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Asparagopsis taxiformis and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations

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The tropical-subtropical red seaweed *Asparagopsis* Montagne (Bonnemaisoniales) constitutes the haploid, gametophytic phase in a heteromorphic diplo-haplontic life cycle. The diploid tetrasporophyte is known as the '*Falkenbergia*' stage. The genus contains two species, *A. armata* and *A. taxiformis*, both present in the Mediterranean Sea where they are regarded as introduced. *A. armata* is morphologically distinct from *A. taxiformis* in that it possesses long stolons bearing harpoon-like hooks. The seemingly morphologically identical '*Falkenbergia*' stages of the two *Asparagopsis* species and phenotypic variation within these species have caused taxonomic confusion. We defined species boundaries in the Mediterranean Sea by inferring phylogenies from sequence data from a variable region in the nuclear LSU rDNA gene, the plastid RuBisCo spacer, and the mitochondrial *cox*2–3 spacer of specimens from the Mediterranean, western Europe and the Canary Islands. Results indicate that *A. armata* and its '*Falkenbergia*' tetrasporophyte are genetically distinct from *A. taxiformis* and its '*Falkenbergia*' phase. No phylogeographic structure was detected within *A. armata,* whereas *A. taxiformis* seems to consist of at least two genetically distinct but morphologically cryptic species, an Atlantic one (from the Canary Islands) and a Mediterranean one. Hypothetical distribution patterns of the two species as reconstructed from critical temperature limits to growth, survival and reproduction and from the summer and winter isotherms in the Mediterranean Sea agree with the actual Mediterranean distribution patterns as gleaned from our data.

Key words: *Asparagopsis armata*, *Asparagopsis taxiformis*, *cox*2–3 spacer, '*Falkenbergia*', Mediterranean, LSU rDNA, phylogeny, RuBisCo spacer

Introduction

Thalli of the rhodophyte genus *Asparagopsis* Montagne (Bonnemaisoniales) are composed of sparsely branched, creeping stolons and erect shoots from which numerous side branches develop in all directions. The latter ramify over and over again giving the thallus a plumose appearance. The ultimate branchlets are filamentous and composed of three cell rows whereas the larger branches consist of a central medullary filament and a gelatinous matrix surrounded by a cortex 3–6 cells thick (Børgesen, 1915).

Two *Asparagopsis* species are currently recognized: *A. armata* Harvey and *A. taxiformis* (Delile) Trevisan (Dixon, 1964; Dixon & Irvine, 1977; Bonin & Hawkes, 1987). *Asparagopsis armata* possesses long hooked stolons (Bonin & Hawkes, 1987), which become entangled among other marine organisms thus permitting thalli to sprawl loosely over large areas. Thalli of *A. taxiformis* grow, instead, on rock or in algal turfs by means of a rhizomatous system; they lack the hooked stolons.

Asparagopsis constitutes the gametophytic (haploid) life stage in a diplohaplontic heteromorphic life cycle (Feldmann & Feldmann, 1939, 1942; Chihara, 1961, 1962). The epiphytic tetrasporophytic '*Falkenbergia*' stage is composed entirely of densely ramified filaments consisting of three cell rows. Feldmann & Feldmann (1942) identified the tetrasporophytes of *A. armata* and *A. taxiformis* as *F. rufolanosa* (Harvey) Schmitz and *F. hillebrandii* (Bornet) Falkenberg, respectively, yet neither they nor later workers mentioned any diagnostic morphological characters or differences in habitat (Dixon, 1964; Dixon & Irvine, 1977). Recently, Ni Chualáin *et al.* (2004) demonstrated morphometric differences between the tetrasporophytes of the two species.

Asparagopsis armata seems to be a temperate species. It is native to southern Australia and New

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Zealand (Horridge, 1951) and is now found from the British Isles, the Canary and Salvage Islands to Senegal as well (Dixon & Irvine, 1977; Price *et al*., 1986). *Asparagopsis taxiformis* has a typical tropical to warm temperate distribution; it abounds throughout the tropical and warm-temperate parts of the Atlantic and Indo-Pacific (Harvey, 1855; Abbott & Williamson, 1974; Price *et al*., 1986; Bonin & Hawkes, 1987; Silva *et al*., 1996). Both species are considered introduced in the Mediterranean Sea (Boudouresque & Verlaque, 2002). *Asparagopsis armata* was first reported from Algeria in 1923 (Feldmann & Feldmann, 1942) and *A. taxiformis* was first collected near Alexandria, Egypt (Delile, 1813), which is also the type locality. The latter species is thus either a Mediterranean native, contradicting Boudouresque & Verlaque's (2002) assessment, or a pre-Lessepsian immigrant since its first record predates the opening of the Suez Canal in 1869. At present, *A. armata* is encountered mainly along western Mediterranean coasts (South & Tittley, 1986; Sala & Boudouresque, 1997) where it is regarded as invasive (Boudouresque & Verlaque, 2002). *Asparagopsis taxiformis* seems to be confined to the eastern Mediterranean (South & Tittley, 1986; Sala & Boudouresque, 1997). The distribution ranges of the two species appear to overlap along the Italian coast because both species have been reported there (Barone *et al*., 2001; D'Archino *et al*., 2003; Furnari *et al*., 2003).

Unfortunately, distribution data of *A. armata* and *A. taxiformis* are potentially unreliable. In many cases taxonomic identifications are based solely on the morphologically similar '*Falkenbergia*' stages (Funk, 1955; Diapoulis & Verlaque, 1981; Athanasiadis, 1987). Even the taxonomic status of the two species is not entirely clear because co-called aberrant morphologies have been reported in both species (Ni Chualáin *et al*., 2004). On the one hand, *A. armata* and *A. taxiformis* may represent extreme morphologies in a continuous range of a single species whereas on the other hand they each could consist of multiple cryptic species. Several cases of cryptic diversity have been recently discovered in red and green algae (Wattier & Maggs, 2001; Kooistra *et al*., 2002; Gabrielson *et al*., 2003; Zuccarello *et al*., 2002, 2003).

In the present study we compare sequence data and morphological information obtained from a series of Mediterranean specimens of *Asparagopsis* spp. and their '*Falkenbergia*' stages to assess (1) if *A. armata* and *A. taxiformis* constitute genetically distinct taxa, (2) if the '*Falkenbergia*' stages can be discriminated using the same genetic markers, (3) if *A. armata* and *A. taxiformis* are each composed of cryptic species, (4) if '*Falkenbergia*' and '*Asparagopsis*' stages from the same collection site belong to the same species, (5) if patterns can be discerned in the Mediterranean distribution of the identified *Asparagopsis* species and (6) if phylogenies inferred from several DNA markers are congruent in the recognition of taxa. Although this study focuses on distribution patterns within the Mediterranean Sea, a restricted number of extra-Mediterranean specimens have been included to identify possible source regions and the extent of geographic variation.

Three DNA markers have been chosen from three distinct compartments of the algal genome to obtain independent phylogenetic information. The markers are the region of the nuclear large subunit rDNA gene spanning the 'D1,' 'D2' and 'D3' hyper-variable domains (Lenaers *et al*., 1989), the chloroplast spacer between the large and small subunits of the ribulose-1-5-bisphosphate carboxylase/oxygenase region (Maggs *et al*., 1992) and the mitochondrial cytochrome oxidase subunit 2–subunit 3 (*cox*2–3) spacer (Zuccarello *et al*., 1999).

Materials and methods

Sample collection and preservation

Specimens were collected from many sites along the Mediterranean and Atlantic coasts of Europe as well as from the Canary Islands (see Table 1 and Fig. 1 for details). From several of these sites, multiple specimens have been included in this study. A clean fragment of each specimen of ca. 1 g wet weight was blotted dry between paper tissues, desiccated immediately in silica gel and stored until DNA extraction. The remainder of the specimen, or a representative part thereof, was dried on herbarium paper or fixed in 1% v/v formalin in seawater to serve as voucher specimen for morphological comparisons. If several morphologically indistinguishable specimens were sampled from a site, only one representative voucher was prepared. Specimens were keyed out using descriptions in Bonin & Hawkes (1987). We only examined the gross morphology of the gametophytes by light microscopy.

DNA extraction and purification

About 100 mg silica gel-dried tissue was ground in liquid nitrogen and added to 700 μl DNA extraction buffer containing 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8, 0.2% w/v PVP, 0.01% w/v SDS and 0.2% β-mercaptoethanol. The mixture was incubated at 65°C for 45 min, vortexing every 5 min. DNA was extracted with an equal volume of chloroform:isoamyl alcohol (CIA; 24 : 1 v/v) and centrifuged in a table-top Eppendorf microfuge (Eppendorf AG, Hamburg, Germany) at maximum speed (14 000 rpm) for 10 min. The aqueous phase was collected, re-extracted with CIA and centrifuged as above. Next, the aqueous phase was mixed thoroughly with NaCl to 1.66 M, mixed with an equal volume of ice-cold 100% isopropanol, then left on ice for 5 min and centrifuged subsequently in a pre-cooled Eppendorf microfuge under

maximum speed for 15 min. DNA pellets were washed in 300 μl 70% v/v ethanol, centrifuged for 10 min and, after decanting the ethanol, allowed to dry in air. DNA pellets were dissolved overnight in 50 μl of sterile water. Quantity and quality of DNA were examined by means of 1% agarose TAE buffer gel electrophoresis against known standards.

PCR amplification and sequencing of DNA marker regions

The 'D1,' 'D2' and 'D3' hyper-variable domains of the large subunit (LSU) rDNA gene (Mitchot & Bachellerie, 1987; Lenaers *et al*., 1989, 1991) were PCR amplified in 30 μl PCR reaction medium containing 10 ng DNA, 3 mM $MgCl_2$, 0.01% BSA, 0.2 mM dNTPs, 1 μM of forward primer D1R, 1 μM of reverse primer D3Ca (Lenaers *et al*., 1989), 1X Roche diagnostics PCR reaction buffer and 1 unit *Taq* DNA polymerase (Roche). PCR cycling comprised a 4-min initial heating step at 94°C, followed by 35 cycles of 94°C for 1 min, 45°C for 1.5 min, 72°C for 2 min and a final extension at 72°C for 5 min.

The chloroplast RuBisCo spacer was PCR amplified in 30 μl volume containing 10 ng DNA, 1.5 μM $MgCl_2$, 0.2 mM dNTPs, 1 μM of forward and reverse primers each, described in Maggs *et al*. (1992), 1X Roche diagnostics PCR reaction buffer, and 1 unit *Taq* DNA Polymerase (Roche). The PCR cycling comprised an initial heating step of 4 min at 94°C, followed by 35 cycles of 93°C for 40 s, 45°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min.

The mitochondrial *cox*2–3 spacer was PCR amplified in 50 μl PCR reaction medium containing 10 ng DNA, 2.5 mM $MgCl_2$, 0.1% bovine serum albumin (BSA; Sigma), 0.2 mM dNTPs, 1 μM of forward and reverse primers each, described in Zuccarello *et al*. (1999), 1X Roche diagnostics PCR reaction buffer (Roche) and 2 units *Taq* DNA polymerase (Roche). The amplification programme included an initial denaturation at 94°C for 4 min followed by 35 cycles of 93°C 1 min, 48°C 1 min and 72°C 1.5 min followed by a final extension cycle at 72°C for 5 min.

Quantity and length of PCR products were examined by 1% gel electrophoresis as described above. Target bands were excised under low UV-light and purified using the QIAEX II Gel Extraction kit 500 (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions. Purified products were sequenced on a Beckman Ceq 2000, using a Dye-terminator cycle sequencing kit (Beckman) according to manufacturer's instructions.

Data analysis

Sequences were assembled using the DNASTAR computer package (Lasergene) supplied with the Beckman sequencer and aligned with Bioedit v. 4.8.5 (Hall, 1999). The alignment was refined by eye. Phylogenetic analyses were conducted using PAUP* 4.0b10 version for Windows (Swofford, 2002). Maximum parsimony (MP) trees were inferred using the heuristic search option, 500 random sequence additions and tree bisection-reconnection (TBR) branch swapping. Characters were unweighted and treated as unordered and gaps were treated as missing data. To assess phylogenetic informativeness of the data, g_1 values of the skewness of distribution of three-lengths among the parsimony trees (Hillis & Huelsenbeck, 1992) were calculated in PAUP*. The significance of the g_1 value was compared with critical values ($p = 0.01$) for four state characters given the number of distinct sequences and the number of parsimony informative sites. Hierarchical Likelihood Ratio Tests (hLRTs) were performed using Modeltest Version 3.06 (Posada & Crandall, 1998) to find the best-fitting parameters (substitution model, gamma distribution, proportion of invariable sites, transition-transversion ratio) for maximum likelihood analysis (ML) given the alignment. ML analyses were performed using heuristic searches and 10 random additions. Bootstrap support for individual clades (Felsenstein, 1985) was calculated with 1000 replicates using the same methods, options and constraints as used in the tree-inferences but with all identical sequences removed. Haplotype networks (gene genealogies) were calculated using the algorithm developed by Templeton *et al*. (1992) deploying the computer program TCS 1.13 (Clement *et al*., 2000).

Results

Morphological data

Gametophytic specimens were separated into two morphologically distinct groups. Thalli in the first group possess modified stolons with apically arranged harpoon-like hooks and lack an obvious rhizomatous system. These thalli fit the description of *A. armata*. Those in the other group lack such hooked stolons but possess a clear rhizomatous system. These keyed out unambiguously as *A. taxiformis*. The side branches along the main axes of specimens in the latter group are generally more densely ramified than those of the first group.

LSU rDNA

The LSU rDNA data matrix comprised eight distinct types among the 21 sequences obtained from *A. armata* and 12 distinct types among the 32 sequences obtained from *A. taxiformis* (Table 2 for length and variation). Maximum likelihood analysis constrained with optimal hLRT parameters (Table 3) resulted in a single ML tree (Fig. 2). Maximum parsimony analysis resulted in a single MP tree (see Table 2 for tree statistics; tree not shown), which was topologically similar to the ML tree. The trees consisted of two clades. One of these included only specimens fitting the morphology of *A. armata* and '*Falkenbergia*' specimens collected from sites where only *A. armata* was found. The other one exclusively contained specimens fitting

Table 1. Gametophytes (G) and tetrasporophytes (T) of *A. taxiformis* and *A. armata* analysed. Genbank Accession Numbers have been assigned to only one sample per distinct sequence

		Sample site	Sample number(s)	Phase	Collection date	LSU rDNA	RuBisCo spacer	*cox*2–3 spacer
Asparagopsis taxiformis	Spain	Tenerife, Canary Is.	80**, 95*, 110***	G	30-05-2002	AY589540	AY589559	AY589524
	Italy	Lerici, La Spezia	1076*·***	T	16-07-1993	AY589545		AY589537
		Elba	479	T	25-08-2003			
		Civitavecchia, Roma	418,	G	15-06-2002			
			1077*	T	30-06-1993	AY589546		
		Pta. S. Pietro, Ischia, Naples	442–444	T	28-01-2003			
		Sant'Anna, Ischia, Naples	169*·**, 170	G	15-06-2002	AY589542	AY589558	
			168*·***	G	15-06-2002	AY589541		AY589527
		Pta. S. Pietro, Ischia, Naples	171*·***, 175	G	19-06-2002	AY589543		AY589528
			174***	G	19-06-2002			AY589529
		Sant'Angelo, Ischia, Naples	32***, 178*, 185, 194	G	22-06-2002	AY589544		AY589526
		Castello, Ischia, Naples	31, 315, 324, 330***	G	28-07-2002			AY589531
		Ischia, Naples	E760	G	not available			
		Capri, Naples	263, 264***	G	04-07-2002			AY589530
		Mergellina, Naples	24*, 30	G	19-04-2002	AY589539		
			10*·***, 21	G	19-04-2002	AY589538		AY589525
			26*	G	19-04-2002	AY589547		
		Capo Posillipo, Naples	383***	G	20-08-2002			AY589535
			391***	G	20-08-2002			AY589536
		Strait of Messina, Sicily	1030	T	16-11-1992			
		Trapani, Sicily	340***	G	20-07-2002			AY589532
			358*·***	G	22-07-2002	AY589549		AY589534
			348***	G	22-07-2002			AY589533
	Tunisia	Mahdia	480–482*	G	06-06-2003	AY589548		
Asparagopsis armata	Ireland	Ard Bay, Galway	373, 374*, 375	G	17-07-2002	AY589556		
	Spain	Oviedo	5***	T	08-09-2002			AY589522
		Novellana	2***	G	11-07-2002			AY589523
		Percebera	1	G	12-07-2002			
	Portugal	Praia Castelo, Albufeira	200, 214, 223*, 233, 254	G	05-06-2002	AY589552		
			228*	G	05-06-2002	AY589550		
			240*	G	05-06-2002	AY589551		
	France	South Marseille	124***, 130, 140*	G	15-05-2002	AY589553		AY589520
		North Marseille	149, 156, 163***	G	15-05-2002			AY589521
		Cassis	268, 277, 283	G	26-06-2002			
		Toulon, Brun Cape	306*	G	26-06-2002	AY589555		
			293*	G	26-06-2002	AY589554		
	Italy	Messina, Sicily	566*·**	T	04-06-1987	AY589557	AY589560	
	Australia	Sydney	E761, E762	G	not available			

Reference sequences for GenBank Accession Number are indicated with * (LSU rDNA), ** (RuBisCo spacer) and *** (*cox* spacer). List of identical sequences per DNA region is available upon request from N.A.

the morphology of *A. taxiformis* and '*Falkenbergia*' specimens collected from sites where only *A. taxiformis* was encountered. The sequences of *A. taxiformis* specimens from the Canary Islands grouped together in a well-supported clade within the group of sequences from the Mediterranean specimens. The LSU rDNA network of *A. taxiformis* (network not shown) revealed several haplotypes one or a few steps away from the dominant one (14 identical sequences). Two steps were detected between the Canary Islands haplotype and the dominant Mediterranean one. Distinct haplotypes were scored within *A. armata* as well as ambiguities due to unresolved genealogies (different branches leading to the same haplotype).

RuBisCo *spacer*

The RuBisCo alignment comprised three different haplotypes among the 37 sequences analysed (see Table 2). Maximum parsimony analysis resulted in a single MP tree (see Table 2 for tree statistics) shown in Fig. 3. Maximum likelihood analysis constrained with optimal hLRT parameters (Table 3) resulted in a single ML tree (tree not shown), which was topologically identical to the MP tree. Again, the two sister species were firmly resolved in two clades, one consisting of *A. armata* and the other one comprising *A. taxiformis*. No intraspecific variation was observed within the clade containing specimens of *A. armata*. In the *A. taxiformis* clade, all sequences from the Canary Islands grouped together in a clade.

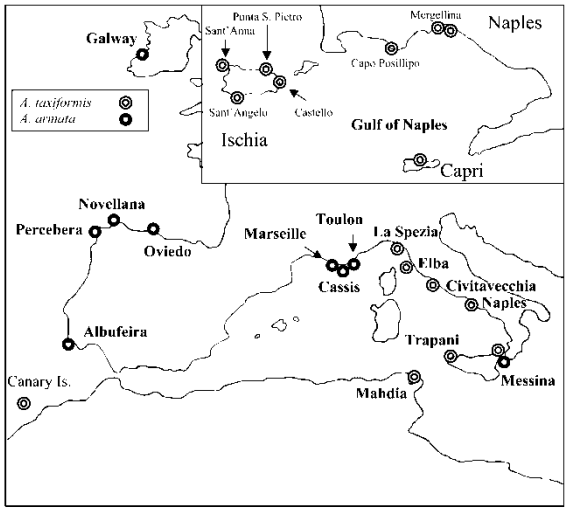

Fig. 1. Sample localities.

Cox*2–3 spacer*

The *cox* alignment revealed four distinct haplotypes among the 21 sequences of *A. armata* and 14 distinct haplotypes among the 39 sequences of *A. taxiformis* (see Table 2). Maximum likelihood analysis constrained with optimal hLRT parameters (Table 3) resulted in a single ML tree shown in Fig. 4. Maximum parsimony analysis resulted in 29 equally most parsimonious trees (see Table 2 for tree statistics; trees not shown), which were topologically similar to the ML tree. The two species again separated into two well-supported clades. The *A. taxiformis* clade consisted of two well-supported lineages, one with all Mediterranean specimens and the other one with the three Canarian specimens. Secondary clades were recovered among the Mediterranean sequences but no geographic patterns were found to correlate with these clades. Results of haplotype network analysis revealed four distinct haplotypes with no ambiguities within *A. armata* (not shown). The dominant one was represented by 18 identical sequences. Thirteen haplotypes, few

Table 2. Sequences and tree statistics

		LSU rDNA	RuBisCo spacer	*cox* spacer
Sequence length	*A. armata*	700 bp	307 bp	365 bp
	A. taxiformis (Canary Is.)	758 bp	303 bp	364 bp
	A. taxiformis (Mediterranean Sea)	758 bp	303 bp	364 bp
	Total alignment	768 bp	308 bp	367 bp
Sequence polymorphism	Number of variable characters	104	30	93
	Parsimony informative sites	84	30	88
	Number of distinct sequences	20	3	18
	g_1 value	− 0.3156	− 0.7067	− 0.3470
	g_1 p = 0.01	− 0.20	− 0.88	− 0.20
	Given # taxa & # characters	15 & 100	5 & 50	15 & 100
Parsimony trees	Length	114 steps	31 steps	104 steps
	Number of trees	1	1	29
	Consistency index	0.982	1.000	0.971

Table 3. Results of hLRTs and – ln likelihood of phylogenies inferred from ML-analyses constrained with optimal hLRT parameters

Alignment	LSU rDNA	RuBisCo spacer	*cox* spacer
Base frequencies			
A	0.2374	0.3898	0.3398
C	0.2085	0.1740	0.1230
G	0.3323	0.1175	0.1176
T	0.2218	0.3186	0.4195
Model*	HKY 85	HKY 85	HKY 85
ti/tv	1.0564	1.9472	0.8717
γ distribution	0.4988	-	-
-ln L of tree	1678.3962	531.0944	929.1726

* See Hasegawa *et al.* (1985)

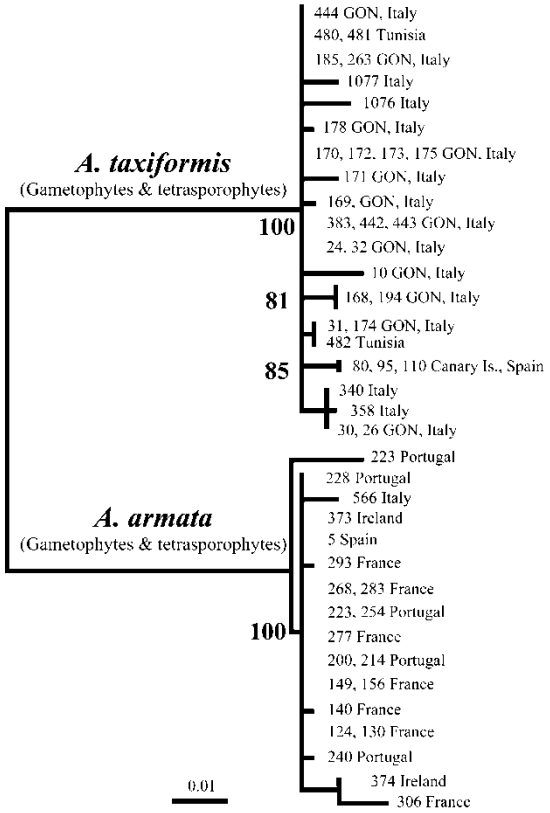

Fig. 2. Midpoint-rooted ML reconstruction of relationships between *A. taxiformis* and *A. armata* gametophytic and tetrasporophytic specimens based on partial LSU rDNA sequences. Only bootstrap values > 80% are indicated. GON denotes Gulf of Naples.

resolved genealogies (although not geographically coherent) and unresolved genealogies were observed within the Mediterranean *A. taxiformis* (Fig. 5). In this network, the Canary Islands haplotype (not shown) differed distinctly from the Mediterranean haplotypes.

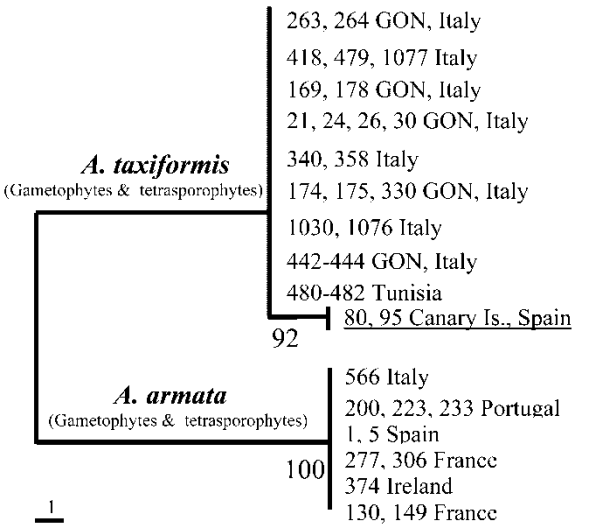

Fig. 3. Midpoint-rooted MP tree based on RuBisCo spacer DNA sequence. Only bootstrap values > 80% are indicated. The Canarian *Asparagopsis* is underlined. GON denotes Gulf of Naples. Scale bar represents 1 change.

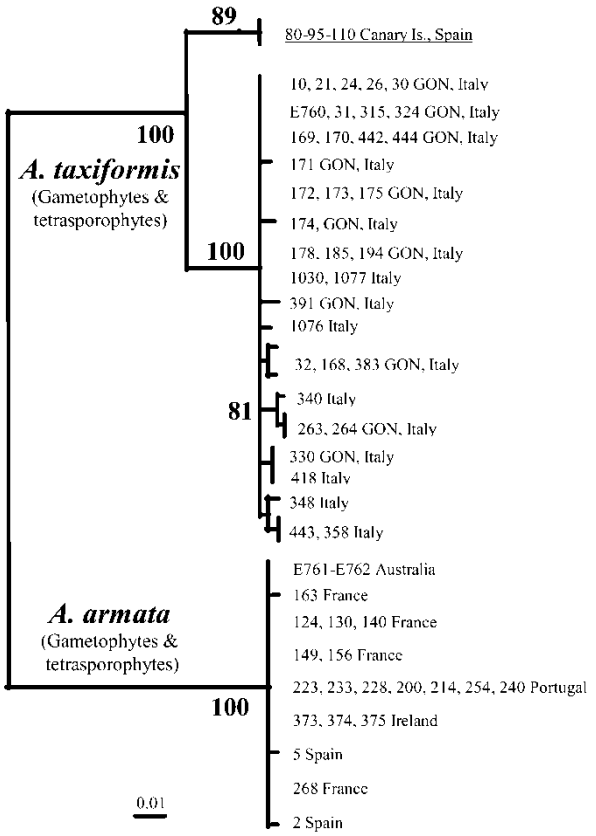

Fig. 4. Midpoint-rooted ML reconstruction based on *cox* spacer sequence data. Only bootstrap values > 80% are indicated. The Canarian *Asparagopsis* is underlined. GON denotes Gulf of Naples.

Discussion

Asparagopsis armata and *A. taxiformis* are, indeed, genetically and morphologically distinct

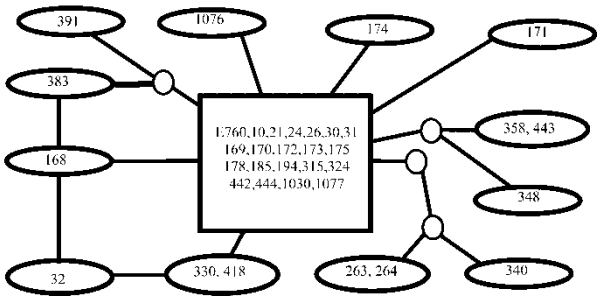

Fig. 5. Haplotype network for *cox*2–3 spacer haplotypes of the Mediterranean *A. taxiformis*. Lines indicate one mutation step; nodes indicate missing haplotypes; reticulations denote unresolved genealogies. The dominant haplotype (group of identical sequences) is surrounded by a rectangle.

species. The existence of two groups of gametophytic thalli based on the presence of distinct morphological characters (long hooked stolons in *A. armata* versus compact rhizoids in *A. taxiformis*; Dixon, 1964; Bonin & Hawkes, 1987) corroborates the division into two groups as revealed by each of the three genetic markers. The two described species definitely do not correspond to extreme growth forms within the plasticity range of a single species.

Genetic identification of tetrasporophytes

The absence of morphological differences between the '*Falkenbergia*' stages of the two species as reported by Feldmann & Feldmann (1942) poses no real identification problem because the thalli are readily identifiable with any of the genetic markers deployed in this study. Our results support those of Ni Chualáin *et al.* (2004) who recovered clear genetic differentiation between tetrasporophytes linked to *A. armata* and those linked to *A. taxiformis*. Ni Chualáin *et al.* (2004) report size differences between sub-apical cells in the '*Falkenbergia*' stages of *A. armata* and *A. taxiformis*. However, these data were collected in thalli grown under comparable conditions in culture and it needs to be assessed if these differences are present also in field samples. Molecular identification may be more expensive but it certainly gives a clear answer.

Cryptic diversity

We did not discover cryptic genetic diversity within Mediterranean and western European *A. armata* and neither did we discover any genetic differentiation between the European specimens and those from Sydney, Australia. Such results are consistent with conclusions based on plastid DNA restriction fragment length polymorphism (RFLP) data in Ni Chualáin *et al.* (2004) that European populations of *A. armata* result from a recent invasion from Australia. Whether *A. armata* consists of a single globally distributed species or of several cryptic ones remains to be uncovered in a far more thorough phylogeographic survey.

All Mediterranean specimens belonging to *A. taxiformis* grouped in a single clade without any clear internal differentiation. A few intraspecific clades were recovered in the LSU tree but these groupings were not recovered in the *cox* tree and *vice versa* suggesting that these patterns are insignificant. The clear genetic distinction in the *cox* marker between Mediterranean and Canarian populations of *A. taxiformis* corroborates results based on RFLP data in Ni Chualáin *et al.* (2004) and suggests that the two are genetically distinct, though closely related, taxa and with apparently identical morphologies. It still needs to be tested if these geographically and genetically distinct populations are reproductively isolated as well.

Ni Chualáin *et al.* (2004) recovered the Canarian genotype also among all their Caribbean specimens whereas the Mediterranean genotype was observed in some of their samples from the Indo-Pacific. However, it is premature to conclude that the Canarian populations are part of an Atlantic genotype and the Mediterranean ones are of an Indo-Pacific origin; their sample set is small and therefore, they may have missed more intricate patterns. In a phylogeographic study on *Cladophoropsis membranacea* (Hofman Bang ex C. Agardh) Kooistra *et al.* (1992) concluded that the Canary Islands and the Mediterranean populations were genetically distinct but they included only a few specimens in their study. Van der Strate *et al.* (2002) included many more specimens from several sites on the Canary Islands revealing the Mediterranean lineage there as well.

Sample coverage of *A. taxiformis* achieved within the West Mediterranean in our study strongly suggests that the 'Mediterranean' genotype is the only one present in this region. Yet, we cannot rule out the co-occurrence of cryptic *Asparagopsis* species within the Mediterranean or elsewhere. RFLP data in Ni Chualáin *et al.* (2004) already hint at the existence of multiple cryptic species in the Indo-Pacific. The DNA markers used in the present study appear to be the right tools for recovering cryptic diversity and reconstructing phylogeographic patterns among the various cryptic species. Other authors have used the same sequence regions to uncover large-scale geographic structure and cryptic species diversity in several other red seaweeds (Van Der Strate *et al.*, 2002a; Zuccarello *et al.*, 2002, 2003).

Coexistence of gametophytes and tetrasporophytes of the same species

Specimens of *Asparagopsis* and '*Falkenbergia*' phases collected from the same sites throughout the Gulf of Naples always belong to *A. taxiformis*. Likewise, all gametophytes and tetrasporophytes collected in Marseilles belong exclusively to *A. armata*. Thus, future population genetic surveys in these regions can assume that gametophytes and tetrasporophytes belong to the same species. Yet, it remains to be checked if this is true elsewhere as the distribution limits of gametophytes and tetrasporophytes belonging to the same species may not be the same. Breeman *et al.* (1988) have demonstrated that the different life stages of the same bonnemaisonialean species possess markedly different temperature tolerance limits for growth, survival and reproduction. For that reason, the tougher phase might show a more extensive distribution, perpetuating itself clonally on the fringes of the species' distribution range.

Distribution of the species in the Mediterranean Sea

Hypothetical distribution patterns of seaweeds can be reconstructed by comparing minimum and maximum temperatures for their growth, survival and reproduction with seawater surface isotherms in the coldest and warmest months (Breeman, 1988). In Bonnemaisoniales in general, and probably also in *Asparagopsis*, the most resilient phase is the tetrasporophyte (Breeman *et al.*, 1988).

The '*Falkenbergia*' stages of *A. armata* show a critical upper survival limit at 25°C and do not grow above 23°C (Ni Chualáin *et al.*, 2004). In the Mediterranean Sea, summer seawater surface temperatures do not rise above 24°C near the Strait of Gibraltar but they do virtually everywhere else (http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html accessed on 14 March, 2004). However, maps in Lipkin & Safriel (1971) provide average temperatures measured through the upper metres of the water column, not on the surface only. According to their map, the water temperature does not rise above 25°C in the Gulf of Lion, in the Ligurian Sea, in the northern Adriatic and in the northern Aegean Sea and it is there where the '*Falkenbergia*' stage of *A. armata* seems to be able to over-summer. In order to complete the life cycle, these tetrasporophytes need short day-lengths (Oza, 1977; Guiry & Dawes, 1992) and temperatures roughly between 17 and 21°C (Guiry & Dawes, 1992; Ni Chualáin *et al.*, 2004). Such conditions are met in autumn all over the western Mediterranean, the Adriatic Sea and the Northern Aegean Sea. The region includes also the aforementioned pockets and, therefore, the only critical factor limiting the distribution of *A. armata* to these northern Mediterranean pockets seems to be a lethal high temperature in summer.

All our Mediterranean specimens of *A. armata*, except one, are from these northern areas (Marseilles, Gulf of Lion), in agreement with records in Sala & Boudouresque (1997). We have no sequence information from specimens from the northern Adriatic but specimens from there belong morphologically to *A. armata* as well (personal communication, A. Falace, Univ. of Trieste). A '*Falkenbergia*' stage of *A. armata* collected at the Strait of Messina (Ni Chualáin *et al.*, 2004; specimen 566) does not fit the model because the local summer sea surface temperature (Lipkin & Safriel, 1971) rises well above the lethal upper temperature of that specimen (Ni Chualáin *et al.*, 2004). The species has never been seen there again after its first observation and collection in 1987 (Barone *et al.*, 2001; personal communication, R. Barone, Univ. of Palermo). It may have been a short-lived founder population that died out the following summer. The first Mediterranean report of *A. armata* in Algeria in 1923 (Feldmann & Feldmann, 1942) appears strange as well due to high summer seawater surface temperatures along southern Mediterranean coasts (http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html; Lipkin & Safriel, 1971). However, along the Algerian coast in particular, summer surface seawater temperatures below 25°C have been recorded (http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html). This would allow the species to survive locally during the summer.

The '*Falkenbergia*' stages of *A. taxiformis* show a critical lower survival limit between 10–13°C and do not grow below (11)–15°C (Ni Chualáin *et al.*, 2004). In the Mediterranean Sea, winter seawater temperatures remain above 13°C except in the Gulf of Lion, the Ligurian Sea, the northern Tyrrhenian, northern Adriatic and the northern Aegean Sea (Lipkin & Safriel, 1971; http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html) and possibly in shallow lagoons elsewhere in the Mediterranean. In order to complete the life cycle, the tetrasporophytes of *A. taxiformis* need short day-lengths (Oza, 1977) and temperatures roughly between 18 and 26°C (Ni Chualáin *et al.*, 2004). Such surface seawater temperature conditions are met anywhere in the Mediterranean in autumn. Even in the aforementioned regions the temperature drops below the reproductive window only from the beginning of November onwards. Therefore, the critical factors keeping *A. taxiformis* outside these regions seem to be a lethal lower temperature in winter.

Indeed, all our Mediterranean specimens of *A. taxiformis* have been collected outside the aforementioned regions. Our specimen from La Spezia (Liguria) seems to represent an exception and might occur at the limits of its distribution. The fact that *A. taxiformis* is the dominant *Asparagopsis* species along most of the Tyrrhenian coast of Italy also fits the expectations.

At this moment both species appear to occur where they are expected to be but that has not always been the case. Since its first record near Alexandria (Delile, 1813), *A. taxiformis* seems to have dispersed slowly throughout the eastern Mediterranean (Dixon, 1964). Funk (1955) reported the '*Falkenbergia*' stage of *A. taxiformis* in the Gulf of Naples during the 1920s but his observation only shows that an *Asparagopsis* species was present at that time. The first unambiguous observations of this species along the Italian coast date from as recently as 2000 (Trapani, Sicily: Barone *et al*., 2001; Procida, Gulf of Naples: D'Archino *et al*., 2003). The species is now common in the Gulf of Naples; its gametophytes and tetrasporophytes cover shallow subtidal turf communities on moderately exposed rocky substrata year-round. Similar westward dispersion has been documented for other macrophytes, e.g. *Halophila stipulacea* (Forsskål) Ascherson (Villari, 1988) while a similar sudden population explosion has been observed in *Caulerpa racemosa* (Forsskål) J. Agardh (Verlaque *et al*., 2003).

Comparison among molecular markers

The three molecular markers reveal different levels of resolution (Table 2). The partial LSU fragment amplified is about twice as long as the *cox*2–3 spacer but it contains about the same number of variable characters and parsimony-informative ones. Thus, our data confirm the observation in Zuccarello & West (2002) that the *cox* marker evolves faster.

The Canarian and Mediterranean *A. taxiformis* clades are well resolved in the *cox* tree whereas in the trees inferred from the RuBisCo and LSU regions the Mediterranean sequences do not form a clade. In the RuBisCo tree the Mediterranean sequences are all the same but in the LSU tree sequences form a grade because the variation among the sequences from the Mediterranean is comparable to the variation between the Mediterranean sequences and the Canary Island ones. As an example, variation in the LSU marker of *A. taxiformis* specimens between Ischia and Posillipo—both in the Gulf of Naples—is as high as among distant Mediterranean samples or between Mediterranean and Canarian sequences.

The high intraspecific variation in the partial LSU rDNA may result from its nuclear nature: it is inherited bi-parentally and affected by recombination processes. Organellar genome markers, in contrast, are strictly clonally inherited. Once daughter populations become genetically isolated, emerging genetic differences can segregate far more rapidly on clonally transmitted genes than on those that undergo recombination processes. Mutations take more generations to be fixed in nuclear genes than in organellar ones due to a larger effective population size of nuclear alleles (Palumbi *et al*., 2001). An extra complication is that rDNA genes occur in at least several hundreds of tandem repeats in each haploid genome (Zimmer *et al*., 1980).

In conclusion, besides the differences in polymorphism and the different levels of resolution revealed by the three markers, all of them are surely suitable for phylogeographic research at a global scale.

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