4.1 ± 1.2 a	5.3 ± 1.8 a	7.7 ± 2.3 b	5.2 ± 1.7 a	6.9 ± 2.4 b
Height where branching begins (cm)	2.4 ± 1.9 a	5.6 ± 1.5 b	0.6 ± 0.4 c	1.8 ± 1.6 a	0.4 ± 0.6 c
Branchlets					
Length (cm)	1.0 ± 0.3 a	2.6 ± 1.3 b	1.3 ± 0.6 a	1.2 ± 0.3 a	0.9 ± 0.3 a
Basal diameter (mm)	0.3 ± 0.1 ab	0.7 ± 0.1 c	0.4 ± 0.1 ab	0.4 ± 0.0 a	0.3 ± 0.0 b

to 5. Amongst the 17, 24 and 8 character traits initially identified from gametophytes, tetrasporophytes and reproductive structures, respectively (Table 1), 6, 15 and 6 were variable

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and either singularly or in combination were useful for delineating Asparagopsis OTUs. Asparagopsis armata gametophytes were clearly distinct by the presence of characteristic harpoon-like lateral branches. Contrary to the characters associated with reproductive structures (Fig. 3), the vegetative characters selected for the gametophytes of2011).

RESULTS

Significant morphological differences were found among gametophytes and tetrasporophytes of the five Asparagopsis OTUs (Table 2). Because the lineages had distinct distribution patterns and often co-occurred, we emphasised the characters useful for discriminating sympatric lineages of A. taxiformis in the Hawaiian Islands and the Mediterranean Sea. The width of the apical cell, thickness of the cell wall and width and length of the axial cell were shown to be diagnostic characters to distinguish tetrasporophytes (Table 2). Conversely, there were no significant differences in the axial cell numbers between ramifications. All isolates had apical dichotomies with ramification angles of approximately 90 degrees, and they had a parallel apical division pattern. Vesicular cells stained blue and were always located next to the main axis. The width of the 10th and 20th cells from the apex was similar among the species and lineages. Where lineages 1 (L1), 2 (L2) and 4 (L4) occurred sympatrically (i.e. the Hawaiian Islands), the diameter of the vesicular cell and the length of the apical cell were characters that distinguished the three lineages (Table 2). For the former character, values were up to 20% and 25% larger in the native

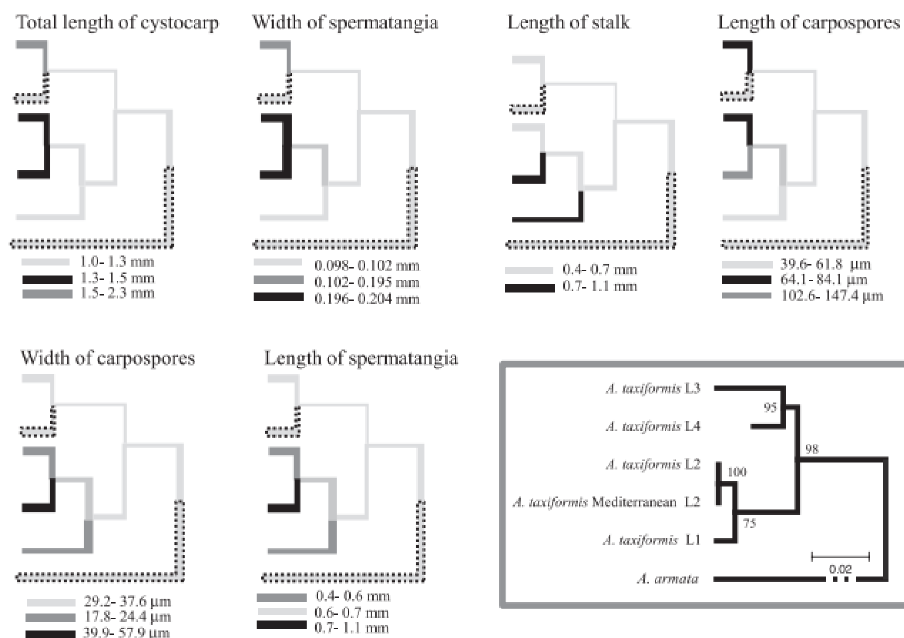

Fig. 3. Morphological character trait reconstruction of reproductive structures (small individual cladograms) based on the character states reported in Table 1, mapped onto the maximum likelihood tree (grey box, scale bar = 0.02 substitutions/site). Character states are colour-coded, and dotted lines on the cladograms denote OTUs not included in the analysis.

lineage 2 when compared to the L4 and L1 lineages, respectively; for the latter character, the lowest values were recovered for L1 as well. In the Mediterranean Sea, invasive lineage 2 (ML2) and lineage 3 (L3) coexist. The characters that distinguished them were the diameter of the vesicular cell (higher in L3); the width and length of the apical cell (wider in L3 and longer in ML2); the thickness of the cell wall (thicker in L3); the distance between the axial cells (greater in L3); the length of the 10th, 40th and 50th cells and the width of the 50th cells (always higher in the case of L3). In addition, the filament's width and the dimensions of the axial cells were statistically different in both lineages, with higher values recorded for L3 (Table 2). Interestingly,

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L2 isolates collected from the lineage's presumed native and introduced ranges (i.e. Hawaii and the Mediterranean Sea, respectively) showed marked differences in all morphological characters except the distance between the axial cells (Table 2). For instance, the diameter of the vesicular cell, the width and length of the apical cell, the width of the cell wall, the width and length of the axial cell and the width of the filament were shown to be larger in Hawaiian populations. In contrast, the height of the 20th, 30th and 40th and the width of the 30th and 40th cells were greater in introduced populations collected from the Mediterranean Sea. All A. taxiformis gametophyte isolates were growing epilithically, attached via rhizoids; they had reddish-coloured thalli with ovate or lanceolate shapes. They were distinguished from A. armata mainly by the absence of harpoonlike lateral branches. The branchlets were opposite and always displayed radially with respect to the main axis. No significant differences were found in the height of the apical cell among the lineages studied. Rhizoids displayed ramifications and bore spine-like structures in all cases. However, statistically significant morphological differences were recovered among sympatric lineages (Table 3). In the Mediterranean Sea, the maximum length and width of the thallus, the height of the thallus where branching began, the length of the branchlets and the basal diameter of the branchlets separated ML2 from L3. All of the aforementioned characters were significantly larger for ML2, in some cases reaching twice the value recovered for L3 (Table 3). In the Hawaiian Islands, useful characters to discriminate sympatric lineages were the length and width of the apical cell and the height of the thallus where branching began. L2 and L4 thalli had similar heights, both being 40% smaller than L1. Furthermore, the width of their apical cells was consistently wider than for L1. Branching in L1 began at a height more than four times higher than in L4 and L2. L4 showed equal width of the main axis to L1 but higher than for L2. The length and the basal diameter of the branchlets were not significantly different among the Hawaiian lineages. Finally, thallus height, basal diameter and length of the branchlets and height where branching begins were greater in ML2 compared to L2; although, L2 had wider apical cells (Table 3). Except for L4, which was not found with reproductive structures, the lineages were monoecious. The diameter of the cystocarps and their total length distinguished the Mediterranean isolates of L2 from L3; these structures were wider and longer in L3 than in ML2. In addition, the width and length of the spermatangia and carpospores had significant diagnostic value with ML2 characterised by considerably wider and longer values (Table 4). In the Hawaiian Islands, the reproductive characters that can be used to distinguish lineages were the width of the spermatangia and the length of the carpospores, which were significantly higher in L2 (Table 4). Interestingly, significant differences were noted among native and introduced isolates of lineage 2 with ML2 exhibiting longer cystocarps and spermatangia as well as wider and longer carpospores (Table 4). Character-state reconstructions of vegetative and reproductive characters over the cox2–3 IGS phylogeny is shown in Figures 3

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to 5. Amongst the 17, 24 and 8 character traits initially identified from gametophytes, tetrasporophytes and reproductive structures, respectively (Table 1), 6, 15 and 6 were variable and either singularly or in combination were useful for delineating Asparagopsis OTUs. Asparagopsis armata gametophytes were clearly distinct by the presence of characteristic harpoon-like lateral branches. Contrary to the characters associated with reproductive structures (Fig. 3), the vegetative characters selected for the gametophytes of A. taxiformis were unable to resolve L1, L3 and L2 (Fig. 4). However, in tetrasporophyte stages, L4 could be distinguished by the length of the apical cells; most importantly, ML2 could be easily identified by three individual characters: the length of the axial cell and the 30th cell from the apex and the thickness of the cell wall (Fig. 5). Based on vegetative character traits, A. armata tetrasporophyte isolates were clearly distinct by the dimensions of the axial cells and the width of the apical cell. Although tetrasporophytes of the four A. taxiformis lineages were distinguishable by one or a combination of multiple characters, both isolates from L2 could be differentiated by the vast majority of the characters considered (Table 2). We were unable to collect data from reproductive structures of L4. Reproductive features such as the length and width of carpospores as well as spermatangial width and the total length of the cystocarps differentiated L1 from the remainder of the OTUs (Fig. 3). Lineage 3 was distinguishable by the length of the spermatangial structures, the total length and the diameter of the cystocarps and the width of carpospores. Finally, the invasive ecotype ML2 was distinct from the remainder of the OTUs by the length of spermatangia and by

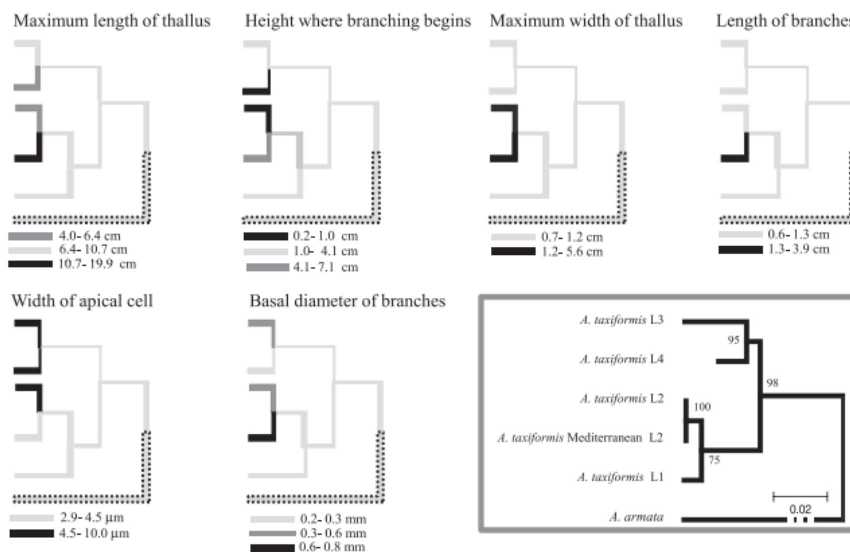

Fig. 4. Morphological character trait reconstruction of gametophyte structures (small individual cladograms) based on the character states reported in Table 1, mapped onto the maximum likelihood tree (grey box, scale bar = 0.02 substitutions/site). Character states are colour-coded, and dotted lines on the cladograms denote OTUs not included in the analysis.

the width and length of carpospores. Interestingly, ML2 showed distinctive differences from L2 in four out of the seven characters analysed.

DISCUSSION

The morphological differences of Asparagopsis OTUs recovered among our field-collected gametophytes and tetrasporophytes were congruent with genetic diversity levels previously reported (Andreakis et al. 2004; 2007a; Nı´ Chuala´in et al. 2004; Sherwood 2008). Our results and conclusions are restricted to the geographical regions where the OTUs occurred in sympatry. Therefore, we could not assess the influence of environmental pressures, such as wave exposure, solar radiation, sedimentation, nutrient richness and salinity, on the stability and diagnostic value

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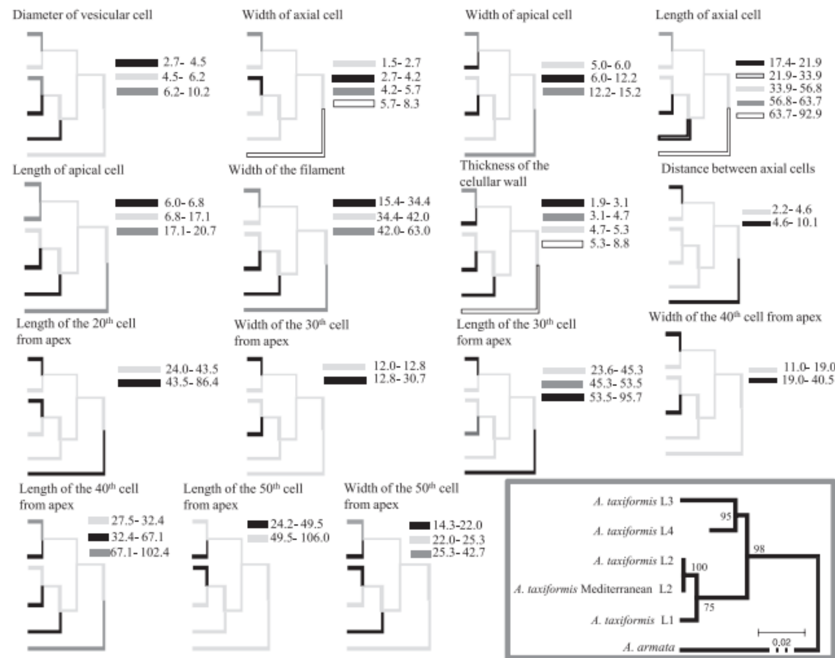

Fig. 5. Morphological character trait reconstruction of tetrasporophyte structures (small individual cladograms) based on the character states reported in Table 1, mapped onto the maximum likelihood tree (grey box, scale bar = 0.02 substitutions/site). Character states are colour-coded.

of morphology at larger geographical scales. We can propose a set of useful diagnostic characters to discriminate between tetrasporophytes of A. armata and A. taxiformis in the Mediterranean Sea, where these two species coexist. Further, a combination of these tetrasporophyte characters can be used to discern the A. taxiformis lineages in the Mediterranean Sea and the Hawaiian Islands (Table 2). Finally, we establish a second character set, associated with reproductive structures, that is capable of discriminating among A. taxiformis cryptic gametophytes occurring in sympatry and between Mediterranean and Hawaiian lineage 2 isolates (Table 4; Figs 3–5). Characteristic tetrasporophyitic morphological differences between A. armata and A. taxiformis mostly pertained to the axial cells and the apical cell; these findings contradict previous observations reporting that the tetrasporophyte stages of A. taxiformis and A. armata were morphologically identical (Feldmann & Feldmann 1939; Dixon 1965; Bolton et al. 2011; Andreakis & Schaffelke 2012). Our data were obtained from field-collected isolates, and they differed from measurements of cultured specimens reported by Nı´ Chuala´in et al. (2004). These incongruences suggest remarkable levels of morphological plasticity of wild plants compared to culture isolates and emphasise the importance of analysing wild material for the rapid identification of Asparagopsis cryptic introductions. Lineage 2 was consistently characterised by significantly larger gametophyte plants, which agrees with Chihara (1962), who described samples from Japan, where L2 has been reported (Andreakis et al. 2007b). Statistically significant differences were recovered for several characters among the lineages of A. taxiformis, such as the width and length of the apical cells or the diameter of the basal branchlets (Table 3). We suggest that these characters are of taxonomic value. Overall, a limited number of diagnostic characters are useful for distinguishing gametophytes, suggesting a broader phenotypic plasticity of this life history stage. For example, we have observed that Mediterranean L2 gametophytes varied greatly in size in the same locality and, depending upon the season, ranging from 8 to 10 cm in winter to . 30 cm in summer. Therefore, molecular analyses will be necessary to distinguish sterile gametophytes. Consistent morphological differences were found between native

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Hawaiian isolates and invasive gametophytic and tetrasporophytic isolates of A. taxiformis L2 collected from the Mediterranean Sea despite their identical nuclear plastid and mitochondrial sequences (Andreakis et al. 2007b; Sherwood 2008). Similar morphological differences have been observed for Codium fragile (Suringar) Hariot, where the invasive C. fragile subsp. tomentosoides (van Goor) P.C. Silva could be distinguished on the basis of utricle dimensions from its noninvasive relative (Trowbridge 1996). Furthermore, the invasive populations of Caulerpa taxifolia (M. Vahl) C. Agardh were differentiated from the noninvasive ones based on both ecological and morphological characters (Wright 2005). Epigenetic variation (e.g. methylation polymorphism) may drastically interfere with the expression of phenotypic traits, and it has been recently considered as a source of morphological diversification between introduced and native populations of genetically homogeneous invasive species (e.g. Schrey et al. 2012; Liebl et al. 2013). Given the previously inferred genetic relationships for Asparagopsis (Andreakis et al. 2007b; Sherwood 2008), we interpret the Mediterranean and Hawaiian populations of lineage 2 as two distinct morphs from within the same genetically homogeneous lineage rather than two biologically distinct species, as was previously proposed (Bouduresque & Verlaque 2010). The systematics of Asparagopsis, including the placement of Bonnemaisonia hamifera Hariot in Asparagopsis (Okamura 1921), is controversial. At the morphological level, identification keys are available only for A. armata and A. taxiformis sensu lato (www.algaebase.org; Guiry & Guiry 2013). Genetic analyses support recognition of the former; whereas, the latter has been identified as a species complex (Andreakis et al. 2004, 2007b; Nı´ Chuala´in et al. 2004; Sherwood 2008; Salvador 2009). However, similar studies have not been performed for two other proposed Asparagopsis taxa (A. sanfordiana f. amplissima and A. svedelli). The morphological descriptions available for A. svedelli and A. sanfordiana f. amplissima do not fit any of the A. taxiformis lineages presented in this study (Setchell & Gardner 1924; Taylor 1945). Whether these two taxa represent new Asparagopsis lineages, ecotype variants of already known Asparagopsis lineages or additional Asparagopsis species can be assessed only by genetically characterising the type specimens or topotype material [e.g. A. sanfordiana f. amplissima has been considered a synonym of A. taxiformis (Bonin & Hawkes 1987)]. The morphological characterisation of the Asparagopsis OTUs presented in this study together with their distinct physiological performance (Zanolla et al. pers. comm.) and their distinct patterns of geographical distribution represent a first step towards a formal delineation of new species within A. taxiformis and further confirm the species status of A. armata. In this sense, as L2 is the only one present in Western Australia (the type locality), we suggest this lineage to be considered as A. taxiformis sensu stricto.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/13–247.1.s1.

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