

**Molecular Phylogeny, Phylogeography and
Population Genetics of the red seaweed genus**

Asparagopsis

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Philosophy

by

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*Che il Mediterraneo sia, quella nave che va da sola,
tra il futuro la poesia, nella scia di quei marinai,
e quell'onda che non smette mai, che il Mediterraneo sia...*

Eugenio Bennato

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THESIS ABSTRACT

The red seaweed genus *Asparagopsis* Montagne (Bonnemaisoniales) was studied with respect to its taxonomy, phylogeny, phylogeography and population genetics. The representatives of this genus, *A. armata* Harvey and *A. taxiformis* (Delile) Trevisan, are notorious invaders. Both species occur worldwide and show disjunct distribution patterns. Such patterns may result from recent jump-dispersal or from fragmentation of once pan-globally distributed species. First, a phylogeographic approach was deployed in order to delineate the taxonomic units in local scale and to assess if European populations of each of the species originated from a single introduction or multiple cryptic ones. Results showed that the two species recognized *A. armata* and *A. taxiformis* are also genetically distinct. *Asparagopsis armata* was found to consist of a single species worldwide, whereas *A. taxiformis* constituted three and probably four morphologically cryptic but genetically distinct lineages. At times, lineages were encountered in sympatry and two of them were detected in the Mediterranean Sea.

In order to confirm distinction between lineages and to assess invasive potential and colonization mechanisms of the species along the western Italian coast, eight nuclear microsatellite markers were identified against the invasive lineage 2 of *A. taxiformis*. The markers cross-hybridised only with lineages 1 and 2. Moreover, it was demonstrated that carpogonia present on many female thalli can affect microsatellite reading patterns because of external (male) allelic contribution. Even after removal of the carpogonia, gametophyte thalli exhibited multiple allelic patterns, which is indicative for polyploidy. The markers were then used to assess genetic structure and diversity within and among Mediterranean populations of *A. taxiformis* lineages 1 and 2. Analyses based on statistics developed for polyploid species showed that the lineage 1-population (HAW) was distinct from Mediterranean lineage 2 populations. A geographically distant Californian lineage 2-population was genetically distinct from the Mediterranean ones as well. The Mediterranean lineage 2-samples showed panmixia. High genotypic diversity, high gene flow, and low differentiation encountered amongst these populations probably are due to a recent invasion of this lineage into the basin.

CHAPTER I - General Introduction

Introduction

Marine animals and macroalgae are generally distributed widely but very few of them if any are truly cosmopolitan. Coral reef organisms for example, are encountered mainly in warm tropical waters whereas kelps dominate communities along temperate rocky shores. Phylogeography, the study of the processes controlling the geographic distribution of genealogical lineages of a single species, seeks to understand the relationship between the evolutionary history of taxa and the distribution patterns of these taxa. In some cases, part of the explanation of distribution patterns is easy because species are often distributed “here and now”. For instance, marine organisms generally do not migrate overland. Yet, there are many cases where such obvious physical barriers are absent, but nevertheless, species do not occur beyond a certain point along the coastline. The most likely reason is that beyond that point, the annual environmental conditions are outside the eco-physiological limits of the species for survival during the unfavourable season, or for growth or reproduction during the favourable season (Breeman 1988).

Historical factors also provide explanations for the present-day distribution patterns of marine organisms. Seaweeds often occur in a region because that is where their ancestors evolved. Moreover, related species may have disjunct, mutually exclusive distribution patterns because of closures of seaways, or climate change fragmentation of their common ancestor's once continuous distribution pattern (*Porphyra* species from the north-east Pacific Ocean, Lindstrom & Fredericq, 2003; north-Atlantic and north-Pacific *Chondrus* species, Lüning *et al.* 1987).

On the other hand, what if the barriers to dispersal are broken or if climate change are reversed? An example is the opening of the Suez Canal permitting range expansion of marine organisms from the Red Sea into the Mediterranean Sea and *vice versa* (Galil and Zenetos 2002; Shefer *et al.* 2004). Range expansion can also result from long-distance dispersal events across regions unfavourable to the existence of the organism. Some of these exotics may arrive by natural means, but intercontinental shipping since the 14th century has increased the chances of dispersal for many marine organisms (Kooistra and Verbruggen 2005). More than ever, in the last century, many exotic species have been introduced in the Mediterranean Sea as hitchhikers in ballast water, and as epiphytes on ship hulls and exotic mariculture organisms (Boudouresque and Verlaque, 2002). Successful long-range dispersal leads to the establishment of a founder population that will expand its distribution range in the recipient region until it meets its limits set by geographical barriers or by its own eco-physiological constraints. Invasions of such exotic species often perturb the regional ecology because of the local biota's initial inability to keep the growth and reproduction of the invader in check. Such undesirable accumulations of biomass can cause also economic damage if the invader affects fisheries and mariculture. Introduction of invasive species represent major threats to the native ones in both the aquatic and terrestrial environments. Together with habitat loss, fragmentation, hunting and pollution some introductions may have catastrophic effects on biodiversity richness and its conservation. In fact, of recent species extinctions for which there is a known cause, 39% have been attributable to the effects of introduced species (Groombridge 1992).

Paradoxically, introduced species keep the composition of the marine flora and fauna in a given region perpetually in flux. In some cases, change can actually be followed,

e.g., the invasion of an exotic species (e.g. *Caulerpa taxifolia*, Jousson *et al.* 2000). However, the explanations for observed distribution patterns are not always that obvious and in addition, time scales at which changes occur are variable. As an alternative, the reconstruction of the phylogeographic relationships is needed to explain what processes most likely gave rise to these patterns.

Phylogeographic relationships among genealogical lineages within species can be studied using sequence data. DNA-marker-regions selected appropriate to the research questions contain a multitude of phylogenetically informative sites, and the information can be evaluated easily based on four discrete character states possible for each site. In addition, a suite of methods is available for phylogeny reconstruction of sequence data. However, these markers generally evolve too slowly to uncover processes that shape populations within one and the same biological species. Population genetic markers such as microsatellites evolve much faster, even to such an extent that they can discriminate between individuals. They also allow estimation of in- and out-breeding levels in sexually reproducing populations, and assess the intensity and directionality of gene flow among populations. With respect to invasive species, population genetic markers can discriminate between founder and donor populations because the former are expected to contain sub-samples of the genetic diversity of the donor population.

The genus Asparagopsis

Red algae (Rhodophyta) constitute one of the major radiations of Eukaryotes with *circa* 6000 species known (Cole and Sheath, 1990; Saunders and Hommersand, 2004). They represent an excellent model for testing evolutionary hypotheses: they comprise a

morphologically heterogeneous group of uni- and multi-cellular marine and freshwater organisms. Some ancestral lineages (*Cyanidium* - adapted to extremophilic habitats) are of controversial taxonomic position (Ciniglia *et al.* 2004). Moreover, ancient representatives have become endosymbionts giving rise to Cryptophytes, Haptophytes and Heterokontophytes (Bhattacharya *et al.* 2004). Among the great controversies in the evolution of these lineages is how many endosymbiosis events occurred and when (Yoon *et al.* 2002, Su *et al.* 2004, Bhattacharya *et al.* 2004). Within the red algae proper, two subclasses are recognized: *Florideophyceae* and *Bangiophyceae* and fundamentally two life history strategies have been described: a triphasic life cycle and a biphasic one (Oliveira & Bhattacharya 2000). Yet, current hypotheses of their evolution and classification inferred from anatomical, life history, and molecular data show disagreement (Freshwater *et al.* 1994).

The target of this PhD-thesis is the red seaweed genus *Asparagopsis* (Bonnemaisoniales). The genus has been studied extensively with respect to its cytology (Svedelius 1933), sexual reproduction (Chihara 1962), and morphology (Bonin and Hawkes 1987). Recently, numerous chemical compounds have been described, which are now being screened for their biomedical applicability (Catayee *et al.* 1980; Caporiccio *et al.* 1984; Haslin *et al.* 2001; Gonzalez del Val *et al.* 2001). The genus consists of two conventional taxonomic units (Fig. 1.1): *A. taxiformis* (Delile) Trevisan and *A. armata* Harvey. *Asparagopsis taxiformis* was first reported at Alexandria (Delile 1813) and *A. armata* was described based on material obtained from Western Australia (Harvey 1855).

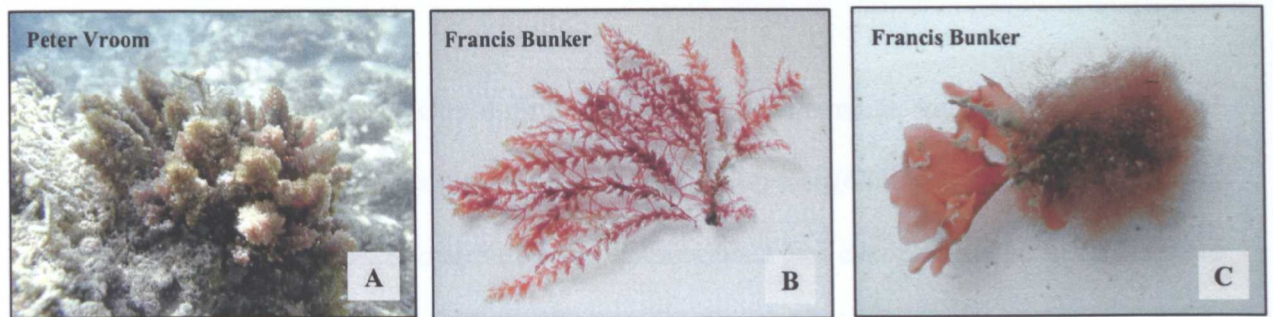


Fig. 1.1. A, *Asparagopsis taxiformis*, B, *Asparagopsis armata*, C, the filamentous “*Falkenbergia*” phase.

Like many other red seaweeds, *Asparagopsis* has a diplo-haplontic life cycle with three stages (Fig. 1.2): gametophytes, carposporophytes, and tetrasporophytes (Chihara 1962; Bonin and Hawkes 1987). The macroscopic gametophytic “*Asparagopsis*-stage” is the morphologically most complex of the three stages, consisting of a rhizoidal part from which several erect, polysiphonous stems arise. These ramify over and over again into trisiphonous ramuli, thus defining the ultimate thallus. The gametophytes produce male gametes on antheridia and female gametes in carpogonia. Male gametes are released in the water column, and if captured by carpogonia, they fuse with female gametes. In Bonnemaisoniales and Ceramiales, spermatangial structures are formed in small patches from surface cortical cells. The procarp is formed within the meristem apically on leading cortical filaments. It consists of a supporting cell and a curved 2-celled carpogonial branch (Cole and Sheath, 1990; Kylin, 1916). A small tuft of nutritive filaments is produced from this supporting (hypogynous) cell before fertilization. After fertilization, the nuclei of all of the cells of the carpogonial branch apparatus enlarge, the carpogonium fuses with the hypogynous cell and the nutritive cells are progressively incorporated into the fusion cell. The supporting cell produces secondary assimilatory filaments and the nuclei in the basal cells of these filaments also enlarge, nearly filling each cell (Cole and Sheath, 1990; Kylin, 1916). Pit connections expand between neighbouring vegetative cells in the axis and basal

cells of the developing pericarp during gonimoblast development, followed by fusions that extend into the vegetative axis as the carposporangia mature (Cole and Sheath, 1990; Kylin, 1916). In *Bonnemaisonia hamifera*, young gonimoblast and candelabra-like fused nutritive filaments surrounded by a pericarp have been observed (Cole and Sheath, 1990; Kylin, 1916). In Gigartinales (*Chondrus crispus* Stackhouse), after presumed fertilization, the carpogonium fuses directly with the supporting cell and deposits the diploid nucleus. After diploidization, the supporting cell continues to enlarge and becomes sub-spherical and filled with many nuclei. The functional supporting cells form numerous enucleate protrusions from the cell surface. They contain a mixture of large and small nuclei. The large nuclei correspond to the haploid vegetative nuclei, originally present in the supporting cell prior to the diploidization. The smaller nuclei, presumably diploid, migrate into the enucleate protrusions and are cut off, one in each gonimoblast initial (Cole and Sheath, 1990; Fredericq *et al.*, 1992).

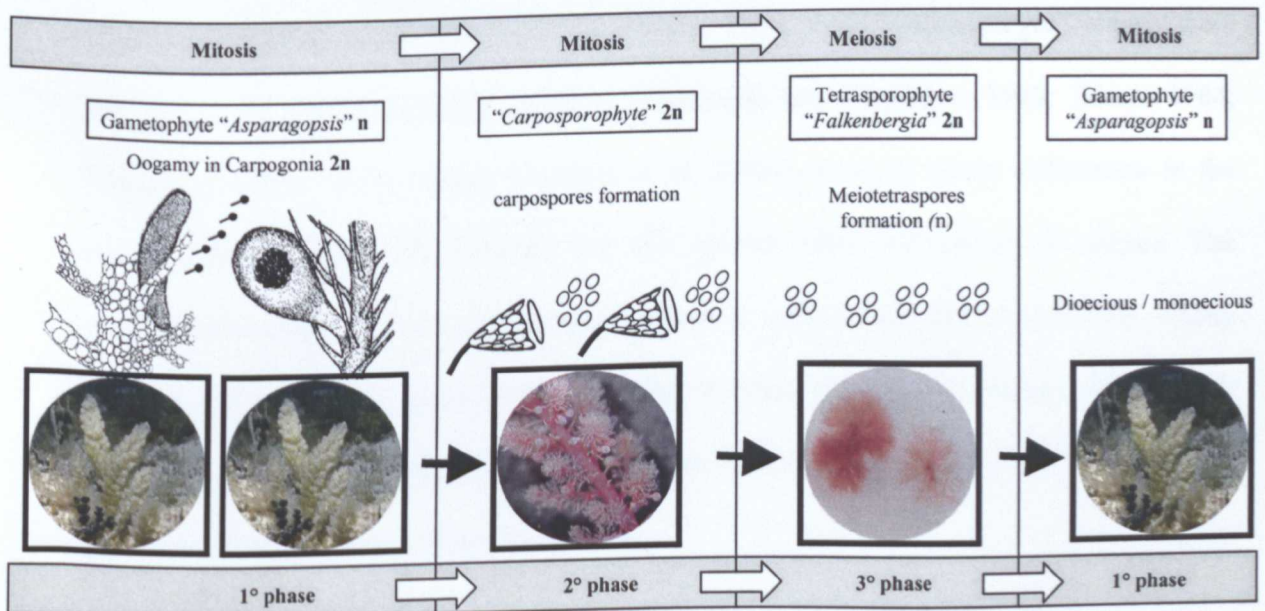


Fig.1.2. The triphasic, heteromorphic, haplo-diplondic life-cycle of *Asparagopsis taxiformis* and *A. armata* (Feldmann and Mazoyer, 1937; Feldmann and Feldmann, 1939a, b, 1942).

The carposporophyte, remains microscopically small and stays attached to the female gametophyte. The carposporophytes in their turn produce carpospores, which are released in the water column, and upon settlement on other macroalgae, develop into tetrasporophytes called "*Falkenbergia*-stage." The latter look like fluffy pom-poms consisting of ramifying tri-siphonal filaments. The tetrasporophytes in their turn form tetraspores, which result from meiosis. These tetraspores are released into the water column and upon settlement on the substratum develop into a new generation of gametophytes (Feldmann and Mazoyer 1937; Feldmann and Feldmann 1939a, b, 1942).

Both *A. armata* and *A. taxiformis* include the "*Falkenbergia*-stage" J. et G. Feldmann (Feldman and Feldman 1939a, 1942) in their life history (Fig. 1.1c), which was considered to be an entirely different species until Feldmann and Feldmann (1939a, b, 1942) clarified the life cycle of *Asparagopsis. Falkenbergia rufolanosa* (Harvey) Schmitz was the original description of the tetrasporophyte of *A. armata* and *F. hillebrandii* (Bornet) Falkenberg was that of *A. taxiformis*. These two "*Falkenbergia*" stages were thought to be morphologically identical (Feldmann and Feldmann 1942; Dixon 1964; Dixon and Irvine 1977), though Chualáin *et al.* (2004) observed subtle differences in the sizes of the terminal cells between the two species when maintained in culture. The *Asparagopsis* stages are morphologically distinct, *A. armata* has spinose branches, highly elongate erect branches, and a sprawling habit in which the spines entangle among other benthic organisms. *Asparagopsis taxiformis* has a more compact rhizoidal system, lacks spiny branches, and forms more patchy tufts.

Since distribution data of the two *Asparagopsis* species (Fig. 1.3) have been based also on records of *Falkenbergia*, they may include erroneous records (Funk 1955; Diapoulis and Verlaque 1981; Athanasiadis 1987; for background information on species distribution, see Bonin and Hawkes 1987; Ní Chualáin *et al.* 2004). Dixon (1964) evaluated several reports on the two species and argued that additional errors may result from misidentification of *A. taxiformis* and *A. armata*.

Asparagopsis taxiformis occurs on reefs and rocky shores throughout the tropical and warm-temperate parts of the Atlantic and Indo-Pacific (Harvey 1855; Abbott and Williamson 1974; Price *et al.* 1986; Bonin and Hawkes 1987; Silva *et al.* 1996).

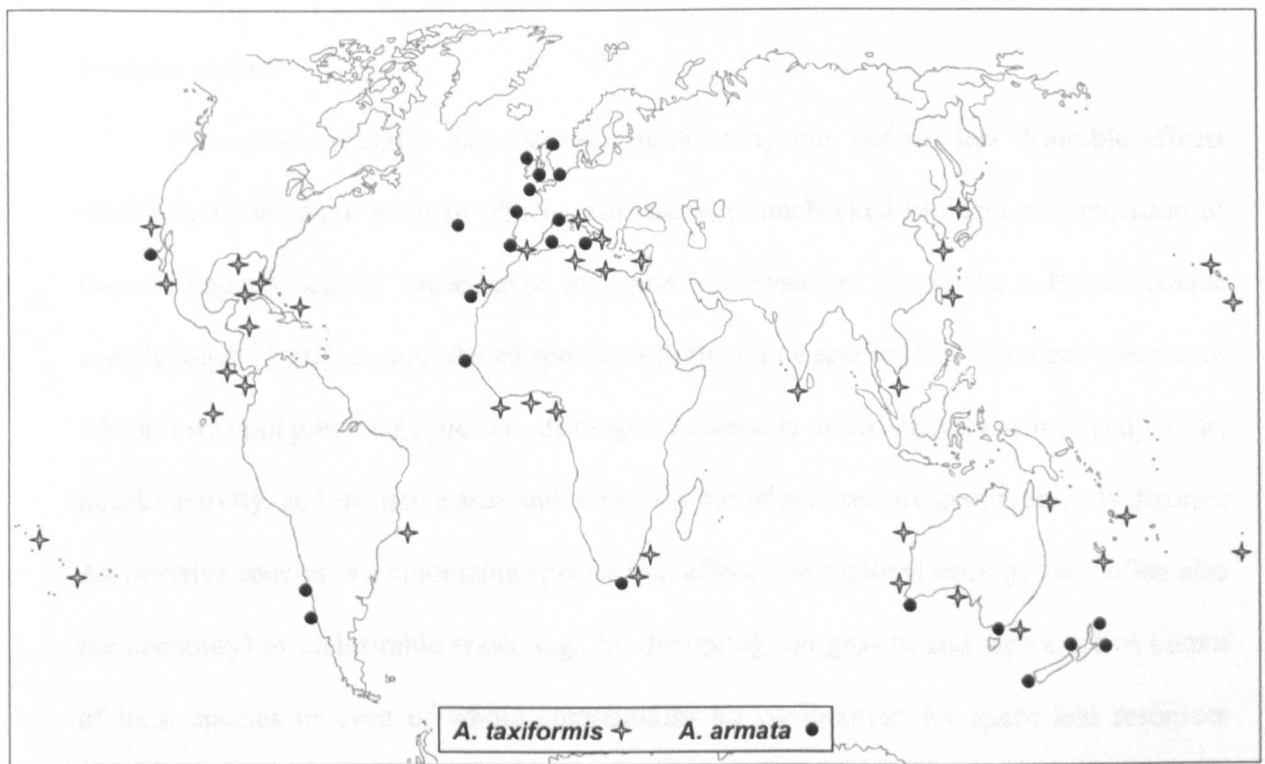


Fig 1.3. World-wide distribution of *A. taxiformis* and *A. armata* based on gametophytes and tetrasporophytes reported in Bonin and Hawkes (1987), Guiry and Dawes (1992), Ní Chualáin *et al.* (2004), this thesis.

Asparagopsis armata seems to be a temperate species. It is native to southern Australia and New Zealand, but is found also along the Chilean coast, in South Africa, California, and in the northern Atlantic (the British Isles, Atlantic France, Spain and Portugal, the Azores, Canary Islands, Salvage Islands, and Senegal (Horridge 1951; Dixon and Irvine 1977; Price *et al.* 1986). In the Mediterranean Sea, *A. armata* is encountered in the western basin (South and Tittley 1986; Sala and Boudouresque 1997), whereas *A. taxiformis* seems to be confined to the eastern Mediterranean (South and Tittley 1986; Sala and Boudouresque 1997). The distribution ranges of the two species appear to overlap along the Italian shores (Barone *et al.* 2001; D'Archino *et al.* 2003; Furnari *et al.* 2003).

Invasive species

Biological invasions may increase biodiversity but various less desirable effects often counteract these positive effects. For instance, unchecked biomass accumulation of the invading species can cause havoc in regional ecosystems. According to Boudouresque and Verlaque (2002), an introduced species is defined as a species that colonizes a new area where it was not previously present, its range extension is linked directly or indirectly to the human activity, and its native area and the newly colonised area are geographically distinct. An invasive species is a colonizing species that affects the regional ecology (and often also the economy) in undesirable ways, e.g., by disrupting the growth and reproductive output of local species or even of whole communities by competition for space and resources (niche displacement, competitive exclusion, or predation) and by genetic competition (hybridization, introgression). Invasions of exotic species have lead in several cases to the

extinction of numerous local species (Mooney and Cleland 2001). Fifty thousand introduced species are believed to exist in the United States alone, where their competitive aptitude promoted at least 958 species to a place on the endangered species list (Costello and Solow 2002). A large number of marine species has been introduced along the European coastline over the last 20 years; many among these are seaweeds (Boudouresque *et al.* 1994).

Both, the actual number of invaders and their impact are underestimated because of the existence of morphologically similar cryptic and/or sibling species (species nearly indistinguishable morphologically but nonetheless reproductively isolated from one another, often thought to be the result of fairly recent differentiation; Lincoln *et al.* 1998). Many recent phylogeographic studies using molecular genetic techniques have demonstrated that many so-called widely distributed taxa, especially those exhibiting disjunctions, are composed of biologically and genetically distinct but morphologically cryptic or pseudo-cryptic species (Gabrielsen *et al.* 2003; Müller *et al.* 2005; Verbruggen *et al.* in press; this thesis). Each of these newly recognized taxa may be widely distributed in their own right but generally have more restricted ranges than the species complex as a whole (Wattier and Maggs 2001, 2003, Gabrielsen *et al.* 2003; Müller *et al.* 2005; Verbruggen *et al.* in press). Many of these phylogeographic studies also detected several cases of cryptic invasions. The invaders were phylogenetically distinct and reproductively isolated from their native look-alikes. In addition, without the use of molecular genetic techniques, it is difficult to detect whether an invasion results from a single introduction of from multiple introductions from different sources (McIvor *et al.* 2001; Saltonstall 2002; this thesis).

Coastal biota are linked to and affected by aquaculture, tourism and fisheries. Thus, any alterations in the local biota by invasive species also affect the economy. Therefore, multidisciplinary research on an international scale is needed to understand these invasions and provide insights that can lead to better management and prevention. From 2002 to 2005, the European Community funded the international, multidisciplinary “ALIENS” project, (Algal Introductions to European Shores). The cooperation included 5 different partners: Universidad de Oviedo (Spain), Queen’s University of Belfast (United Kingdom), Centro de Siências do Mar, Faro (Portugal), the Université de Marseille (France), and the Stazione Zoologica A. Dohrn, Naples, (Italy). The research objectives were a) identification of ecological causes underlying the success of invasive seaweeds along European shores, b) estimation of the level of alien introductions and evaluation of ecosystem susceptibility to future invasions and, c) assessment of genetic variability and phylogeographic patterns in different spatial scales of target invasive species in order to estimate persistence and expansion capabilities of the populations and, eventually, uncover cryptic invasions (Guala *et al.* 2003).

The Mediterranean Basin

The Mediterranean Sea is connected with the Atlantic Ocean through the Strait of Gibraltar and with the Indian Ocean through the Red Sea and the Suez Canal. Despite its modest surface (0.82% of the world’s oceans) and volume (0.32%), it contains more than 8500 marine macro-algal and animal species (an estimated 4-18% of marine species worldwide) (Spanier and Galil 1991; Bianchi and Morri 2000). Many of these are considered Mediterranean endemics (Bianchi and Morri 2000). This rich biota may result from its

geological history, a series of unique ecological and climatic conditions, and anthropogenic activities (Spanier and Galil 1991; Bianchi and Morri 2000; Galil and Zenetos 2002).

The Mediterranean Basin is a remnant of the late Tethys, a tropical ocean that once connected the Atlantic and Indo-Pacific Ocean and divided Pangea into two supercontinents: Laurasia and Gondwana (*ca.* 200 Ma). During the Miocene (*ca.* 10 Ma), sea-floor spreading pushed Africa and Eurasia against one another thereby severing the connection between the Mediterranean and the Indian Ocean through what is now Syria and the Mesopotamian basin. At the end of Miocene (*ca.* 6 Ma) the connection with the Atlantic was closed and, consequently, the Mediterranean Sea evaporated into a salt desert with a few hyper-saline lakes. This period is referred to as the Messinian salinity crisis and it led to the demise of the Tethyan biota in the basin. The re-opening of the Strait of Gibraltar in late Pliocene (*ca.* 5 Ma) repopulated the Mediterranean with a biota of Atlantic provenance. The alternation of cool and warm periods during the Pleistocene (*ca.* 2 Ma) must have affected the Mediterranean biota in the sense that many cool-temperate species may be remnants from the latest Pleistocenic cool period whereas many species that are also found in the Caribbean may have immigrated during the current interglacial period, which commenced about 10,000 year ago (Spanier and Galil 1991; Bianchi and Morri 2000; Galil and Zenetos 2002). The most recent geographical change affecting the biota of the Mediterranean Sea was the opening of the Suez Canal in 1869. Not surprisingly, this event has resulted in the entry to over 300 species originating from the Red Sea and the Indo-Pacific Ocean (Lessepsian immigrants), and their settlement, mainly in the warmer Eastern part of the basin (Spanier and Galil 1991; Bianchi and Morri 2000; Galil and Zenetos 2002).

The history of Asparagopsis in the Mediterranean

Boudouresque and Verlaque (2002) reported that 85 marine macrophytes have probably been introduced into the Mediterranean Sea, nine of which show invasive behaviour, including *Asparagopsis taxiformis* and *A. armata*. The former species was first collected near Alexandria, Egypt (Delile 1813), which is also the type locality of the species. It appears as if *A. taxiformis* is a Mediterranean native because it was detected there prior to the opening of the Canal. But if that is so, then it needs to be explained why it behaved so unobtrusively until recently when it suddenly expanded its range towards the Northwest, showing invasive behaviour (Boudouresque and Verlaque 2002). Such a peculiar behaviour may result from a cryptic invasion. *Asparagopsis armata* was first reported in Algeria in 1923 (Feldmann and Feldmann 1942). Today, this species abounds along the French, northern Spanish and northern Adriatic shores of the Mediterranean, and shows invasive behaviour and great biomass production (Boudouresque and Verlaque 2002).

Molecular systematics and phylogeography

Molecular systematics infers phylogenies (genealogical histories) of a series of taxa that can be illustrated as trees. Trees describe differences among particular DNA sequence regions called markers. Tree reconstructions can include phylogeographic data all the way from the eukaryotic crown groups down to the level of genetically distinct but morphologically cryptic species depending on the DNA-markers used. Phylogenies at higher taxonomic levels need to be inferred from slowly evolving markers such as highly

conserved gene regions whereas those near the species level require fast evolving markers such as introns, spacers, pseudo-genes, or regions without functional constraints.

Macro-algal phylogenies have been particularly fruitful, although mainly inferred from a limited number of DNA regions such as the plastid *rbcL* gene and spacer, the mitochondrial *cox2-3* spacer or the nuclear ribosomal DNA and spacers (Wattier and Maggs, 2001; 2003). At higher taxonomic levels molecular phylogenies inferred from the above markers are able to clarify relationships among groups whose morphology did not provide clear genealogical insights. Near the species-level, the lack of available markers some times has been particularly problematic and only recently, universal primers for the amplification of coding and non coding regions of the chloroplast genome of red and green algae have been developed (Provan *et al.* 2004). Molecular phylogenetic approaches however, have identified cryptic and semi-cryptic species where phenotypic plasticity and lack of morphological characters plagued biologically meaningful circumscription of taxa (Guiry 1992; van Oppen *et al.* 1996). Moreover, these molecular methods are useful for the detection of cryptic invasions [e.g., cryptic *Phragmites australis* (Saltonstall 2002), invasive *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman *et* Boudouresque, (Verlaque *et al.* 2003, 2004), *Asparagopsis*, this thesis].

Phylogeography involves tracking species genealogies over the geographic landscape (Arbogast and Kenagy 2001). Molecular phylogenies are inferred from a series of geographically defined specimens, but taxon names at the end tips of the inferred tree are replaced with names of collection sites. If geographic groupings are recovered in the reconstructed phylogeny, then these patterns are compared with hypotheses about the geological history of the region. For example, let us consider a phylogeny of a group of

marine benthic species and each species contains several clades in which one containing Atlantic sites is sister to another with exclusively Indo-Pacific sites. We can assume a common cause of this repetitive pattern, namely vicariance of once circum-tropical species into disjunct Atlantic and Indo-Pacific daughters. Ancient and modern dispersal events can also be elucidated from tree topologies. For instance, if an Atlantic clade is embedded in an Indo-Pacific grade, then the Atlantic clade probably arose through a dispersal event from the Indo-Pacific into the Atlantic.

Molecular phylogenies can also reveal short periods of rapid diversification, which appear in the phylogenetic reconstruction as unresolved polytomies. If the diversification co-occurs with the acquisition of evolutionary novelties and/or occupation of novel niches, then we speak of adaptive radiation. Molecular phylogenies can also be useful for the estimation of the evolutionary age of lineages. In the so-called molecular clock approach, several clades in a phylogeny are dated because they can be linked to vicariant events or first appearances of taxa in the fossil record. The remainder of the clades can then be dated as well by means of extra- or interpolation.

Phylogenetic analysis markers

Reconstructions of genealogies are preferentially obtained from multiple genetic markers because phylogenies based on single sequence markers may not always accurately reflect species genealogies. Moreover, they are generated preferentially with markers from different compartments of the genome (the nucleus, the mitochondria, and, in plants, the plastids). In the thesis presented here, 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 enzyme (Maggs *et al.* 1992) and the mitochondrial cytochrome oxidase subunit 2 - subunit 3 (*cox 2-3*) spacer (Zuccarello *et al.* 1999) have been selected. These three regions are known to evolve rapidly and to be suitable for phylogenetic inference near the species-level. They have been selected from the nuclear, plastid and mitochondrial genomes in order to obtain independent phylogenetic information and take advantage of the distinct characteristics and inheritance modalities of each of the three genomes.

Nucleotide sequences of the ribosomal DNA (rDNA) genes provide a set of conserved as well as hyper-variable regions. These regions exhibit mutation rates suitable for inferring phylogenetic relationships over several taxonomic levels. For example, the nuclear small subunit rDNA gene sequence evolves relatively slowly and it is useful to infer phylogenies among distantly related organisms. The mitochondrial 16S rRNA evolves more rapidly and may be useful at the ordinal and family level while the nuclear ITS1, ITS2 and IGS (Internal transcribed spacer 1, 2 and intergenic spacer) is the fastest evolving region and may vary significantly at the intra-generic and intra-specific level. The nuclear large subunit rDNA gene fragment spanning the “D1,” “D2” and “D3” hyper-variable domains (Lenaers *et al.* 1989) for example was found to be suitable at the species level in dinoflagellate and diatom phylogenies.

The LSU rDNA gene region used in the present study is approximately twice as long as the chloroplast and the mitochondrial regions but exhibits a lower mutation rate. Nevertheless, a single synapomorphy is enough to provide statistical support for a clade

(Hare 2001). The nuclear large subunit ribosomal DNA (LSU-rDNA) gene is inherited biparentally and is affected by recombination processes whereas the plastidial and mitochondrial markers are uniparentally (maternally) inherited in red algae, as in plant and animal species (Coyer *et al.* 2002; William *et al.* 2004). Although the LSU gene is organised in clusters of hundreds to thousands of tandem repeats, all of its copies should remain similar due to the homogenization effect of concerted evolution (Zimmer *et al.* 1980). Nonetheless, intra and inter-individual variability has been found to be common in algae, higher plants and animals (Famà *et al.* 2000; Harris and Crandall 2000; Gandolfi *et al.* 2001). Reasons are manifold, e.g., partial homogenization, polyploidy, ancestral polymorphism, and hybridization.

In red algae, the chloroplast spacer between the large and small subunits of the ribulose-1-5-bisphosphate carboxylase/oxygenase enzyme (Maggs *et al.* 1992) and the mitochondrial cytochrome oxidase subunit 2 - subunit 3 (*cox 2-3*) spacer (Zuccarello *et al.* 1999) are found to be informative within species and populations. Both sequences span intergenic spacers anchored in conserved gene domains and vary in length from species to species. Molecular data from organellar genomes have proved to be extremely useful in evolutionary studies because these genomes are uni-parentally inherited, exhibit no crossing over and display neutral mutation rates different from those encountered at the nuclear DNA. Chloroplast DNA is extensively used in plant phylogeny and in some cases its intraspecific variation is sufficient even for the estimation of gene flow among populations (McCauley 1995). In animals, mitochondrial DNA is the tool of choice for phylogenetic inferences at different taxonomic levels. The chloroplast and mitochondrion

genomes are haploid and, therefore have a reduced effective population size. Moreover, contrary to the state of affairs in higher plants, algal mitochondrial DNA exhibits an elevated mutation rate. Moreover it is believed to accumulate synonymous substitutions several times faster than the plastid algal DNA (Wolfe 1996; Ballard 2004).

Population genetics markers

Microsatellites or simple sequence repeats (SSRs) are highly mutable loci distributed throughout the genome (Ellegren, 2000a, 2000b; Sibly *et al.* 2001, 2003).

The popularity of microsatellite DNA markers is not unexpected considering the following features: they are single locus, co-dominant, highly polymorphic, with respect to the number of simple repetitive sequences, and they produce reliable and reproducible results. Microsatellites follow Mendelian inheritance laws and simple models of evolution. The flanking regions of the microsatellites are selected among the highly conserved and unique, in order to allow the design of highly specific primer pairs (Weber and May 1989). A suitable number of loci and the homogeneous distribution of the genetic variability in the population is required and it is critical in order to correctly estimate population genetic parameters (Kimura and Ohta 1973). Microsatellite markers are considered as the markers of choice in studies focusing on population-genetic structure, test for parentage and relatedness, exploration of levels and patterns of genetic diversity within and among populations, phylogeographic patterns and, discovering of recent evolutionary histories of populations (Zhang and Hewitt 2003). Yet, evolutionary relationships among microsatellite allelic frequencies are not without problems. It is now accepted that differences in allele

size result from slipped-strand mispairing events, leading to the deletion or insertion of repeat units during mitosis. However, these events may not be related directly to evolutionary divergences among phylogenetic lineages and populations (Eisen 1999). Studies addressing the directionality of the microsatellite mutation process reported a bias toward addition of repeats suggesting that these markers have the tendency to expand. In fact, examples of microsatellites exhibiting a deletion bias are rare and are limited to a few loci in multilocus studies (for a review see Weetman *et al.* 2002). What is neither entirely clear, is their neutrality, their homoplasmy level, and their constancy of mutation rates. Moreover, differences in alleles length can be produced by variation in number of repeats, base variation within the repeated region, and by insertions and deletions in the flanking region (Zhang and Hewitt 2003).

Issues that can be addressed in *Asparagopsis* species using microsatellites are a) the genetic contribution of haploid and diploid phases, b) the effect of clonal and sexual reproduction in populations, c) the influence of the generation time and of the distinct life stages to the population genetics structure. Microsatellites have not been deployed often in macroalgal population genetic studies because they are apparently less abundant and/or less polymorphic compared to the ones isolated from animal and plant species (Olsen *et al.* 2002; Wang *et al.* 1994, this thesis). Thus in algae, they may require a considerable amount of time and economic resources to identify and standardize them (Zane *et al.* 2002; Squirrell *et al.* 2003). Furthermore, relevant scoring problems are encountered when satellites are tested in populations of species with haplo-diplondic isomorphic (haploid and diploid phases, morphologically indistinguishable) life strategies (Van der Strate *et al.* 2002).

Analysis of microsatellite loci is based on the estimated genetic variability among populations gathered from the computation of genetic distances between individuals of populations. The distance calculations are usually based on the proportion of shared alleles, they generally follow the infinite allele model, they assume independence of the allelic variants and they ignore mutational processes thus including homoplasy in the calculation. Alternative analysis strategies imply that distance calculations are based on the stepwise mutation model. This model considers that small rather than large changes may occur in the number of microsatellite repeats. However, the distance calculation methodologies are mainly developed for diploid organisms and hence are not suitable for species with different ploidy level (Bruvo *et al.* 2004). Different procedures have been proposed to calculate relative distances between microsatellite genotypes that specifically permit analysis of polyploid species and take under account the coexistence of individuals with different ploidy levels in the same matrix (Samadi *et al.* 1999; Zhang *et al.* 1999; Espinosa and Noor 2002; Bruno *et al.* 2004).

Use of the markers

In the present study, the three aforementioned phylogenetic analysis markers were used to infer phylogenies from the same set of geographic samples. The phylogenies were then compared with each other to search for possible topological incongruences. Then, the phylogenies were used to test the taxonomic delineation of the two species within the genus *Asparagopsis*, and to identify cryptic diversity in *A. taxiformis*. The trees were used also to determine how many independent cryptic species of *A. taxiformis* have invaded the

Mediterranean Sea and where these invaders came from. Molecular clock methods were deployed to estimate the age of dichotomies in the tree topologies.

The nuclear microsatellite loci developed in this study were treated as dominant markers and the obtained multilocus genotypes were compared using an index of similarity based on shared allelic variants, developed for DNA fingerprinting (Lynch 1990). The resulting distance matrixes were then used to analyse clonal diversity, population genetics parameters, persistence and expansion capabilities of populations and finally draw trees and networks underlying genetic relationships between and among populations and individuals.

Research strategy

Asparagopsis taxiformis and *A. armata* are believed to be introduced in the basin and to have originated from the Indo-Pacific Ocean. Both show invasive behaviour; they expand rapidly and compete with local biota. Their distribution range appears to overlap but records are potentially unreliable because their taxonomic status is not entirely clear and reports are based often on morphologically identical “*Falkenbergia*” phases.

In **Chapter II**, sequences from nuclear, plastid and mitochondrial molecular markers and macroscopic morphological characters are compared from Mediterranean gametophytes and tetrasporophytes of *Asparagopsis* spp. in order to a) delineate the taxonomic units within the genus, b) assess whether “*Falkenbergia*” and “*Asparagopsis*” from the same collection site belong to the same species and c) uncover phylogeographic structuring within *A. taxiformis* and *A. armata* at a local scale. A few extra-Mediterranean specimens have been included to identify possible source regions and the extent of geographic variation of the species.

In Chapter III, geographic samples have been obtained from throughout the distribution range of the genus to uncover sequence differences and phylogeographic structure using the same molecular markers as in Chapter II. Contrasting intra-specific phylogeographic patterns, inferred by means of median joining networks, have been found within the two species. Incongruences among the three distinct topologies are explained and a molecular clock approach is applied to estimate genome-specific substitution rates and the minimum age of the cryptic lineages recovered within *A. taxiformis*. Four cryptic lineages were defined in *A. taxiformis*.

Chapter IV, deals with the development and use of population genetic markers in *A. taxiformis* cryptic lineage two. Because the life-cycle in *A. taxiformis* exhibits phases with different ploidy levels and because fecund carpogonia were found in almost all individuals analysed, I evaluated whether external genetic signals in mature (fertilized) carpogonia affect the genetic patterns of female gametophyte thalli. The results demonstrate that *A. taxiformis* is polyploid. Recommendations are made for future population genetics studies of algal species with similar life cycles.

In Chapter V, the microsatellite markers developed against the invasive Indo-Pacific Mediterranean lineage 2 are used to: a) reveal genetic structure and levels of gene flow between the later and the Into-Pacific lineage 1 in which microsatellites found to cross-hybridise, b) uncover intra-lineage genetic variability in spatial scale c) calculate gene-flow levels among populations within lineage 2 and d) predict dispersal capabilities of the species, as well as intensity and success of colonization of new areas

**CHAPTER II - *Asparagopsis taxiformis* and *Asparagopsis armata*
(Bonnemaisoniales, Rhodophyta): genetic and morphological
identification of Mediterranean populations¹**

Abstract

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 are 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. I 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 *cox2-3 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

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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 my data.

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 and Irvine 1977; Bonin and Hawkes 1987). *Asparagopsis armata* possesses long hooked stolons (Bonin and 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 and Feldmann 1939, 1942; Chihara 1961, 1962). The epiphytic tetrasporophytic "*Falkenbergia*" stage is composed entirely of densely ramified filaments consisting of three cell rows. Feldmann and 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 and 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 Zealand (Horridge 1951) and is now found from the British Isles, the Canary and Salvage Islands to Senegal as well (Dixon and 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 and Williamson 1974; Price *et al.* 1986; Bonin and Hawkes 1987; Silva *et al.* 1996). Both species are considered introduced in the Mediterranean Sea (Boudouresque and Verlaque 2002). *Asparagopsis armata* was first reported from Algeria in 1923 (Feldmann and 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 and Verlaque (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 and Tittley 1986; Sala and Boudouresque 1997) where it is regarded as invasive (Boudouresque and Verlaque 2002). *Asparagopsis taxiformis* seems to be confined to the eastern Mediterranean (South and Tittley 1986; Sala and 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 and 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 and Maggs 2001; Kooistra *et al.* 2002; Gabrielson *et al.* 2003; Zuccarello *et al.* 2002, 2003).

In the present study I 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 (*cox2-3*) spacer (Zuccarello *et al.* 1999).

Materials and Methods

Sample collection and preservation

Specimens were collected from several sites along the Mediterranean and Atlantic coasts of Europe as well as from the Canary Islands (see Table 2.1 and Fig. 2.1 for details). From some 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 and Hawkes (1987). Only the gross morphology of the gametophytes was examined in light microscopy.

Sample site	Sample n°	Life stage	Collection date	LSU rDNA	RuBisCo spacer	cox spacer
<i>Asparagopsis taxiformis</i>						
Spain	Tenerife, Canary Is.	G	30-05-2002	AY589540	AY589559	AY589524
Italy	Lerici, La Spezia	T	16-07-1993	AY589545		AY589537
	Elba	T	25-08-2003			
		G	15-06-2002			
	Civitavecchia, Roma	T	30-06-1993	AY589546		
		T	28-01-2003			
	P.ta S. Pietro, Ischia, Naples				AY589542	AY589558
		G	15-06-2002		AY589541	AY589527
	Sant'Anna, Ischia, Naples				AY589543	AY589528
		G	19-06-2002			AY589529
	P.ta S. Pietro, Ischia, Naples				AY589544	AY589526
		G	22-06-2002			AY589531
	Sant'Angelo, Ischia, Naples					
		G	28-07-2002			
	Castello, Ischia, Naples					
	G	not available				
Ischia, Naples	E760	G	04-07-2002			AY589530
Capri, Naples	263,264***	G				
	24*,30			AY589539		
Mergellina, Naples	10***,21	G	19-04-2002	AY589538		AY589525
	26*			AY589547		
Capo Posillipo, Naples	383***	G	20-08-2002			AY589535
	391***					AY589536
Strait of Messina, Sicily	1030	T	16-11-1992			
	340***					AY589532
Trapani, Sicily	358***	G	22/07/2002	AY589549		AY589534
	348***					AY589533
Tunisia	Mahdia	G	06-06-2003	AY589548		
<i>Asparagopsis armata</i>						
Ireland	Ard Bay, Galway	G	17-07-2002	AY589556		AY589522
Spain	Oviedo	T	08-09-2002			
		G	11-07-2002			AY589523
	Novellana	G	12-07-2002			
	Percebera	G				

Portugal	Praia Cascielo, Albufeira	200,214,223*,233,254			AY589552
		228*	G	05-06-2002	AY589550
		240*			AY589551
France	South Marseille	124***,130,140*	G	15-05-2002	AY589553
	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*			AY589554
Italy	Messina, Sicily	566***	T	04-06-1987	AY589557
Australia	Sydney	E761,E762	G	not available	AY589560

Table 2.1. List of specimens of gametophytes (G) and tetrasporophytes (T) of *A. taxiformis* and *A. armata* used in the phylogenetic analysis. GenBank Accession Numbers have been assigned to only one sample per distinct sequence. Reference sequences for GenBank Accession Number are indicated with * (LSU rDNA), ** (RuBisCo spacer) and *** (cox spacer).

DNA extraction and purification

About 100 mg silica gel-dried tissue was ground in liquid nitrogen and added to 700 μ l 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 minutes. 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 μ l 70% v/v ethanol, centrifuged 10 min and, after decanting the ethanol, allowed to dry in air. DNA pellets were dissolved overnight in 50 μ l of sterile water. Quantity and quality of DNA were examined by means of 1% agarose TAE buffer gel electrophoresis against known standards.

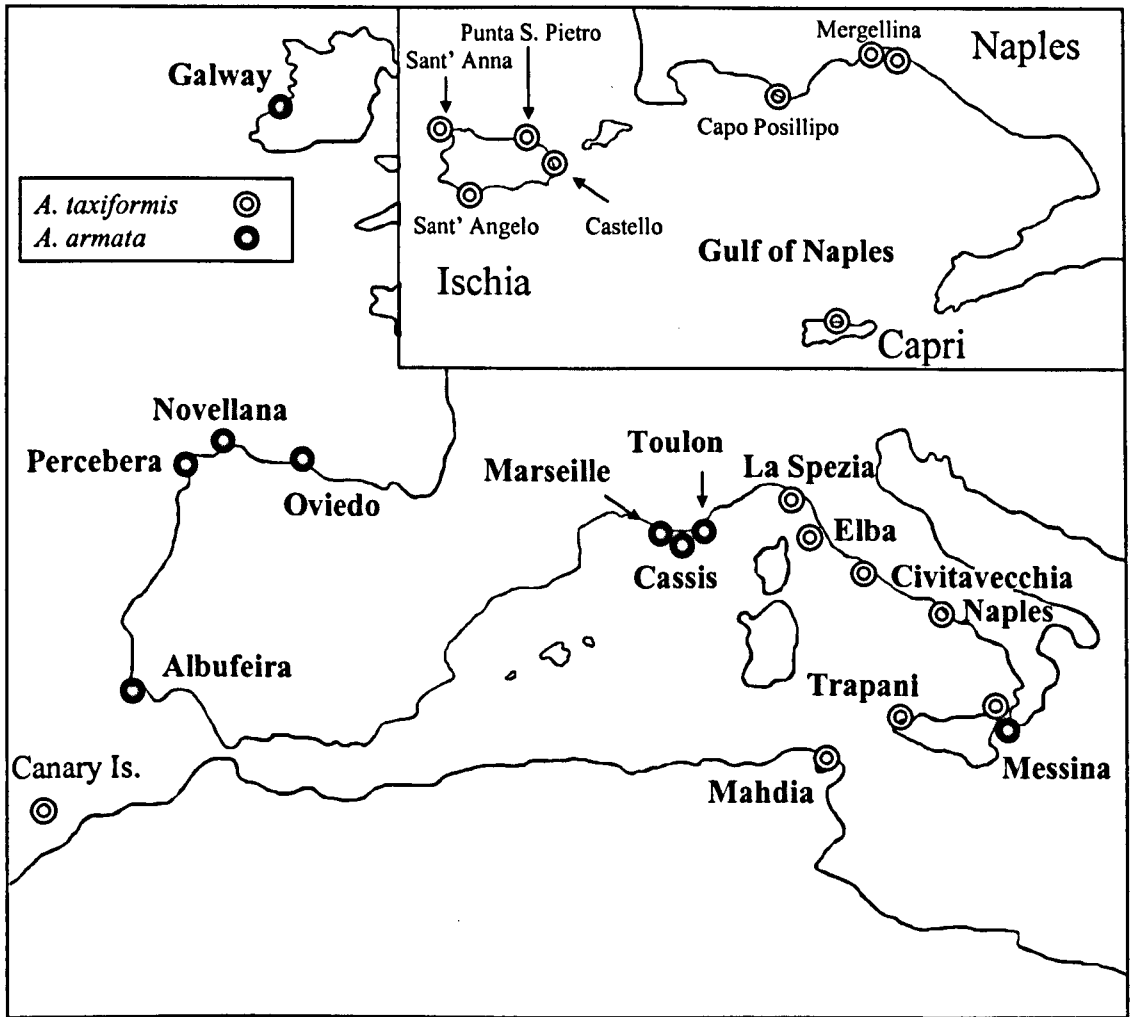


Fig. 2.1. Sample localities.

PCR amplification and sequencing of DNA marker regions

The "D1," "D2" and "D3" hyper-variable domains of the large subunit (LSU) rDNA gene (Mitchot and Bachellerie 1987; Lenaers *et al.* 1989, 1991) were PCR amplified in 30 μ l PCR reaction medium containing 10 ng DNA, 3 mM MgCl₂, 0.01 % BSA, 0.2 mM dNTPs, 1 μ M of forward primer D1R, 1 μ M 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 μ l volume containing 10 ng DNA, 1.5 μ M MgCl₂, 0.2 mM dNTPs, 1 μ M 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 *cox2-3* spacer was PCR amplified in 50 μ l PCR reaction medium containing 10 ng DNA, 2.5 mM MgCl₂, 0.1 % bovine serum albumin (BSA; Sigma), 0.2 mM dNTPs, 1 μ M of forward and reverse primers each, described in Zuccarello *et al.* (1999), 1X Roche diagnostics PCR reaction buffer (Roche Diagnostics, GmbH, Mannheim, Germany) 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 and 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 and 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 ten random additions. Bootstrap support for individual clades (Felsenstein 1985) was calculated on 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 possessed modified stolons with apically arranged harpoon-like hooks and lack an obvious rhizomatous system. These thalli fitted the description of *A. armata*. Those in the other group lacked such hooked stolons but possessed a clear rhizomatous system. These keyed out unambiguously as *A. taxiformis*. The side branches along the main axes of specimens in the latter group were 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* (see Table 2.2 for length and variation). Maximum likelihood analysis constrained with optimal hLRT parameters (Table 2.3) resulted in a single ML tree shown in Fig. 2.2. Maximum parsimony analysis resulted in a single MP tree (see Table 2.2 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 the morphology of *A. taxiformis* and "*Falkenbergia*" specimens collected from sites where only *A. taxiformis* was encountered.

		LSU rDNA	RuBisCo spacer	<i>cax</i> spacer
Sequence length	<i>A. armata</i>	700 bp	307 bp	365 bp
	<i>A. taxiformis</i> (Canary Is.)	758 bp	303 bp	364 bp
	<i>A. taxiformis</i> (Mediterr. Sea)	758 bp	303 bp	364 bp
	total alignment	768 bp	308 bp	367 bp
Sequence polymorphism	# variable characters	104	30	93
	parsimony informative sites	84	30	88
	# distinct sequences	20	3	18
	g_i value	-0.3156	-0.7067	-0.3470
	$g_i p=0.01$	-0.20	-0.88	-0.20
	given # tax and # char	15 and 100	5 and 50	15 and 100
Parsimony trees	Length	114 steps	31 steps	104 steps
	# of trees	1	1	29
	consistency index	0.982	1.000	0.971

Table 2.2. Sequences and tree statistics

alignment	LSU rDNA	RuBisCO spacer	<i>cax</i> 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

Table 2.3. Results of hLRT's and $-\ln$ likelihood of phylogenies inferred from ML-analyses constrained with optimal hLRT parameters. * see Hasegawa *et al.* (1985)

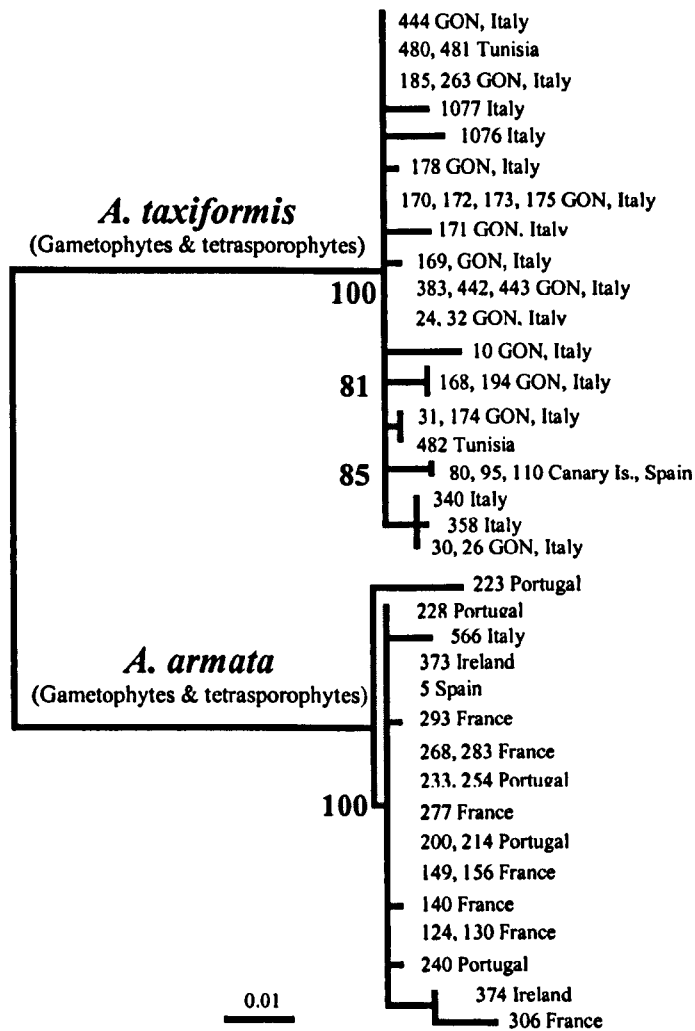


Fig. 2.2. Midpoint rooted ML reconstruction of relationships between *A. taxiformis* and *A. armata* gametophytic and tetrasporophytic specimens based on partial LSU rDNA sequences. Only bootstrap values > 80 % are indicated. GON denotes Gulf of Naples.

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 2.2). Maximum parsimony analysis resulted in a single MP tree (see Table 2.2 for tree statistics) shown in Fig. 2.3. Maximum likelihood analysis constrained with optimal hLRT parameters (Table 2.3) 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 intra specific 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 distinct clade.

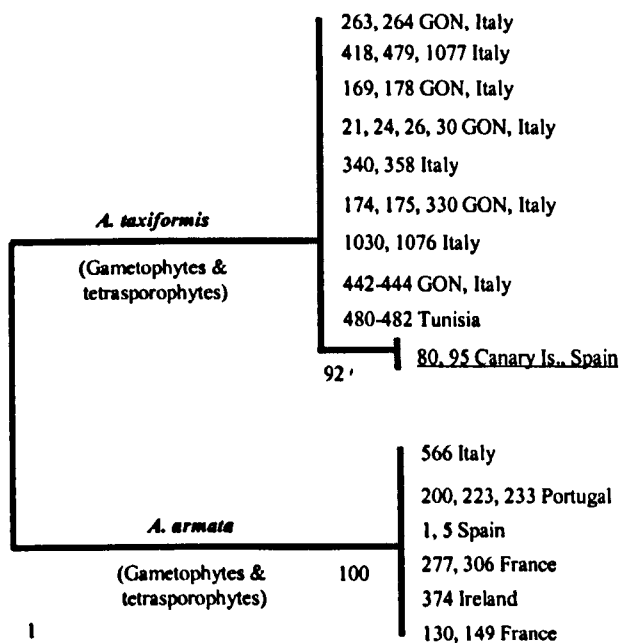


Fig. 2.3. Midpoint rooted MP tree based on RuBisCo spacer DNA sequence. Only bootstrap values > 80 % are indicated. The Canarian *Asparagopsis* is underlined. GON denotes Gulf of Naples.

cox2-3 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 2.2). Maximum likelihood analysis constrained with optimal hLRT parameters (Table 2.3) resulted in a single ML tree shown in Fig. 2.4.

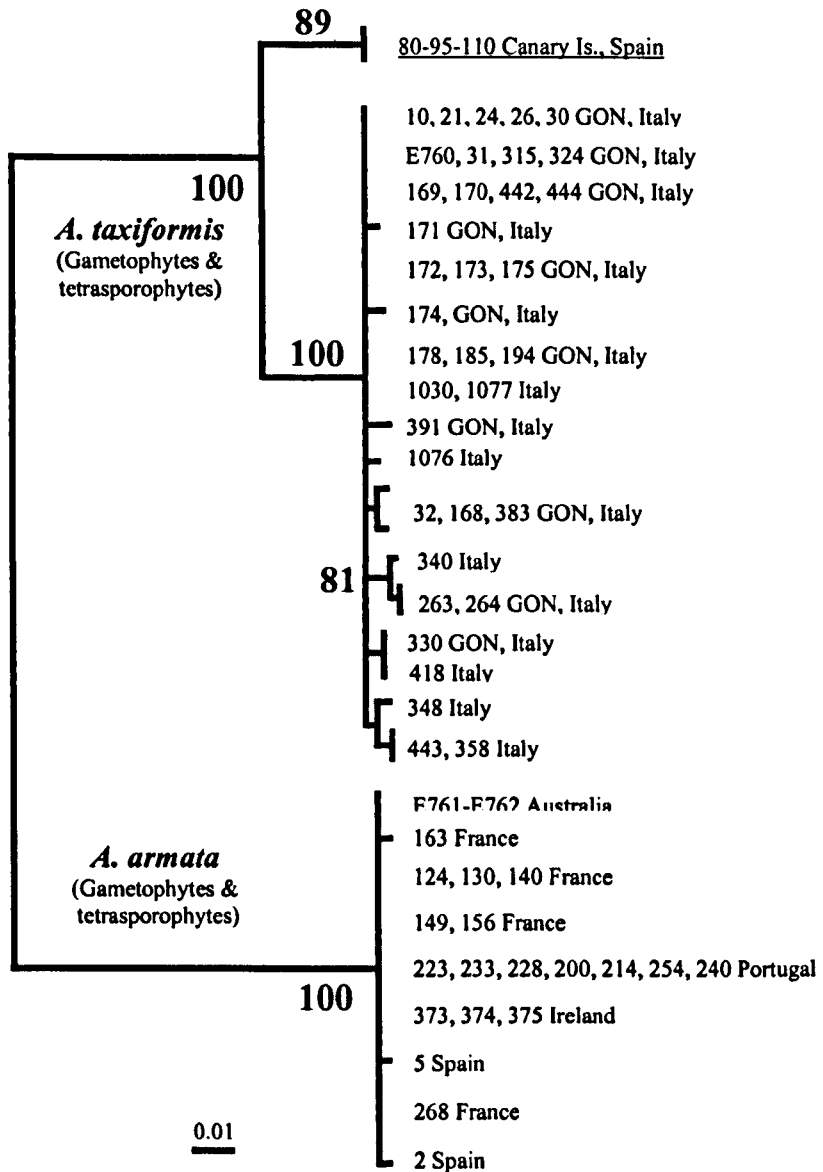


Fig. 2.4. Midpoint rooted ML reconstruction based on *cox* spacer sequence data. Only bootstrap values > 80 % are indicated. The Canarian *Asparagopsis* is underlined. GON denotes Gulf of Naples.

Maximum parsimony analysis resulted in 29 equally most parsimonious trees (see Table 2.3 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 resolved genealogies (although not geographically coherent) and unresolved genealogies were observed within the Mediterranean *A. taxiformis* (Fig. 2.5). In this network, the Canary Islands haplotype (not shown) differed distinctly from the Mediterranean haplotypes.

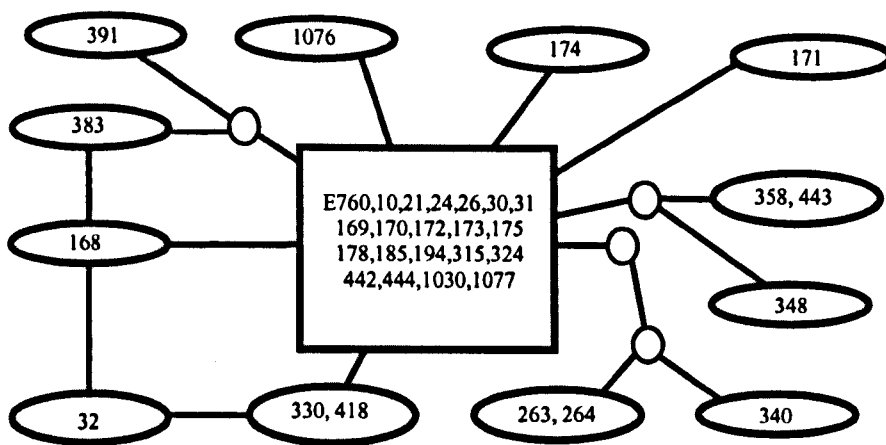


Fig. 2.5. Haplotype network for *cox2-3* spacer haplotypes of the Mediterranean *A. taxiformis*. Lines indicate one mutation step; nodes indicate missing haplotypes; reticulations denote unresolved genealogies. The dominant haplotype (group of identical sequences) is surrounded by a rectangle.

Discussion

Asparagopsis armata and *A. taxiformis* are, indeed, genetically and morphologically distinct species. The existence of two groups of gametophyte thalli based on the presence of distinct morphological characters (long hooked stolons in *A. armata* versus compact rhizoids in *A. taxiformis*; Dixon 1964; Bonin and 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 and Feldmann (1942) poses no real identification problem because the thalli are readily identifiable with any of the genetic markers deployed in this study. My results support those in Ni Chualáin *et al.* (2004) who recovered clear genetic differentiation between tetrasporophytes linked to *A. armata* and those 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

I did not discover cryptic genetic diversity within Mediterranean and western European *A. armata* and neither did I discover any genetic differences 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) Børgesen, 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.* (2002a) 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 this study strongly suggests that the “Mediterranean” genotype is also the only one present in this region. Yet, it 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, for the moment 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 else². However, maps in Lipkin and Safriel (1971) provide average temperatures measured through the upper meters 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

² http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html, accessed on 14 March 2004

order to complete the life cycle, these tetrasporophytes need short day-lengths (Oza 1977; Guiry and Dawes 1992) and temperatures roughly between 17 and 21°C (Guiry and 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 the Mediterranean specimens of *A. armata*, except one are from these northern areas (Marseilles, Gulf of Lion), in agreement with records in Sala and Boudouresque (1997). There is no sequence information available 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 and 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 extirpated the following summer. The first Mediterranean report of *A. armata* in Algeria in 1923 (Feldmann and Feldmann 1942) appears strange as well due to high summer seawater surface temperatures along southern Mediterranean coasts³ (Lipkin and Safriel (1971). However, along the Algerian coast in

³ http://www7320.nrlssc.navy.mil/global_nlom/globalnlom/med.html

particular, summer surface seawater temperatures below 25°C have been recorded⁴. 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 Sea⁴ (Lipkin and Safriel 1971) 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 the Mediterranean specimens of *A. taxiformis* have been collected outside the aforementioned regions. The 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.*

⁴ http://www7320.nrlssc.navy.mil/global_nlom/globalnlonm/med.html

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 1920’s 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 2.2). The partial LSU fragment amplified is about twice as long as the *cox2-3 spacer* but it contains about the same number of variable characters and parsimony informative ones. The LSU rDNA gene region is inherited bi-parentally and affected by recombination processes.

Thus, my data confirm the observation in Zuccarello and 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 sequences the Mediterranean sequences do not form a clade. In the RuBisCo tree the Mediterranean sequences are all the

same but the LSU 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 intra specific 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, if used all together in order to obtain information from all three genomes, are surely suitable for phylogeographic research at a local and global scale.

CHAPTER III - Phylogeography of the Invasive Seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) Reveals Cryptic Diversity⁵

Abstract

The rhodophytan seaweed *Asparagopsis armata* Harvey is distributed in the northern and southern temperate zones, and its congener *A. taxiformis* (Delile) Trevisan, abounds throughout the tropics and subtropics. Here I determined intraspecific phylogeographic patterns to compare potential causes of the disjunctions in the distributions of both species. I obtained specimens throughout their ranges and inferred phylogenies from nuclear, plastid and mitochondrial markers: the hyper-variable domains D1-D3 of the nuclear rDNA LSU, the spacer between the large and small subunits of RuBisCo (a chloroplast marker), and the mitochondrial *cox* 2-3 intergenic spacer, respectively. The *cox* spacer acquired base changes fastest and the RuBisCo spacer was the slowest. Median joining networks inferred from the sequences revealed lack of phylogeographic structure in the introduced range of *A. armata*, corroborating the species' reported recent introductions. *Asparagopsis taxiformis* consisted of three nuclear, three plastid and four mitochondrial genetically distinct, lineages (1-4). Mitochondrial lineage 3 is found in the western Atlantic, the Canary Islands and the eastern Mediterranean. Mitochondrial lineages 1, 2, and 4 occurred in the Indo-Pacific, but one of them (lineage 2) was also found in the central Mediterranean and Southern Portugal. Phylogeographic results suggested separation of Atlantic and Indo-Pacific lineages resulting from the

⁵ This Chapter has been submitted to Molecular Ecology as: N Andreakis, G Procaccini CA Maggs and WHCF Kooistra. Phylogeography of the Invasive Seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) Reveals Cryptic Diversity

emergence of the Isthmus of Panama, as well as dispersal events post-dating the closure event, such as the invasion of the Mediterranean Sea by mitochondrial lineages 2 and 3. Molecular clock estimates using the Panama closure event as a calibration for the split of lineages 3 and 4 suggested that *A. taxiformis* diverged into two main cryptic species, (1+2 and 3+4), about 8.5 to 9.6 Ma, and that the separation of the mitochondrial lineages 1 and 2 occurred 3-3.7 Ma.

Introduction

Seaweed distributions have generally been considered to be extensive but evaluation of their patterns is often affected by an overly broad view of species circumscriptions. Recent phylogeographic approaches have shown that many so-called widely distributed taxa, especially those exhibiting disjunctions, are composed of biologically and genetically distinct but morphologically cryptic or pseudo-cryptic species. Each of these newly recognized taxa may be widely distributed in its own right but is generally seen to have a more restricted range than the species complex (Wattier and Maggs 2001; Zuccarello *et al.* 2002, 2003; Kim and Kawai 2002; Kooistra *et al.* 2002; Gabrielsen *et al.* 2003; Montresor *et al.* 2003; Van der Strate *et al.* 2002; De Clerck *et al.* 2005; Müller *et al.* 2005; Sarno *et al.* 2005; Verbruggen *et al.* 2005, in press).

The red seaweeds *Asparagopsis armata* Harvey and *Asparagopsis taxiformis* (Delile) Trevisan constitute such widely distributed taxa with multiple disjunctions (Dixon 1964; Dixon and Irvine 1977; Bonin and Hawkes 1987). The gametophytic *Asparagopsis* stage and the tetrasporophytic *Falkenbergia* stage constitute different phases in the life

cycle of the same species (Feldmann and Feldmann 1939, 1942; Chihara 1961, 1962; Ní Chualáin *et al.* 2004). *Asparagopsis armata*, and its tetrasporophyte phase formerly known as *F. rufolanosa* (Harvey) Schmitz, abound in temperate zones. The species' distribution shows discontinuities between northern and southern Indo-Pacific populations and between those in the Indo-Pacific and Atlantic, including the north-western Mediterranean Sea. *Asparagopsis armata* is believed to be a native of southern Australia and New Zealand (Horridge 1951) from where it was exported to western Europe, the northern Mediterranean, Japan and the US west coast during the 20th century (Feldmann and Feldmann 1942; Dixon 1964; Dixon and Irvine 1977; Price *et al.* 1986; Farnham 1994; Boudouresque and Verlaque 2002). Given such a recent invasion history, one would not expect any phylogeographic structure among populations of this species. *Asparagopsis taxiformis*, including its tetrasporophyte phase, formerly known as *Falkenbergia hillebrandii* (Bornet) Falkenberg, occurs along the coasts of tropical and warm temperate seas, and likewise, shows disjunct Atlantic, Mediterranean, and Indo-Pacific populations (Price *et al.* 1986; Bonin and Hawkes 1987; Silva *et al.* 1996; Boudouresque and Verlaque 2002). The disjunctions in *A. taxiformis* may result from recent introductions, from fragmentation of a once continuous tropical distribution range, or from a combination of these two possibilities. If the pattern results from fragmentation, then each disjunct group of *A. taxiformis* might consist of a cryptic species (Andreakis *et al.* 2004; Ní Chualáin *et al.* 2004). Both *A. armata* and *A. taxiformis* are thus prime targets for a worldwide comparative phylogeographic survey.

To date, genetic studies on the two species (Andreakis *et al.* 2004; Ní Chualáin *et al.* 2004) have not revealed genetic differentiation in *A. armata* but it was detected in *A.*

taxiformis. Andreakis *et al.* (2004) uncovered genetic differences between Mediterranean and Canary Islands populations using direct sequencing of nuclear, plastid and mitochondrial DNA regions, and Ní Chualáin *et al.* (2004) revealed ecophysiological and genetic dissimilarities between Atlantic and Indo-Pacific-Mediterranean isolates using restriction fragment length polymorphism (RFLP) of chloroplast DNA. To further elucidate intraspecific phylogeographic patterns in these two species on a world-wide scale, I added many more samples originating from throughout the genus distribution range and sequenced a nuclear (Lenaers *et al.* 1989), a plastid (Maggs *et al.* 1992), and a mitochondrial DNA marker (Zuccarello *et al.* 1999a), all known to evolve rapidly. I inferred median joining networks (Bandelt *et al.* 1999), and assessed if their topology was congruent for the three markers. A molecular clock approach (Sarich and Wilson 1973) was used to estimate the minimum age of cryptic lineages recovered within the species. According to this approach the evolutionary age of these distinct genealogical lineages can be estimated by calibrating their DNA sequence distances against first appearance dates of some of these lineages in the fossil records or against a large scale paleogeological event (Sarich and Wilson 1973). This procedure puts a timeline along the evolution of a whole group of taxa (Yoon *et al.* 2002, Su *et al.* 2004) and allows a comparison of substitution rates among different DNA markers as well as among markers in different genomes (nuclear, mitochondrial and plastid in plants). However, the methodology is somewhat controversial. Substitution rates are stochastically constant but some sequences evolve significantly slower or faster than the average. In addition, substitution rates have been found to differ from lineage to lineage depending on e.g., generation time, growth rate, mutation rate, effectiveness of DNA-repair mechanisms and population size.

Here substitution rates of the three molecular markers were determined by using the emergence of the Isthmus of Panama 3.1 to 3.5 Ma (Coates and Obando 1996) to calibrate the separation of Indo-Pacific and Atlantic populations of *A. taxiformis*. Several authors have used this closure event to calibrate molecular clocks in tropical marine taxa (Lessios 1979; Keigwin 1982; Duque-Caro 1990*a,b*; Knowlton *et al.* 1993; Knowlton and Weigt 1998; Lessios *et al.* 1999; Lessios *et al.* 2001; Wares 2001; Marko 2002; Zuccarello and West 2002). I use the date of the event as a minimum age because separations of amph-Isthmian species pairs with short larval dispersal times often pre-date the emergence of the Isthmus of Panama (Craig *et al.* 2004; Williams and Reid 2004).

Materials and methods

Specimens and their sample sites are listed in Table 3.1. Sample preservation, DNA extraction, PCR amplification, sequencing, and sequence alignments were carried out as described in Chapter II (Andreakis *et al.* 2004). Sequence reactions were obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City CA) and purified in automation using a robotic station “Biomek FX” (Beckman Coulter, Fullerton CA). Products were analysed on an Automated Capillary Electrophoresis Sequencer “3730 DNA Analyzer” (Applied Biosystems). Sequences of the nuclear large subunit rDNA gene spanning the D1, D2 and D3 hyper-variable domains, (LSU rDNA) (Lenaers *et al.* 1989), the chloroplast spacer between the large and small subunits of the ribulose-1-5-bisphosphate carboxylase/oxygenase region, (RuBisCo spacer) (Maggs *et al.* 1992) and the mitochondrial cytochrome oxidase subunit 2 - subunit 3 spacer, (*cox 2-3* spacer) (Zuccarello *et al.* 1999a) were used as markers (see Chapter II, Andreakis *et al.*

2004). Initial phylogenies were inferred in PAUP* 4.0b10 (version for Windows; Swofford 2002) using the neighbour joining algorithm and Kimura-2-parameter pairwise distances. Bootstrap analyses (1000 replicates) were obtained using the same settings. The trees were used for sample identification because tetrasporophyte stages of *Asparagopsis* species are morphologically similar (Ní Chualáin *et al.* 2004), and they were utilized to determine the position of the root in intraspecific haplotype networks (see below). Relationships among sequences within *A. armata* and within *A. taxiformis* were inferred using the Median Joining algorithm (MJ) (Bandelt *et al.* 1999). The method identifies groups of closely related haplotypes for the plastid and mitochondrial sequences and similar clusters of genotypes for the partial LSU rDNA sequences. Then “median vectors” are introduced to connect the haplotypes or genotypes into a tree or network. Median vectors can be interpreted biologically as existing non-detected haplotypes or genotypes (Bandelt *et al.* 1999). In a few sequences, ambiguities were observed at low frequencies. A minor “G” peak under a dominant “A” peak was treated as “A” in the network analyses.

	Sample site	Sample n°	Life stage	Collection date	LSU rDNA	RuBisCo spacer	cox spacer
<i>Asparagopsis taxiformis</i>							
U.S.A	Key Largo, Florida	985	T	01-08-1991	DQ228909		DQ228887
	Catalina Is., California	494, 497, 498	G	26-08-2003		DQ228873	
	Hawaii	997	T	02-07-1991			
	Kaneohe Bay, Hawaii	491, 492	G	16-08-2003			
	Ka'alaui, O'ahu, Hawaii	35****, 51*, 55	G	15-05-2002	DQ228914		DQ228892
		70			DQ228915		
	Kawaihae, North Kohala, Hawaii	260	G	28-05-2002			
	Lele'wi State Beach, Hawaii	261	G	24-05-2002			
	Ka'alawai, Honolulu, Hawaii	262	G	31-05-2002			
Panama	Isla Perico, Balboa	372	T	02-05-1999			DQ228893
	Isla Naos, Balboa	368	T	30-05-2002			DQ228884
		367	T	30-05-2002	DQ228919		DQ228885
Mexico	Puerto Morales, Quintana Roo, Yucatan	999	T	27-07-1980			
Spain	Tenerife, Canary Is.	80** , 95* , 110****	G	30-05-2002	AY589540	AY589559	AY589524
	Las Palmas Canary Is.	1075	T	05-10-1993			
Brasil	Praia Fortaleza, Sao Paolo	256, 258****	G	29-05-2002			DQ228891
	Ilha Anchieta, Sao Paolo	257****, 259	G	28-05-2002			DQ228890
Puerto Rico	La Parguera	986	T	17-03-1991	DQ228910		DQ228886
	Luna Reef, La Parguera	421	G	25-06-2002			
	Cabo Rojo, Lighthouse	420*, 422	T	11-01-2002	DQ228911	DQ228874	
Croatia	Dubrovnik-Porparela (old Harbour)	487, 489****	G	18-10-2003			DQ228895
		488			DQ228906		DQ228896
Italy	Elba	479	T	25-08-2003			
	Lerici, La Spezia	1076*, ***	T	16-07-1993	AY589545		AY589537
	Civitavecchia, Roma	418	G	15-06-2002			DQ228899
		1077*	T	30-06-1993	AY589546		
	P.ta S. Pietro, Ischia, Naples	442*, ***	T	28-01-2003	DQ228908		DQ228897
		443	T	28-01-2003			DQ228898
		444	T	28-01-2003	DQ228907		
	Sant'Anna, Ischia, Naples	169** , ** , 170	G	15-06-2002	AY589542	AY589558	

		168*,***						AY589541	AY589527
	P.ta S. Pietro, Ischia, Naples	171*,***,173,175	G	19-06-2002				AY589543	AY589528
		172						DQ228912	
		174						DQ228913	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					AY589539	AY589525
		10*,***,21		19-04-2002				AY589538	
		26*						AY589547	
	Capo Posillipo, Naples	383***	G	20-08-2002					AY589535
		391***							AY589536
	Strait of Messina, Sicily	1030	T	16-11-1992					AY589532
	Trapani, Sicily	340***	G						AY589534
		358*,***		22/07/2002				AY589549	AY589533
		348***							
	Faro	484-486	G	10-04-2004					DQ228894
Portugal	Little Armstrong Bay, Rottnest I.	1045	T	26-11-1992					
W. Australia	South Pacific Ocean	445	G	08-01-2003					
Norfolk Island	Hon Tre, Nha Thrang, Khan Hoa Province	1040	T	06-03-1992				DQ228916	
Vietnam	Hinomi-saki, Taisha, Shimane Pref.	1032	T	01-05-1992				DQ228917	
Japan	Komesu, Itoaman, Okinawa Pref.	1031	T	22-03-1992				DQ228918	
New Caledonia	Passé Mato	423	G	12-09-2002					DQ228883
Sri Lanka	Dikwella village	483	G	17-08-2003					
Tunisia	Mahdia	480-482*	G	06-06-2003				AY589548	DQ228888
Lebanon	Barbara	496***	G	21-05-2004					DQ228889
		495							
<i>Asparagopsis armata</i>									
U.S.A	San Diego, California	996	T	1972					
UK	Portelet Bay, Jersey, Channel Is.	1034	T	10-1992					DQ228882
	Archirondel, Jersey, Channel Is.	1035		26-10-1992					
	Horses Ledges, Shanklin, Is. of Wight	1029		24-09-1992					DQ228881
	Archirondel, Jersey, Channel Is.	125		Unknown				DQ228905	
Ireland	Ard Bay, Galway	373,374*,375	G	17-07-2002				AY589556	
	St John's Point, W. Donegal	137	T	Unknown					

	New Quay, Co. Clare	510	T	06-02-1985	
Spain	Oviedo	417***	T	08-09-2002	AY589522
	Arledo	1028	T	10-1992	
Portugal	Puerto di Porticella, Ovimana, Asturias	1026	T	30-08-1992	
	Novellana	414	G	11-07-2002	AY589523
	Perecebra	413	G	12-07-2002	
	Praia Castelo, Albufeira	200,214***,223*,233,254	G		DQ228876 DQ228878 AY589552 AY589550 AY589551
France	Faro	228	T	02-2003	DQ228877
	South Marseille	446	T	02-2003	DQ228879
	Roscoff	447	G	15-05-2002	DQ228901 DQ228902 AY589553 DQ228904
	Villefranche-sur-mer	124***,130,140*	T	Unknown	AY589520
	North Marseille	984	T	01-09-1980	
	Cassis	980	G	15-05-2002	AY589521
	Toulon, Brun Cape	156,163***	G	15-05-2002	DQ228875 DQ228880
Italy	Messina, Sicily	149	G	26-06-2002	
	Sydney	268***,277*,283	G	26-06-2002	DQ228900 AY589555 AY589554 AY589557 AY589560
Australia	Sorrento Back Beach, Victoria	306*	G	26-06-2002	
	Robinson Crusoe Is.	293*	T	04-06-1987	
Chile		566***	G	Unknown	
		E761,E762	T	28-10-1987	DQ228903
		604	T	Unknown	
		979	T	Unknown	

Table 3.1. List of specimens of *Asparagopsis taxiformis* and *A. armata* used. "G" denotes gametophyte and "T" tetrasporophyte. For each group of identical sequences only a single representative has obtained a GenBank accession number. Samples of the same geographic location have been gathered together in a row. In such cases, the reference strain for the GenBank Accession Number of the LSU rDNA is indicated with a "***", for the one of the RuBisCo spacer with a "****" and for that of the *cox 2-3* spacer with a "*****".

Two methods were used to test for significant deviations from stochastic substitution rates in sister lineages: the relative rate test (RRT; Li and Bousquet 1992; Robinson *et al.* 1998) as implemented in the RRTree software (version 1.1; Robinson-Rechavi and Huchon 2000) and the likelihood ratio test (LRT; Felsenstein 1981). Both tests were employed because the LRT does not need outgroups but it requires a tree topology, whereas the RRT is independent of topology but needs outgroups (Posada 2001). The branch-length test (BLT) in the LinTree software (Takezaki *et al.* 1995) was used to eliminate sequences with evolution rates significantly different from the average. Hierarchical Likelihood Ratio tests (hLRTs) were performed using Modeltest (Version 3.06; Posada and Crandall 1998) to determine the best-fitting evolution model for each of the three alignments. Maximum Likelihood (ML) calculations were constrained with the obtained Modeltest parameters for the best-fitting evolution model. For the LRT, clock-enforced ML trees and non-enforced trees were calculated using the quartet puzzling method (Strimmer and von Haeseler 1996) as implemented in the TREE-PUZZLE package (version 5.0; Muse and Weir 1992; Schmidt *et al.* 2002). ML analyses were performed in the same computer package using the heuristic search option and ten random sequence additions. Identical sequences were removed prior to the analysis. Gaps were treated as missing data. The Chi-square test was performed to test whether likelihood values of trees with and without the molecular clock enforced were significantly different. The degrees of freedom were set equal to the number of distinct sequences minus two.

Molecular clock estimates were made for *A. taxiformis* for each of the three markers after removal of significantly different evolving sequences according to the BLT results. The substitution rate of each marker was calculated as follows: the average p-

distance between two clades with known date of separation was obtained by averaging all p-distances between pairs of sequences from across that dichotomy. The p-distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared and it does not make any correction for multiple substitutions at the same site, substitution rate biases or differences in evolutionary rates among sites (Nei and Kumar, 2000). As calibration point I used the emergence of the Isthmus of Panama at 3.1-3.5 Ma before present (Coates and Obando 1996). The percentage sequence divergence rate (r) of each marker was calculated according to the formula $r = d/2t$ where d is the average (pair-wise) p-distance between the Atlantic lineage 3 and the Indo-Pacific lineage 4 and t is the hypothesized time elapsed since their divergence (i.e., 3.1-3.5 Ma coinciding with the closure of the Isthmus of Panama). According to the above formula and assuming rate constancy over time, the average sequence divergence among lineages is divided by two times the time since they split in order to obtain an overall calibrated sequence divergence rate.

Results

Asparagopsis armata

The *cox 2-3* spacer network (Fig. 3.1a) revealed relationships among 12 haplotypes from 25 specimens (Table 3.2) with a dominant haplotype in 12 specimens central in the network, and 11 infrequent haplotypes one or few base changes away from it. Unresolved genealogies are shown as reticulations. The partial LSU gene network was completely resolved (Fig. 3.1b). Among the 34 sequences analysed (Table 3.2) there was a frequent

genotype (25 samples) and nine less common genotypes one or a few steps away from it. The RuBisCo spacer showed no haplotype variation among the 24 specimens screened (Table 3.2), and is illustrated as a single circle (Fig. 3.1c). Collection sites of the specimens have been mapped in Fig. 3.1d. Neither of the networks showed any phylogeographic structure, and the topology of the *cox* 2-3 spacer network was not congruent with that of the LSU marker.

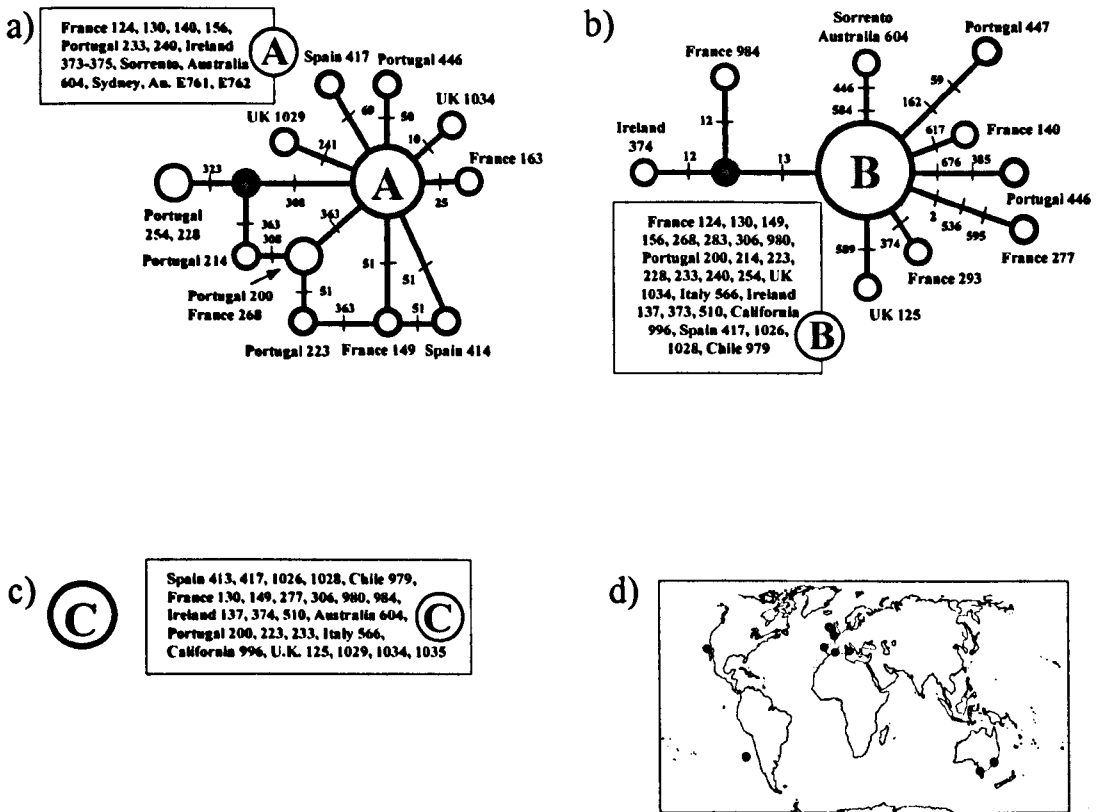


Fig. 3.1. Median joining networks of a) *cox* 2-3 spacer, b) hyper-variable region of LSU rDNA, and c) RuBisCo spacer of specimens of *Asparagopsis armata*. Circles represent haplotypes or, in case of the LSU, genotypes; circle size is proportional to number of specimens. Specimen numbers and their collection sites are given next to each type. Frequent haplotypes are indicated with uppercase letters inside the circle and samples sharing this type have been listed in boxes. Bars across lines connecting haplotypes denote base changes in the sequence alignment at positions indicated by numbers. Fig. 1d. Collection sites of *A. armata* specimens indicated on a world map.

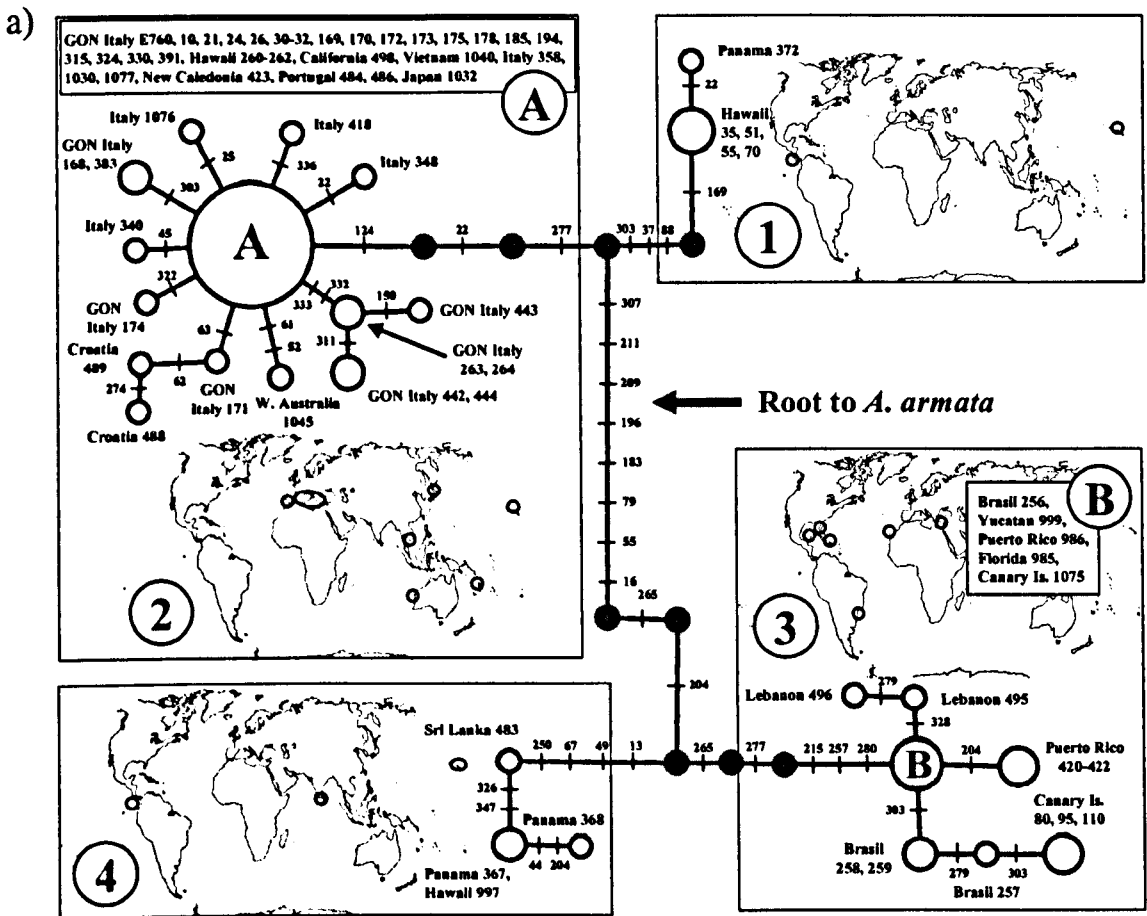
<i>A. armata</i>	<i>cox</i>	LSU	RuBisCo
# sequences included	25	34	24
# characters in alignment of sequences	365	702	303
# variable characters in alignment	8	19	0
# parsimony informative characters	3	2	0
# haplotypes (genotypes) among sequences	12	10	1
<i>A. taxiformis</i>			
# sequences included	72	62	62
# characters in alignment of sequences	364	759	307
# variable characters in the alignment	41	32	6
# parsimony informative characters	32	10	5
# haplotypes (genotypes) among sequences	26	25	4

Table 3.2. Sequences and alignment statistics for the *cox* 2-3 spacer, the LSU rDNA gene and the RuBisCo spacer of *A. armata* and *A. taxiformis*.

Asparagopsis taxiformis

The completely resolved *cox* 2-3 spacer network (Fig. 3.2a) linked 26 haplotypes obtained from 74 samples analysed (Table 3.2). Four distinct haplogroups were each represented by a frequent haplotype accompanied by one to several others up to a few base changes away from the central one. Haplogroups have been referred to as lineages 1, 2, 3 and 4. Lineage 1 contained two haplotypes among five sequences from the Pacific and Indian Oceans including Pacific Panama and Hawaii. Lineage 2, which was sister to lineage 1, included the vast majority of sequences obtained, with one common haplotype (32 specimens) and 13 other haplotypes one or a few steps divergent. This lineage contained specimens from the Indo-Pacific Ocean, the central Mediterranean and southern Portugal. Lineages 3 and 4 were sisters to each other. Within lineage 3, the most frequent

haplotype was encountered in five specimens and the six other haplotypes were one or a few steps away from the dominant one. Specimens in lineage 3 were obtained from Atlantic localities and from the Lebanon (eastern Mediterranean). Lineage 4 contained three closely related haplotypes, found in specimens from Sri Lanka, Hawaii and Pacific Panama. The origin of the network (Fig. 3.2a) rooted with *A. armata* is situated on the vertical branch connecting lineages 1 and 2 with lineages 3 and 4.



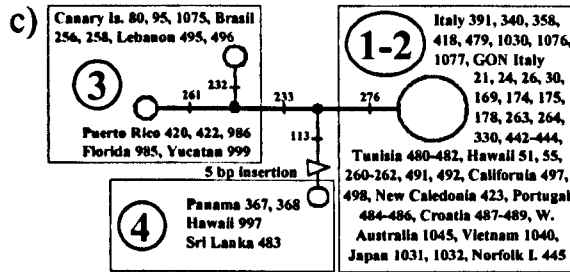
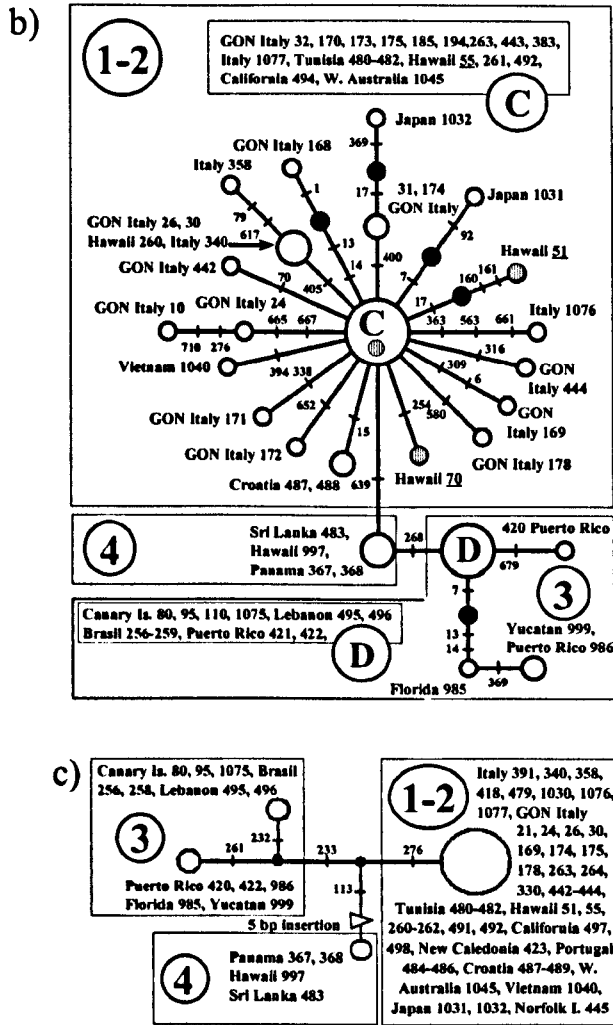


Fig. 3.2. Median joining networks of a) *cox 2-3* spacer, b) hyper-variable region of LSU rDNA, and c) RuBisCo spacer of specimens of *Asparagopsis taxiformis*. Figure legend as in Fig. 1. In Fig. 2a, collection sites of specimens in each group have been indicated on world maps. In Fig. 2b, an open circle denotes that the *cox 2-3* spacer of the sample is recovered in lineage 2; a hatched circle and an underlined sample number denote that its *cox 2-3* spacer groups in lineage 1.

The partial LSU gene revealed 25 genotypes among the sequences obtained from 62 specimens tested (see Table 3.2). The inferred MJ network (Fig.3.2b) was resolved completely. The most frequent genotype was shared among 18 specimens and several less frequent genotypes were up to four base changes away from it. All samples belonging to

this LSU group were found in lineages 1 and 2 in the *cox* 2-3 spacer network (Fig. 3.2a), but their topologies were not congruent because the LSU sequences of Hawaiian specimens 51, 55 and 70 (lineage 1 in the *cox* spacer network) were scattered among those recovered in lineage 2 in Fig. 3.2a. Therefore, the upper cluster of genotypes is not well separated and is referred to as “lineages 1+2”. Samples 997 (Hawaii), 367, 368 (Pacific side of Panama) and 483 (Sri Lanka) shared identical LSU genotypes. The *cox* haplotypes of these specimens grouped together in lineage 4, but their shared LSU genotype (also marked lineage 4) was just a single step away from the dominant one in lineages 1+2. Specimens in mitochondrial lineage 3 (Fig. 3.2a) shared related nuclear genotypes (Fig. 3.2b) at least two steps away from the most frequent one of lineage 1+2 and at least one step from those in lineage 4. Therefore, this LSU group is referred to as lineage 3.

The RuBisCo spacer network (Fig. 3.2c) revealed the relationships among four haplotypes from the 62 specimens analysed (Table 3.2). All samples in mitochondrial lineages 1 and 2 shared a single RuBisCo haplotype, referred to as lineages 1+2. Mitochondrial and nuclear lineage 3 was also seen in the RuBisCo spacer network. Specimens from the Canary Islands, Lebanon and Brazil shared a haplotype two base changes away from the other, Caribbean haplotype. Mitochondrial and nuclear lineage 4 samples shared a single RuBisCo spacer haplotype characterized by a 5-base-pair insertion.

Rate constancy among lineages

Taking sequences of *A. armata* as reference, none of the mitochondrial, nuclear and plastid lineages within *A. taxiformis* revealed significant mutation rate differences for any

of the markers used. ML trees were inferred using optimal modeltest estimates, with and without clock-enforcement. Results of the likelihood ratio test, (LRT; Table 3.3, test performed with all lineages), showed no significant differences between ML values with and without the clock enforced in either the nuclear and the plastid datasets.

Table 3.3. Likelihood ratio tests (LRT) constrained with optimal Modeltest parameters for *A. taxiformis* *cox* 2-3 spacer, LSU rDNA gene and RuBisCo spacer alignments. Tests for the *cox* 2-3 spacer alignment were performed with all four lineages*, with all four lineages and *A. armata* as outgroup**, with all four lineages after the branch length test***.

Marker	-ln L clock-enforced	-ln L no clock	2x-ln LR	df	P	Clock rejected?
<i>cox</i> 2-3 spacer*	844.92	798.80	92.24	27	0.000	yes
LSU rDNA*	1270.60	1255.54	30.12	32	0.562	no
RuBisCo spacer*	454.17	451.00	6.34	4	0.175	no
<i>cox</i> 2-3 spacer						
Lineage 3 vs. 4	583.47	568.07	30.81	11	0.001	yes
4 Lineages**	1094.08	1079.86	28.44	28	0.441	no
4 Lineages***	756.52	745.97	21.09	20	0.392	no

***Significantly different evolving sequences were excluded, LRT reruns revealed not significant differences between a clock-enforced and a non-enforced tree at the 5% level either with or without sequences of *A. armata*. Thus, a constant rate of evolution was assumed.

In the case of the mitochondrial data (*cox* 2-3 spacer), the -ln likelihood of the clock-enforced tree was significantly worse ($p < 0.05$). A clock-enforced tree was also rejected if only sister lineages 3 and 4 were included in the computation ($p < 0.05$). If *A. armata* was included in the tree calculations, then the clock-enforced tree was not significantly worse (Table 3.3). The branch length test (BLT) on the mitochondrial alignment of *A. taxiformis* showed that the evolution rate of the *cox* spacer sequences from samples 263, 442 and 443 (in lineage 2), 421, 422 (in lineage 3), and 367 and 483 (in

lineage 4) was significantly different from the remainder. After exclusion of these sequences the test was repeated and no additional outliers were detected. Reruns of the LRT on the remaining sequences revealed no significant difference between a clock-enforced and a non-enforced tree at the 5% level, either with or without sequences of *A. armata* and, therefore, a constant rate of evolution was assumed (Table 3.3). RRT statistics and modeltest estimates are not shown.

Relative rates among the three markers

The mitochondrial region accumulated changes almost 14 times faster than the LSU region and four times faster than the RuBisCo spacer (Table 3.4).

	bp	Average # diff. \pm SE	p-distance \pm SE	Substitutions x (site ⁻¹ y ⁻¹)	% divergence per Ma \pm SE
<i>cox</i> 2-3	364	13.83 \pm 3.23	38.0 \pm 9.3 $\times 10^{-3}$	61.3–54.3 $\times 10^{-10}$	0.613 \pm 0.143 – 0.543 \pm 0.127
LSU	758	2.10 \pm 0.96	2.8 \pm 1.5 $\times 10^{-3}$	4.47–3.96 $\times 10^{-10}$	0.045 \pm 0.020 – 0.040 \pm 0.018
RuBisCo	307	3.00 \pm 1.46	9.8 \pm 5.0 $\times 10^{-3}$	15.76–13.96 $\times 10^{-10}$	0.157 \pm 0.076 – 0.140 \pm 0.067

Table 3.4. Relative substitution rates calculated from average sequence differences between lineages 3 and 4 for the *cox* 2-3 spacer, the LSU rDNA gene and the RuBisCo spacer within *A. taxiformis*. It is assumed that the separation between the lineages resulted from the rise of the Panama Isthmus 3.1 - 3.5 Ma B.P. (Coates and Obando 1996).

The LSU marker was about twice as long as the *cox* 2-3 spacer, but contained about the same number of variable sites. Yet, the number of parsimony-informative sites in the LSU alignment was only a third of those of the *cox* 2-3 spacer (Table 3.2). The shorter RuBisCo spacer showed fewer variable sites and only about half the number of parsimony-

informative ones in comparison to the LSU alignment. The LSU region appeared to evolve faster than the RuBisCo spacer but had no enhanced phylogenetic information (Table 3.2).

Molecular clock reconstructions

I assumed that the Atlantic lineage 3, is the only lineage of Atlantic origin according to these data. The latter lineage diverged from the Indo-Pacific lineage 4 approximately 3.1 to 3.5 Ma (Table 3.4). Estimates of genealogical ages of pairs of lineages within *A. taxiformis* are given in Table 3.5. Separation of lineages 1 and 2 from lineages 3 and 4 took place *ca.* 8.5 to 9.6 Ma, and lineages 1 and 2 separated about 3.0 to 3.7 Ma.

Lineages	Average % divergence \pm SE	Time since split (Ma)
<i>cox2-3</i> spacer		
1 vs 2	1.92 \pm 0.61	3.13 \pm 0.99 - 3.54 \pm 1.12
3 vs 4	3.8 \pm 0.93	6.2 \pm 1.51 - 7 \pm 1.71
1-2 vs 3-4	5 \pm 1.01	8.16 \pm 1.65 - 9.21 \pm 1.86
LSU rDNA		
3 vs 4	0.28 \pm 0.15	6.2 \pm 3.33 - 7 \pm 3.75
1-2 vs 3-4	0.39 \pm 0.15	8.67 \pm 3.33 - 9.75 \pm 3.75
RuBisCo spacer		
3 vs 4	0.98 \pm 0.49	6.2 \pm 3.1 - 7 \pm 3.5
1-2 vs 3-4	0.90 \pm 0.43	5.73 \pm 2.73 - 6.43 \pm 3.07

Table 4.5. Mean percent divergence between lineages recovered from the *cox 2-3* spacer, the LSU rDNA gene and the RuBisCo spacer within *A. taxiformis* and divergence dates between nodes calculated assuming that the split between lineage 3 and 4 occurred at 3.1 to 3.5 Ma B.P. The average % divergence of the lineages considered is divided by the calibrated % divergence rate for that marker as given in the last column in Table 3.4.

Discussion

Congruence among three markers

Although the three markers used in this study are all reported to be suitable for intraspecific phylogeographic studies (Lenaers *et al.* 1989; Maggs *et al.* 1992; Zuccarello *et al.* 1999a; Zuccarello and West 2002; Andreakis *et al.* 2004), they show marked differences in their substitution rates and their phylogenetic resolution. Between as well as within the two species the *cox* spacer was the most variable and the RuBisCo spacer the least. Zuccarello and West (2002) observed the same trend in the red alga *Bostrychia calliptera* (Montagne) Montagne. The region of the LSU marker used in the present study differs slightly from that used by Zuccarello and West (2002) but the trend is the same. Phylogenetic signal from the three markers in the present study corroborates results obtained among geographic populations of *B. calliptera* by Zuccarello and West (2002).

Phylogeography of A. armata

All specimens of *A. armata* included in this study are interpreted as a single biological species, with a single plastid haplotype and lack of congruence between the nuclear and mitochondrial network. Australian, Chilean and Californian sequences are recovered among the European ones in the nuclear and mitochondrial networks. This absence of any congruence is expected in view of the known history of this species' introductions in various regions as well as its invasive nature (see introduction of this Chapter). The considerable haplotype variation within introduced European populations suggests that this species arrived from the donor region by means of large contingents of immigrants and/or multiple introduction events.

Phylogeography of A. taxiformis.

Asparagopsis taxiformis diverged into two main mitochondrial lineages at about 8.5 to 9.6 Ma (Table 3.5): sister lineages 1+2 and sister lineages 3+4. Lineages 3 and 4 are resolved in all markers, whereas lineages 1 and 2 are only resolved at the mitochondrial level. Here, I interpret lineages 1+2, 3, and 4 as three biologically distinct but morphologically cryptic species. Cryptic lineages are common in many algal taxa (see introduction of this Chapter), and many of these lineages have also been shown to be reproductively incompatible, ecophysiologicaly distinct, and/or allopatric (Zuccarello *et al.* 2002, 2003; Kooistra *et al.* 2002, De Clerck *et al.* 2005).

Lineages 1 and 4, distinguishable by organellar haplotypes and nuclear genotypes, are sympatric at the Pacific entrance of the Panama Canal. Such distinct patterns for all three markers would collapse if individuals from these two lineages interbred freely. The possibility for interbreeding between lineages 1 and 4 cannot be excluded completely because one of them may, in theory, have been introduced recently in the region, and time elapsed since the introduction may not have been sufficient to generate any mixing. Lineages 3 and 4 differ for all three markers but reproductive isolation cannot be ascertained because I have not encountered them in sympatry. Indo-Pacific lineages 1 and 2 probably constitute a single biological species because they share a single RuBisCo haplotype and the differences observed in the *cox* spacer are not recovered with the LSU marker. Samples separating into two distinct groups (lineages 1+2) in the *cox2-3* spacer network do not separate into two such groups in the LSU rDNA gene network. Distinct *cox* haplogroups, such as those of lineages 1 and 2, can occur in a single biological species and even within a single population. In this study, mitochondrial genes describe intraspecific

divergence events in *A. taxiformis* better than nuclear genes. On average, mitochondrial genes have higher mutation rates than their nuclear and plastid relatives (William *et al.* 2004). There is also a higher fixation rate of the mutations because the mitochondrial genome is haploid, and is generally maternally inherited in red algae, as in plant and animal species (Coyer *et al.* 2002; William *et al.* 2004). Moreover, the effective population size (N_e) of a mitochondrial gene is about one-quarter that of a nuclear one (William *et al.* 2004). Thus mitochondrial genes are differentially dispersed in red algae than nuclear and plastid genes and this may be the reason for the discrepancies observed in the phylogenetic patterns.

Asparagopsis taxiformis shows clear phylogeographic structure among the cryptic species. Lineages 1 and 4 are distributed in the Indo-Pacific Ocean. Lineage 2 is also Indo-Pacific but is present in the central Mediterranean and southern Portugal as well. Lineage 3 is restricted to the Atlantic Ocean and to the eastern Mediterranean coast. A previous report that the Vietnamese isolate 1040 of *A. taxiformis* belongs to the Atlantic lineage is based only on growth response curves (figs 8-9 in Ní Chualáin *et al.* 2004), and the present study shows that this isolate groups clearly with lineage 2 in Fig. 3.2a.

Hybrid origin and incomplete lineage sorting of lineages 1 and 2

The high intraspecific variation among the LSU rDNA sequences in lineage 1+2 of *A. taxiformis* (Figs. 3.2a and 3.2b) is consistent with recent hybridization events involving two LSU genotype lineages, not yet homogenized by concerted evolution. High intraspecific rDNA variation has been encountered also in many other widely distributed marine species, particularly those with a history of hybridization events, such as corals

(Vollmer and Palumbi 2002; Miller and Van Oppen 2003; Vollmer and Palumbi 2004) and the green alga *Caulerpa racemosa* (Forsskal) J. Agardh (Famà *et al.* 2000; Durand *et al.* 2002). Unresolved phylogenetic signal of the nuclear marker may result also from incomplete lineage sorting of ancestral polymorphisms through genetic drift. In this case symplesiomorphies from a polymorphic ancestor are still shared between the lineages after a rapid speciation event. Incomplete lineage sorting has been documented in numerous species, e.g., cichlid fishes as incongruences in genealogies for nuclear loci (Takahashi *et al.* 2001). The insufficient phylogenetic signal may result also from extensive homoplasy because the LSU rDNA marker exhibits regions of high variability (changes are not randomly distributed). Random incomplete sorting of ancestral polymorphisms, homoplasy, or both, have been responsible for incongruence between phylogenies inferred from nuclear and mitochondrial markers in stiff-tailed ducks (McCracken and Sorenson 2005). On the other hand the unresolved genealogy between lineages 1+2 observed in the RuBisCo spacer is attributed to its slower mutation rate uncovered in this study.

Molecular clock calculations in A. taxiformis

If the *cox* haplotype groups of lineages 3 and 4 of *A. taxiformis* separated as a result of the emergence of the Isthmus of Panama 3.1-3.5 Ma, then mitochondrial lineages 1 and 2 separated anywhere between about 3 and 3.7 Ma and the split between lineages 1 + 2 and lineages 3 + 4 is assumed to have happened at *ca.* 8.5 to 9.6 Ma. The average distance between these two pairs of lineages, about 5%, was generated over a period of 8.5 to 9.6 Ma (Table 3.5) implying approximately 0.6% *per* Ma for the *cox* marker.

The percentage divergence for the *cox* spacer in *A. taxiformis* (Table 3.4) is consistent with the one obtained from *Bostrychia calliptera* (Montagne) Montagne Zuccarello and West 2002). Zuccarello and West used the same calibration point as in this study and obtained similar values (0.6-0.51% Ma⁻¹, table 3 in Zuccarello and West 2002). Yet, the rate of the *cox* spacer in *A. taxiformis* is about half that estimated for mitochondrial regions of marine animals, e.g., 0.9% Ma⁻¹ for the mitochondrial 16S ribosomal gene of the copepod *Eurytemora* (Lee 2000), and 0.67-1.21% Ma⁻¹ across all sites of the mitochondrial *cox1* among bivalve molluscs in the family Arcidae (Marko 2002). Whereas plant mitochondrial genes are believed to accumulate synonymous substitutions about three times slower than plastid genes (Wolf 1996), the red algal mitochondrial *cox* spacer accumulates mutations almost four times faster than the plastid RuBisCo spacer.

Timing of the divergence between A. taxiformis lineages

Here I assume that the closure of the Central American seaway by the emergence of the Isthmus of Panama 3.1 to 3.5 Ma BP (Coates and Obando 1996) terminated exchange between the tropical Atlantic and the tropical Indo-Pacific Ocean, leading to the separation of Atlantic lineage 3 from Indo-Pacific lineage 4. The subsequent intensification of the cold Benguela current system (Marlow *et al.* 2000; Weigelt and Uenzelmann-Neben 2004) could also be involved in this separation. Similar partitions of pan-tropical taxa into Atlantic and Indo-Pacific cryptic and pseudo-cryptic species have been demonstrated in the green algal genus *Halimeda* Lamouroux (Kooistra *et al.* 2002; Verbruggen and Kooistra 2004) and the red algal genera *Bostrychia* Montagne (Zuccarello *et al.* 1999b; Zuccarello

and West 2003), *Caloglossa* (Harvey) G. Martens (Kamiya *et al.* 2000; Zuccarello *et al.* 2000; West *et al.* 2001), *Spyridia* Harvey (Zuccarello *et al.* 2002) and *Grateloupia* C. Agardh (De Clerck *et al.* 2005). The separation of lineages 3 and 4 is unlikely to be the result of more ancient events such as the closure of the Tethys Sea 13-15 Ma in the Middle East (Por 1978; Sonnenfeld 1985) because the Central American seaway was open at that time and the presence of all three Indo-Pacific mitochondrial lineages of *A. taxiformis* on a strictly oceanic group of islands like Hawaii demonstrates the ability to migrate across vast oceanic expanses. There is no reason why such long distance migration would have halted at the Central American seaway.

Cryptic invasion of A. taxiformis in the Mediterranean

Two distinct *A. taxiformis* lineages coexist in the Mediterranean Sea: the central Mediterranean specimens included in Andreakis *et al.* (2004) that group with Indo-Pacific ones in lineage 2 and the Lebanese samples that group with Atlantic accessions in lineage 3. The co-occurrence of these two genetically distinct lineages in the basin indicates that at least one results from a recent immigration. Similar immigrations have been uncovered in *Polysiphonia harveyi* Bailey (McIvor *et al.* 2001) and in *Caulacanthus ustulatus* (Turner) Kützing (Rueness and Rueness 2000). *Asparagopsis taxiformis* remained confined to the eastern Mediterranean until the mid-20th century but then it suddenly expanded northwest (Boudouresque and Verlaque 2002). The scenario resembles that of the apparently sudden range expansion of *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman *et al.* Boudouresque in the Mediterranean and Canary Islands (Verlaque *et al.* 2003, 2004).

Mediterranean specimens of lineage 3 appear to be of Atlantic provenance, having invaded the eastern part of the Mediterranean only recently. Immigration must have happened in any case following the re-opening of the Strait of Gibraltar 5 Ma (Briggs 1974, Bianchi and Morri 2000) and most likely after the last glacial maximum: survival of *A. taxiformis* in the Mediterranean basin during this cold period is unlikely because minimum winter temperatures experienced during that period were below minimum survival temperatures of lineage 3 (Ní Chualáin *et al.* 2004). Lineage 3 immigrated into the Mediterranean from the Atlantic either in early historical times, well before the opening of the Suez Canal, or recently by an accidental introduction. The first report of *A. taxiformis* in the Mediterranean Sea, near Alexandria, Egypt (Delile 1813) before the opening of the Suez Canal in 1869, seems to support the first scenario. Support for this scenario can be obtained by sequencing the type material of *A. taxiformis* collected in Alexandria (Delile 1813). Both hypotheses would require a more extensive sampling in the area, in order to be tested. The discovery of Atlantic lineage 3 in the Eastern-most part of the Mediterranean makes ecophysiological sense. The Caribbean specimens of lineage 3 tested by Ní Chualáin *et al.* (2004) showed a much higher minimum temperature for survival than lineage 2 isolates so these former specimens could survive throughout the year only in the extreme south-eastern Mediterranean. It should be noted, however, that I lack direct ecophysiological data for the Lebanese population itself.

Lineage 2 probably colonized the Mediterranean from the Indo-Pacific, most likely via the Suez Canal (Por 1978). The high diversity observed at the LSU locus among the Mediterranean samples may result from the fact that each individual already possesses hundreds to thousands of LSU copies or that other bio-geographical locations have not been

sampled sufficiently. In any case, the founders could already have carried a large sample of the Indo-Pacific LSU diversity whereas they might, by chance, have carried only lineage-2-type mitochondria. Nonetheless, there must have been a large contingent of founder individuals or multiple immigrations from the Indo-Pacific into the Mediterranean because also the mitochondrial haplotype diversity in the Mediterranean is quite high. The patterns therefore suggest that the Mediterranean populations of lineage 2 result from range expansion from the Red Sea through the Suez Canal. I predict that the Red Sea populations will show high diversity in the mitochondrial marker as well but that lineage 1 is lacking there. A pre-Suez Canal invasion of lineage 2 is unlikely because all early connections established between the 13th and 8th centuries BC between the Red Sea and the Nile were freshwater (Por 1978). Also here, sequencing the type material of *A. taxiformis* collected in Alexandria (Delile 1813) could help in testing the hypothesis.

Lineage 2 has apparently now been introduced from the Mediterranean into the Atlantic where *A. taxiformis* was first detected near Faro in 2004 (Mata and Santos personal communication). However, I cannot exclude entirely that lineage 2 is not already introduced in the Atlantic coast of Africa. The genetic characterization of *A. taxiformis* in the area is based solely on a few specimens from the Canary Islands (Table 3.1), all belonged to the Atlantic lineage 3 in all three markers (Fig. 3.2). Lineage 2 may be present in Atlantic coast of Africa but this is not affecting its essentially indo-Pacific nature. Increasing sample coverage for *Cladophoropsis membranacea* (C. Agardh) Børgesen (Kooistra *et al.* 1992; Van der Strate *et al.* 2002) and *Caulerpa racemosa* var. *cylindracea* (Verlaque *et al.* 2003, 2004) in the Canary Islands recovered Mediterranean populations

where earlier studies including only a few specimens from each region missed these populations.

Conclusions

Results of this phylogeographic study confirm that both *A. armata* and *A. taxiformis* have expanded their range recently by means of invasions. The observed genetic diversity of the founder populations of *A. armata*, for both the mitochondrial and nuclear markers, suggests that it arrived by means of multiple introductions or as large contingents of individuals. Results confirm the existence of multiple, Indo-Pacific lineages and one Atlantic lineage in *A. taxiformis*. Indo-Pacific lineage 1+2 may result from a hybridization event, ancestral polymorphism and/or incomplete lineage sorting because it shows high diversity in the nuclear marker and two distinct groups in the mitochondrial one. Both the genetically diverse Indo-Pacific lineage 2 and the Atlantic lineage 3 have invaded the Mediterranean in historic times. Notably, the Mediterranean samples of the Indo-Pacific lineage 1+2 possess only one group of mitochondrial haplotypes (lineage 2 in the *cox*-haplotype network). The Atlantic lineage 3 could have arrived first in the Mediterranean but stayed confined in the South-eastern part because of its sensitivity to low winter seawater temperatures. The Indo-Pacific lineage 2 could have arrived more recently but its lower minimum temperature for survival permitted occurrence further to the north-west thus explaining the sudden range expansion of the species into the north-western Mediterranean in the second half of the 20th century. Its appearance on the south coast of Portugal shows that this lineage has now also established a bridgehead in the eastern Atlantic.

**CHAPTER IV - Microsatellite markers in an invasive strain of
Asparagopsis taxiformis (Bonnemaisoniales, Rhodophyta): insights in
ploidy level and sexual reproduction**

Abstract

Eight polymorphic nuclear microsatellite markers were identified in a strain of *Asparagopsis taxiformis*, and were applied to address population genetic structure. Previous work demonstrated four haplotype lineages at the mitochondrial cox spacer. Lineage 1 was strictly Indo-Pacific, sister lineage 2 was Indo-Pacific - Mediterranean, and lineages 3 and 4 were of Indo Pacific and Atlantic-Eastern Mediterranean origin, respectively. In this pilot study I tested the microsatellites in 15 tetrasporophytes sampled from a population off Elba (Italy), 15 gametophytes from Mergellina (Naples, Italy), 15 gametophytes from Catalina Island (California; all in lineage 2), and 15 gametophytes from Oahu Island (Hawaii, lineage 1). The markers worked in all of these but failed consistently in thalli of the remaining two cox-lineages and in *A. armata*, the sister species of *A. taxiformis*. Since many female thalli in the Mediterranean samples contained carpogonia, genotyping was performed on these supposedly haploid thalli and their diploid carpogonia, separately. As expected, external allelic contribution was detected in the carpogonia. However, even after removal of the reproductive structures, gametophyte thalli exhibited patterns consisting of up to three alleles in all of the tested populations indicating polyploidy. Up to 13 out of 15 individuals per population had a unique genotype

suggesting high intra-population variation. Results showed high genetic similarity between the two Mediterranean populations, lower similarity between these two and the Californian one within the same *cox*-lineage, and lowest similarity between these three and the Hawaiian population belonging to *cox*-lineage 1.

Introduction

Microsatellite markers are widely used in population genetic studies because of their high levels of polymorphism, their co-dominant pattern of inheritance, and consequently, the straightforward statistics needed for data evaluation (Goldstein and Schlotterer 1999).

In marine algae, genetic diversity at the population level has been explored for several species, e.g., in the seaweeds *Cladophoropsis membranacea* (Chlorophyceae, Van der Strate *et al.* 2000), *Ulva intestinalis* (as *Enteromorpha*) Linnaeus (Chlorophyceae, Alström-Paraport and Leskinen 2002), *Ascophyllum nodosum* (Phaeophyceae, Olsen *et al.* 2002), *Fucus* spp. (Phaeophyceae, Coyer *et al.* 2002a; Engel *et al.* 2003), *Postelsia palmaeformis* (Heterokontophyta, Whitmer 2002) and *Gracilaria chilensis* (Rhodophyta, Guillemin *et al.* 2005), in the marine coccolithophorid *Emiliana huxleyi* (Iglesias-Rodriguez *et al.* 2002), and in the diatoms *Ditylum brightwellii* (Ryneronson and Armbrust 2000; 2004), *Pseudo-nitzschia pungens* and *Pseudo-nitzschia multiseriata* (Bacillariophyceae, Evans and Hayes 2004, Evans *et al.* 2004). Results from these studies suggest that microsatellite loci are less abundant and/or less polymorphic than in animals or higher plants (Olsen *et al.* 2002; Wang *et al.* 1994). Additional problems are encountered when alleles are scored in diplo-haplontic isomorphic algal species because recovery of a

homozygous genotype in a thallus signifies that that thallus is either haploid or homozygous diploid (Van der Strate *et al.* 2002).

Here I developed microsatellite markers for the tropical to subtropical Rhodophyte *Asparagopsis taxiformis* (Delile) Trevisan . The species has a triphasic, diplo-haplontic, anisomorphic life cycle with haploid gametophytes (*Asparagopsis*-stages) alternation with minute carposporophytes growing on the female gametophytes, and morphologically distinct tetrasporophytes (*Falkenbergia*-stages; Feldmann and Feldmann 1939, 1942; Chihara 1961, 1962). Phylogeographic studies by Ní Chualáin *et al.* (2004) revealed cryptic diversity within *A. taxiformis* and in this thesis (Chapter III) four distinct mitochondrial lineages have been uncovered in this species. One of these (their lineage 2) occurs in the central Mediterranean where it shows recent range expansion and invasive behaviour. In this thesis, it has been hypothesized (Chapter III) that this lineage is of Indo-Pacific origin.

The present report focuses on the Indo-Pacific Mediterranean invasive lineage 2. Initial reasons for developing nuclear polymorphic microsatellite markers were: a) to assess if the four mitochondrial lineages were discernable with these markers as well, b) to uncover intra-lineage genetic variability at various temporal and spatial scales, c) to predict dispersal capability, intensity and success of colonization of new areas and ultimately d) to explain the recent expansion of lineage 2 in the Mediterranean Sea.

However, in the course of the work I encountered two factors affecting the resulting patterns, namely a) evidence of differences in ploidy levels due to polyploidy or due to heterocaryotic cells and b) the presence of carposporophytes, which develop from fertilized carpogonia on female gametophytes. In sexually reproducing gametophytes,

carposporophytes also carry paternal alleles, which affect genotyping if these structures are not removed. Multiple peaks still present in haploid individuals after the removal of mature carposporophytes probably signify polyploidy. If this interpretation is correct, then this report is the first to detect polyploidy and estimate the ploidy level in algae using microsatellites.

Materials and methods

Collection of material and unialgal culture

To obtain unialgal thallus for microsatellite development, a gametophyte thallus was sampled from ca. 1 m depth at San Pietro (Ischia, Gulf of Naples, Italy). Immature branches developing from the rhizomes were cut from the thallus, blotted briefly on tissue paper, washed repeatedly by shaking vigorously in each of a series of 10 mL-volumes of filter sterile seawater to remove any unattached organisms. Each tip was then incubated in 1 mL filter sterile seawater (SSW) in a multi-well plate (Falcon, Becton-Dickinson Labware, New Jersey, USA) on a North-facing windowsill at 20°C. After three days, the plantlets were blotted again, transferred in fresh SSW and tips with a length of 1 mm were cut from the newly developed growth. These tips were washed as aforementioned, and incubated as described before but now in 1 mL autoclaved *f/2* growth medium (1 ml of NaNO₃ (75.0 g/L dH₂O), 1 ml of NaH₂PO₄·H₂O (5.0 g/L dH₂O), 1 ml of *f/2* Trace Metal Solution, 0.5 ml of *f/2* Vitamin Solution and filtered seawater up to 1.0 L; Guillard, 1975). After two weeks, growing thalli were screened and unialgal ones were transferred to 250 mL *f/2* growth medium in sterile glass Erlenmeiers and incubated on a windowsill under daylight and room temperature conditions.

For microsatellite genotyping, 15 thalli were collected at each of the following sites: Elba (Italy), Mergellina, (Naples, Italy), Catalina Island (California; all lineage 2), and Oahu Island, (Hawaii; lineage 1). Twenty specimens of *A. taxiformis* lineage 3 (collected in Canary Islands) and five of lineage 4 (collected in Panama and Hawaii) and ten specimens of *A. armata* (from Ireland and France) were tested for evaluating cross-hybridization of the microsatellite loci. Lineage designation is based on mtDNA *cox* spacer haplotype groups as described in Chapter III.

DNA extraction

DNA extraction was carried out as described in Chapter II (Andreakis *et al.* 2004) from: a) fresh frozen at -20° C thalli and b) from silica gel-desiccated thalli conserved in room temperature. For the construction of an enriched genomic library, DNA was isolated from 5 gr. fresh unialgal tissue. For microsatellite genotyping, DNA was extracted from approximately 30 mg of tissue sampled directly from field material. Carposporophytes were removed using a binocular microscope.

Microsatellite development

Total DNA was digested separately with blunt cutters *Alu* I, *Hae* III and *Rsa* I restriction enzymes according to the manufacturer's instructions and phenol/chloroform purified. Fragments of 300 to 800 bp were excised from a 0.8% agarose gel for the enrichment procedure with (AT)₁₅ (AC)₁₅ and (AG)₁₅ biotinylated oligonucleotides. The enrichment protocol followed the method of Kijas *et al.* (1994), which implies the use of streptavidin-coated magnetic particles and biotinylated probes (PRIMM srl, Milan, Italy).

The resulting single stranded DNA enriched fragments were amplified, double-digested with *Eco* RI/*Hind* III endonucleases, ligated into the *Eco* RI/*Hind* III digested pUC19 vector and transformed into DH5 α chemically competent cells. A polymerase chain reaction (PCR) based method (Isaksson and Tegelstrom 2002) was used to estimate the presence of microsatellite motifs: recombinant clones were picked up with a sterile tip or toothpick and dipped into a PCR tube prepared with 10 μ l of PCR mixture containing 1X PCR buffer, 60 μ M each of dNTPs, 400 nM each of the two vector specific primers, 400 nM of the microsatellite oligo and 0.5 units of *Taq* polymerase (BIOGEM). After a 5-min denaturation step at 94°C, 30 cycles were performed as follows: 30 s at 94°C, 30 s at 50°C, 45 s at 72°C, and finally a 10-min extension step at 72°C. PCR products were then separated on a 2% agarose gel and visualized by Et-Br staining. Cloned fragments lacking a microsatellite region were expected to give a single amplification product resulting from the amplified poly-linker region of the vector and the inserted fragment. Contrary, multiple bands were expected from cloned fragments including microsatellite motifs. Nearly 650 positive colonies were screened and clones showing multiple bands were then grown in liquid media overnight for plasmid DNA isolation and sequencing. Microsatellite primer pairs from suitable sequences were designed with the help of the interactive web interface of the primer 3 software (Rozen and Skaletsky 1998).

PCR optimization and microsatellite amplification and detection

Microsatellite amplifications were conducted in a 10 μ L reaction mixture containing approximately 5 to 10 ng of template DNA, 5 mM MgCl₂, 1X PCR buffer, 0.2 mM of each dNTP, 0.4 μ M of each primer and 0.4 units of *Taq* DNA polymerase (BIOGEM).

Locus (GenBank #)	Core sequence	Primers 5' to 3'	Size (bp)	°C	# alleles
AT-1 (xxxxx)	(CA) ₇ TC(ACC) ₃ (GCA) ₃	F:CCCAGATACGCACCTCCAG R:TGACGGGCTGGTTTAGTAGG	178	57°	4
AT-2 (xxxxx)	(GGGAGT) ₄ (GAG) ₄	F:CAGACGGGAGAGGAAGAATG R:TCTACTCCCCTTCGAAACCA	214	58°	2
AT-3 (xxxxx)	(TCGG) ₂ C(CG) ₃ A(CA) ₃ T(AC) ₄	F:CGATACGGAGTTTGGTCAGC R:TGTGTGAGTCTGAGCGATCC	153	58°	3
AT-5 (xxxxx)	(GT) ₃ C(TG) ₄ C(TG) ₃	F:ACTTGTGGGTTCTCCA R:AGAGATCGAACGCAGGACAT	153	59°	4
AT-7 (xxxxx)	(GT) ₇	F:ATATCGATCGGTGAGGAACG R:TTCGTTCAGTGACGGCATA	116	57°	6
AT-11 (xxxxx)	(GC) ₄	F:ACTTCGGCTTTCACCTCGTA R:TCGTGAAGCCTCTTTTGGAT	154	59°	-
AT-23 (xxxxx)	(ACGT) ₂ (GC) ₃ (CG) ₂	F:AGTTAGCTATTAATGGTTGTTCTGC R:CAATTCGACGGACGGTAAA	180	59°	4
AT-26 (xxxxx)	(GAGAAG) ₄ GAGA(GGGAGAAGGAGA) ₄	F:ACACGTCCAGAGGGACTGG R:CCTCATTCTCTACCAAAGC	183	60°	7
AT-30 (xxxxx)	(ACC) ₃ CACT(GACC) ₄	F:CCATTCACTGACCCAGAGGT R:CTCGGTACCCACGAAGGATA	205	57°	7

Table 4.1. Core sequence, forward and reverse PCR primer sequence for each of the nine microsatellite loci used in this study. Size refers to the originally cloned allele. °C refers to the optimal primer annealing temperature. F denotes forward primer; R, reverse primer. # alleles refers to the total number observed among the 60 specimens from the four populations tested.

Initial denaturation was 4 min in 94°C followed by 20 to 35 cycles of: 40 s at 94°C, 40 s at the primer annealing temperature (Table 4.1), 40 s at 72°C and a final extension of 5 min at 72°C. 10 µL formamide dye was added to the PCR product and the final mixture was denatured for 5 min at 94°C. Products were separated by running 5 µL in a 6% denaturing polyacrylamide electrophoresis gel, and visualized by a DNA silver staining system (PROMEGA). To further ensure that PCR products were the cloned microsatellite loci, target bands were run on 1% agarose TAE buffered gel against known standards, excised under low UV-light, purified using the QIAEX II Gel Extraction kit 500 (Qiagen GmbH, Hilden, Germany) and sequenced using the locus-specific primer pair. Sequence reactions

were obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City CA) and purified in automation using a robotic station “Biomek FX” (Beckman Coulter, Fullerton CA). Products were analyzed on an Automated Capillary Electrophoresis Sequencer “3730 DNA Analyzer” (Applied Biosystems). For genotyping, 0.5-1.0 μ L of PCR products were loaded on a 96-wells plate with 0.2 μ L Size Standard (Beckman, Coulter, Fullerton CA) and 10 μ L formamide. Allele detection was conducted using an automated sequencer (CEQ 2000XL DNA Analysis system, Beckman Coulter). Finally, electropherograms were analyzed with the software Beckman CEQ 2000 v3.0. Amplifications were replicated several times in order to check reliability of results.

Analysis of banding patterns

Each amplicon variant was treated as an allele and scored as present/absent. A pairwise distance matrix was created according to the Lynch similarity index (1990), developed for DNA fingerprinting. Number of distinct genotypes and genetic diversity (Nei 1987) were computed using the GenoType and GenoDive programs (Meirmans and Van Tienderen 2004). Pairwise Nei’s distance (1973) among populations was obtained with GenALEX 6 software (Peakall and Smouse 2005).

Results

To select suitable microsatellites, 150 recombinant clones containing an insert of appropriate length (average size of 300 bp) were sampled and sequenced. About 70% of the sequenced clones contained small or imperfect dinucleotide repeats, the vast majority of which were AG or AC rich. In some cases mini-satellite motifs (6 to 110 bp fragments

repeated 2 to 4 times) were present instead of microsatellites. Specific primers were designed for 16 of the sequenced clones found to have perfect or imperfect microsatellite motifs of a suitable length. Eight of them showed reliable and consistent amplification results and were used for further analysis. One of them was characterized by a pure dinucleotide repeat (AT-7) while the other six contained multiple or interrupted repeated regions (Table 4.1). A ninth locus (AT-11) failed to amplify in more than 50% of the specimens, and was not used. The selected primer pairs (Table 4.1) generated products with DNA from thalli belonging to mitochondrial cox-haplotype lineages 1 and 2, Products were of the expected size and showed the repeated motif when sequenced. All primer pairs failed to give amplification products from DNA isolated from thalli belonging to co-generic *A. armata* or to *A. taxiformis* mitochondrial lineages 3 and 4. Initial scores with loci AT-7 and AT-30 deployed on gametophyte thalli showed multiple alleles, and at times several additional small peaks. I realized that carposporophytes present in many of the gametophyte thalli could explain the multiple peaks. So, I compared the genotypes for loci AT-7 and AT-30 of four thalli bearing carposporophytes with the genotypes of these thalli devoid of carpospores and the genotypes of the four sets of carpospores. For both loci, carposporophytes exhibited the alleles present in the cleaned maternal gametophytes, and in two of the four thalli examined at least one extra allele of reliable molecular size (Fig. 4.1). All further experiments were carried out with tetrasporophytes (the sample of the population from Elba) or with gametophytes from which carposporophytes had been removed prior to DNA extraction. In all the samples, the resulting scores showed between one and three peaks in the eight loci analyzed (Table 4.2).

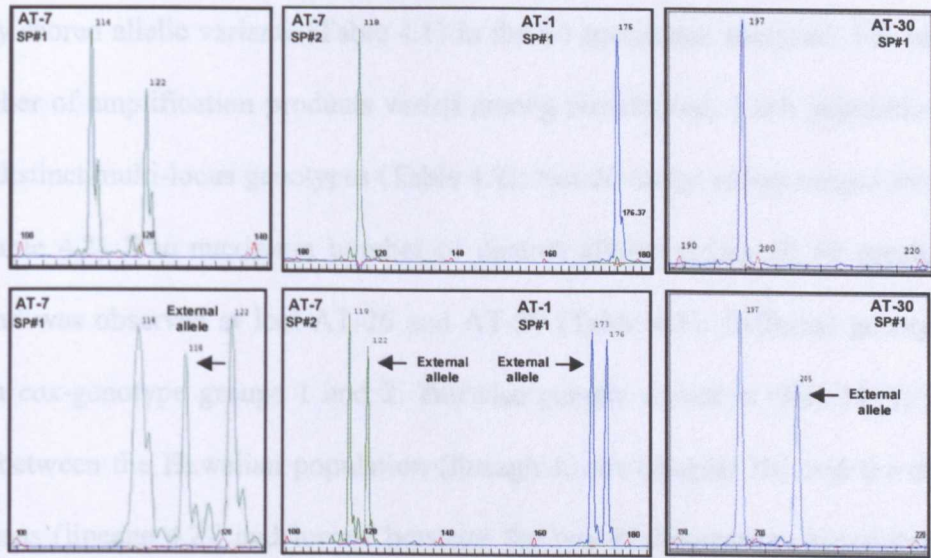


Fig. 4.1. Single locus genetic profiles of an *A. taxiformis* gametophyte. DNA was extracted and PCR amplified at loci AT-1, AT-7 and AT-30 from clean thalli, (upper panels), and from only mature carpogonia (lower panels). Arrows indicate supernumerary, probably paternal alleles. Specimens utilized (SP#1 and SP#2 are from Mergellina population).

pop	#	S	L	# of distinct amplification products per locus								D	Div
				AT-1	AT-2	AT-3	AT-5	AT-7	AT-23	AT-26	AT-30		
ELB	15	T	2	2	1	1-2	1	2-3	1-3	3	1-2	10	0.83
MER	15	G	2	1-2	1	1-2	1	2-3	1-2	3	1-2	12	0.89
CAT	15	G	2	1	1	1	1	1	1-2	2-3	1-2	9	0.84
HAW	15	G	1	1-2	1	1-2	1-2	1	1	3	1-3	13	0.91

Table 4.2. Number of amplification products for each locus and in each population. ELB (Elba Is. Italy), MER (Mergellina, Naples, Italy), CAT (Catalina Island, California), HAW (Hawaii, USA). S, life stage; T, tetrasporophyte; G, gametophyte; L, mitochondrial lineage (see Chapter III); D, distinct genotypes found per population; Div = Nei's (1987), genetic diversity.

Length differences among alleles of the same locus could always be referred to addition/deletion of one or more repeated units. The eight polymorphic loci gave a total of

37 easily scored allelic variants (Table 4.1) in the 60 specimens analyzed. For each locus, the number of amplification products varied among populations. Each population showed several distinct multi-locus genotypes (Table 4.2); Nei diversity values ranged from 0.83 to 0.91 (Table 4.2). The maximum number of distinct alleles across all 60 specimens was seven and was observed at loci AT-26 and AT-30 (Table 4.1). Different genotypes were found in *cox*-genotype groups 1 and 2. Pairwise genetic distances (Nei 1978) were also highest between the Hawaiian population (lineage 1, see Chapter III) and the other three populations (lineage 4.2), and lowest between the two Mediterranean populations (Table 4.3).

L	Pop	ELB	MER	CAT	HAW
2	ELB	-			
2	MER	0.034	-		
2	CAT	0.405	0.369	-	
1	HAW	0.774	0.678	0.420	-

Table 3. Pairwise Nei's (1973) genetic distance among the four populations analyzed. L, mtDNA lineage (see Chapter III). ELB (Elba Is. Italy), MER (Mergellina, Naples, Italy), CAT (Catalina Island, California), HAW (Hawaii, USA).

Discussion

Eight polymorphic nuclear microsatellite markers were identified in the Indo-Pacific Mediterranean strain of *A. taxiformis*. Most of the selected loci were characterized by interrupted repeats, confirming the results obtained in the only published study on microsatellites isolation from red algae, namely in *Gracilaria chilensis* (Guillemin *et al.* 2005). In that species, only one of the six selected loci showed a pure dinucleotide repeat, while complex repeated sequences characterized the others. Interrupted repeats are

expected to have lower mutation rates than pure ones (e.g. Sainudiin *et al.* 2004), but nevertheless, the loci developed here showed sufficient variation for meaningful genetic analysis given the up to thirty seven distinct alleles detected among the 60 specimens analyzed, and between 9 and 13 distinct genotypes in the four populations of 15 individuals, each.

Two major problems were encountered during microsatellite selection and testing for polymorphism. The first was the presence of carposporophytes on mature thalli of *A. taxiformis*, which contain external and thus potentially different alleles. The second was the presence of multiple alleles in thalli even if carposporophytes were removed.

The complete disappearance of alleles in thalli from which carposporophytes were eliminated in most of the experiments performed, demonstrated that the removal was effective. The detectable signal of paternal genotypes in thalli adorned with carposporophytes demonstrates that parental genotypes can affect allele scoring in seaweeds with a similar life history. Nonetheless, the results show that sexual reproduction occurs in *A. taxiformis*, and that male gametes constitute a significant vehicle of gene flow, though the actual distance over which they can travel still remains to be uncovered. Gametes and tetraspores are believed not to travel effectively very far (Santelices 1990). Yet, dislodged *Asparagopsis* thalli drift and the abundance of floating gametophytes in late spring-summer suggest that they are a major source of dispersal. These floating thalli can shed their carpospores and male gametes along the way. Likewise, dislodged and drifting tetrasporophytes can shed their carpospores along the way. Of course, the effective contribution of these drifting thalli to gene flow needs to be investigated in detail.

The multiple allelic variants encountered in seven of the markers in carposporophyte-free thalli are consistent with the existence of polyploidy, though I was aware of the remote possibility of genotyping errors in microsatellite scoring (Hoffman and Amos 2005). Two factors seem to confirm the above interpretation. First, I obtained consistent results in all the cases when experiments were repeated. Second, multi-allelic profiles were consistent for each locus within the same lineage, independently from the site where the samples were collected. Moreover, allele scores from carposporophytes invariably included the alleles observed in the maternal thallus from which carposporophytes were removed. According to the number of allelic variants encountered in my results I can conclude that *A. taxiformis* mtDNA lineage 1 and 2 is composed of gametophytes that are at least triploid because these thalli exhibit up to three distinct alleles for most of the loci tested. However, the assessment of the precise ploidy level is very problematic with this kind of data (Bruvo *et al.* 2004) also in relation to the possibility of sympatry of individuals with different ploidy levels (Trewick *et al.* 2002).

Polyploidy can originate through allo-polyploidization or endo-polyploidization. Allopolyploidy (i.e. the co-presence of several genomes of different origin in the same cell) could result from hybridization between different lineages. Cross-hybridization of microsatellite loci in the two mtDNA sister lineages 1 and 2 (Chapter III; this Chapter) suggests that homologous sets of chromosomes belonging to the two lineages may have associated through hybridization and introgression events. Endo-polyploidy (i.e. the multiplication of DNA and chromosomal number without cell division) was the mechanism proposed in brown (Garbary and Clarke 2002), green (Hinson and Kapraun 1991) and red algae (Golff and Coleman 1990). Variations in DNA content can happen via endo-

reduplication or during mitosis (Goff and Coleman 1986, 1990). Both mechanisms could account for the ploidy levels found in the analysis. Moreover, Kapraun (2005) reported variability in the number of nuclei within cells and in the nuclear DNA content of different cells in the same thallus of several red algae suggesting that endo-redublication and endo-ployploidization are relatively easy. Multiple alleles however, may be the result of heterocaryotic cells formed during the carposporophyte development (see introduction). Such cells contain haploid nuclei, from one parental lineage, and true diploid nuclei from the fusion of both parental lineages nuclei. In *Chondrus* spp. for example, after presumed fertilization, haploid medullary cells are transformed in multinucleate diploid cells containing also haploid nuclei. Numerous enucleate protrusions are created in all sides of the cell some of which receive a diploid nucleus. The result is the creation of a diploid tissue from where the gonimoblast filaments born (Cole and Sheath, 1990; Fredericq *et al.* 1992). The removal of the already mature carposporophyte at this point will not eliminate the presence of multiple alleles.

The existence of more than two alleles for each single-locus-amplification prevented the use of statistics for co-dominant markers and forced me to use approaches standardized for dominant markers. Nevertheless, I was able to derive preliminary results from my set of data. First, microsatellite markers detected higher genetic variation and resolution within *A. taxiformis* lineage 2, than obtained with mtDNA marker (Chapter III). The similarity between the Mediterranean populations and the California population is congruent with findings obtained with the *cox* marker because the three populations all belong to lineage 2 whereas the more distantly related Hawaiian population belongs to *cox* lineage 1. Moreover, failure of the microsatellite primers to generate any products with

thalli in lineages 3 and 4 corroborated the distinction of lineage 3 and 4 from lineages 1 and 2 and confirms the existence of cryptic diversity in *A. taxiformis* reported by Ní Chualáin *et al.* (2004) and by Andreakis *et al.* (2004), Chapter III of this thesis.

In conclusion, microsatellite markers can be used in algae to uncover different ploidy levels. High intra-population genetic variability, consistent differentiation among distinct localities and supernumerary alleles, found in mature carposporophytes, demonstrated input of external genotypes by means of sexual reproduction. Finally, I point out that, because of the possible presence of external alleles in the mature thalli, attention must be paid in population genetic studies of algal species with similar reproductive strategies.

CHAPTER V - Microsatellite diversity in the Mediterranean invasive seaweed *Asparagopsis taxiformis* (Bonnemaisoniales Rhodophyta).

Abstract

Gametophytes of the invasive Indo-Pacific Mediterranean lineage 2 of the rhodophytan *Asparagopsis taxiformis* form conspicuous, dense stands from 0.5 to 30 m deep in the central Mediterranean Sea. Recently eight polymorphic microsatellite markers have been developed against this lineage, though they function also with individuals of the Indo-Pacific lineage 1. The markers revealed high levels of genetic variability within populations and showed multiple bands even in the gametophytes, the latter was interpreted as evidence for polyploidy. Here I used these microsatellites to assess if genetic differentiation exists between lineages 1 and 2 as well as among Mediterranean populations in lineage 2. Genetic structure exists only among geographically distant populations as demonstrated by only modest levels of gene flow or even a lack thereof. The Hawaiian population (HAW; lineage 1) was distinct from populations belonging to the Indo-Pacific Mediterranean lineage 2. The latter lineage shows a clear distinction between geographically distant populations (California vs Mediterranean Sea). All Mediterranean populations analysed within lineage 2 exhibited high genotypic diversity, high gene flow and low differentiation amongst each other. This result corroborates the recent invasion of the lineage. Bayesian population clustering indicated that the Mediterranean sites were structured panmictically in eight weak clusters without any geographical structure typical

for rapidly expanding populations. Moreover, populations reproduce predominantly sexually; clonal reproduction does not contribute significantly to genetic isolation by distance at a local scale. Therefore, the observed genetic patterns among Mediterranean populations corroborate with the current invasive behaviour of the species in the Mediterranean Sea.

Introduction

Population genetic structure results from interactions between demographic, ecological and genetic factors of the species and the environment in which the species live. Marine algae constitute particularly interesting but also challenging targets for population genetic studies because of their complex life cycles and the dynamics of the marine environment (Norton *et al.* 1996). Distinct life stages (e.g., gametophytes, tetrasporophytes) are often phenotypically identical; both form macroscopic thalli growing side by side (Van den Hoek *et al.* 1995; Van der Strate *et al.* 2002). Or they differ morphologically to such an extent that they are not even recognized as belonging to the same species (e.g. Porphyra-Conchocelis; Mastocarpus-Petrocelis; Asparagopsis-Falkenbergia). For instance, one stage can be microscopically small and elusive. Moreover, propagules of the different stages often have different dispersal potentials.

In algae, molecular systematics and phylogeography constitute powerful approaches for elucidating contemporary geographical patterns of evolutionary lineages within species and species complexes (Arbogast and Kenagy 2001). In some cases, the comparison of phylogeographic patterns of so-called cosmopolitan taxonomic groups have revealed cryptic vicariance events and previously unrecognized biogeographic patterns (Wattier and

Maggs 2001; Van der Strate *et al.* 2002; Gabrielsen *et al.* 2003; Zuccarello and West 2003; Verbruggen *et al.* 2005; De Clerck *et al.* 2005; this thesis).

Systematics and reproduction of red algae has been extensively studied (Saunders and Hommersand 2004). Intra-individual variation in nuclear DNA content with emphasis to the extent of ploidy levels, GC content and genome complexity in target taxa is also widely studied. Moreover, the first attempts to genetically modify commercially important seaweeds are now in progress (Garbary and Clarke 2002; Kapraun 2005). Population genetic structure and measurements of gene flow in algae was first studied by isozyme electrophoresis (Sosa and Lindstrom 1999). However the low resolution power of the method often hindered the results of these studies. Nuclear microsatellite markers have been used to explore population structure, mating system, dispersal and genetic contribution of different life phases in only few species i.e., *Ulva intestinalis* (as *Enteromorpha*) (Chlorophyceae, Alström-Paraport and Leskinen, 2002), *Ascophyllum nodosum* (Phaeophyceae, Olsen *et al.* 2002), *Fucus* spp. (Phaeophyceae, Coyer *et al.* 2002a; Engel *et al.* 2003), *Postelsia palmaeformis* (Heterokontophyta, Whitmer 2002) and *Gracilaria chilensis* (Rhodophyta, Guillemin *et al.* 2005). Results from these studies suggest that microsatellite loci are less abundant and/or less polymorphic in algae than in animals or higher plants (Olsen *et al.* 2002; Wang *et al.* 1994). Moreover co-dominant microsatellites are not appropriate for species in which haploid and diploid individuals cannot be distinguished due to isomorphy (Van der Strate *et al.* 2002). In *Cladophoropsis membranacea*, Van der Strate *et al.* (2000), explored the contribution of the two different life phases and clones of the species to the population genetic pool as well as the effect of dispersal on the population structure at different spatial scales. Wallace *et al.* (2004) by

using four microsatellite loci uncovered evidence of hybridization and introgression between *Fucus vesiculosus* and *F. spiralis*, two morphologically plastic species. Recently, microsatellites have been developed against the invasive brown seaweed species *Undaria pinnatifida* (Daguin *et al.* 2005), to assess mechanism of introduction, local recruitment and reproduction strategies in newly introduced populations.

In the present study I used eight polymorphic nuclear microsatellite markers recently developed against a Indo-Pacific Mediterranean strain of the tropical to subtropical rhodophyte *Asparagopsis taxiformis* (Delile) Trevisan. This morphologically defined species consists of three and probably four cryptic species (Chapter III). One of these (lineage 2) occurs in the central Mediterranean where it expanded rapidly in recent years by forming conspicuous populations and by colonising habitats occupied by species of *Cystoseira* C. Agardh (Barone *et al.* 2003; Chapter II in this thesis). *A. taxiformis* populations in the Gulf of Naples consist of conspicuous patches between 0.5 and 30m depth (Flagella *et al.* 2005). *A. taxiformis* reproduces clonally through fragmentation of the tetrasporophytic and the gametophytic phases, and sexually via a triphasic, haplo-diplondic, heteromorphic life cycle (Feldmann and Feldmann 1939, 1942). Moreover, gametophytes of the Indo-Pacific/Mediterranean lineage 2 of *A. taxiformis* have been reported to be dioecious, with spermatangia and cystocarps on different thalli (Barone *et al.* 2003), while Australian plants have been consistently reported as monoecious (Adams 1994). Mating system and reproductive strategies can thus strongly affect the distribution of the genetic variation, the demography and the dispersal capabilities of the species.

The microsatellite markers developed against *Asparagopsis taxiformis* (see Chapter IV) showed high genetic variability within and between lineages 1 and 2. Preliminary

results also showed that sexual reproduction is active in *Asparagopsis* populations, and that both lineages were polyploid (Chapter IV). In this Chapter my aims are to a) assess levels of population genetic variability in *A. taxiformis*, b) study genetic differentiation and structure among populations within and between lineages 1 and 2 of *A. taxiformis*, c) infer dispersal capabilities and directionality of gene flow within the Mediterranean Sea and, in particular the Gulf of Naples, in order to clarify the partition of genetic diversity in the studied area.

Materials and Methods

Sample collection and storage

A total of 319 specimens of *Asparagopsis taxiformis* collected from 12 Mediterranean and three extra-Mediterranean populations were included in the analysis (see Table 5.1 and Fig. 5.1). Most samples consisted of gametophytes. Only 24 individuals were “*Falkenbergia*-stage” and since the ones of *A. taxiformis* are morphologically indistinguishable from those of *A. armata*, they were identified using the mtDNA sequence markers used in Chapter II and III. Almost all samples grouped within mtDNA lineage 2 (Chapter III). Only specimens from Ka’alawai (Hawaii) belonged to mtDNA lineage 1. In almost all sites eight samples were collected randomly in each of three quadrats of about 25 square meters (Fig. 5.1 and sampling strategy in appendix 3).

Pop	Locations	# specimens	Life stage	L	Collection date
ADR	Dubrovnik-Portopetra, Croatia	8	G	2	18-10-2003
PAN	Pantelleria, Italy	24	G	2	20-05-2004
TRAP	Trapani, Sicily, Italy	24	G	2	22-07-2002
TUN	Mahdia, Tunisia	12	G	2	06-06-2003
MER	Mergellina, Naples, Italy	24	G	2	19-04-2002
CAP	Capo Posillipo, Naples, Italy	24	G	2	20-08-2002
SAG	Sant'Angelo, Ischia, Naples, Italy	24	G	2	22-06-2002
CAS	Castello, Ischia, Naples, Italy	24	G	2	28-07-2002
PUP	Punta Pizzago, Procida, Naples Italy	24	G	2	25-03-2003
PSP	P.ta S. Pietro, Ischia, Naples, Italy	24	G	2	28-01-2003
ELB	Elba, Italy	24	T	2	25-08-2003
FRA*	Marseille, France	24	G	2	15-05-2004
POR*	Sagres, Faro, Portugal	11	G	2	25-04-2005
CAT	Santa Catalina Is., California, USA	24	G	2	14-06-2004
HAW	Ka'alawai, O'ahu, Hawaii, USA	24	G	1	15-05-2002

Table 5.1. List of abbreviated population names, geographic locations and collection date. "G" denotes gametophyte, "T", tetrasporophyte, "L" mitochondrial lineage according to results reported in Chapter III. *Recently colonized sites.

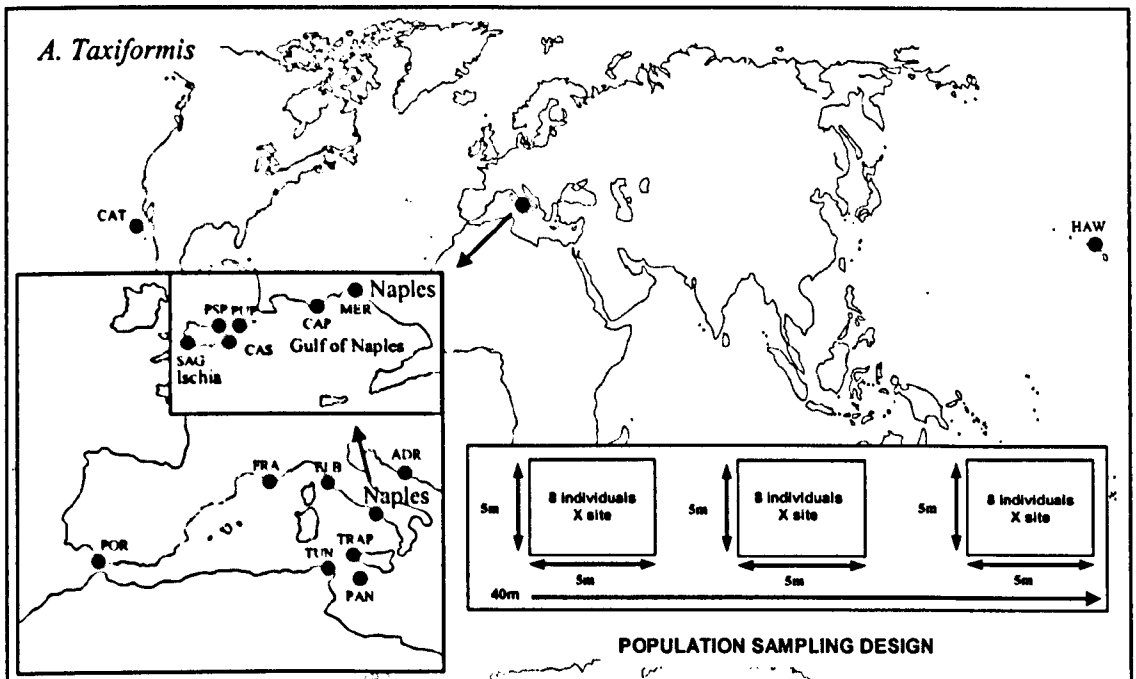


Fig. 5.1. Map indicating geographic localities of the populations of *Asparagopsis taxiformis*, and the sampling design.

Reciprocal distance between quadrats was 10 to 15m. In Croatia (ADR), Tunisia (TUN) and Portugal (POR) individuals were collected within a single quadrat. Samples were washed in dH₂O and either desiccated in silica gel or frozen at -80°C before DNA extraction.

DNA extraction

In order to exclude the presence of external alleles (see chapter IV), small portions (0.5 cm) of gametophytic thallus from each individual was checked under a binocular microscope for the presence of carpogonia. It is impossible however to assess whether diploid tissue is still present or not in the thallus (see chapter IV). Frozen or silica gel-desiccated tissue was either thawed or re-hydrated in dH₂O before analysis. Selected portions of carpogonia-free thalli were crushed with a micro-pestle in a 0.5 mL PCR tube containing 50 µl of 1x Roche diagnostics PCR reaction buffer. The mix was heated for 5 min at 95°C and centrifuged for 3 min in a table-top Eppendorf microfuge (Eppendorf AG, Hamburg, Germany) at maximum speed (14,000 rpm). One µl of the aqueous phase was used as a template for PCR amplifications. The DNA extracted this way was amplifiable for more than four months if stored at 4 C°.

Eight variable microsatellite loci, selected from the invasive Indo-Pacific Mediterranean lineage 2 of *A. taxiformis*, were used in the present analysis. For PCR conditions and fragment analysis see Chapter IV. Amplification patterns were highly reproducible. Allele detection was conducted using an automated sequencer (CEQ 2000XL DNA Analysis system, Beckman Coulter) and electropherograms were analyzed with the Beckman CEQ 2000 v3.0 software.

Scoring of polymorphisms

In polyploid species, assessment of the number of alleles of the same size in heterozygous individuals is not possible rendering correct identification of the genotype difficult (Espinoza and Noor 2002; Bruvo *et al.* 2004). Therefore, the number of detected alleles is often lower than the ploidy level of the species, making it also impossible to detect null alleles due to the lack of meiotic segregation (Samadi *et al.* 1999; Bruvo *et al.* 2004). To complicate matters even further, individuals with different ploidy levels may co-occur in the same population (Trewick *et al.* 2002; Wilmhoff *et al.* 2003). Hence, polyploidy can significantly affect estimates of population genetic parameters when using co-dominant markers such as microsatellites.

The existing statistics for microsatellites has been developed principally for diploid organisms and hence is not suitable for species with different ploidy levels (Bruvo *et al.* 2004). It is based on the proportion of shared alleles; it assumes that variability of loci follows the infinite allele model, that allelic variants are independent, and it ignores mutational processes. Different strategies have been proposed for the population genetic analysis of polyploid species from multilocus microsatellite genotypes. Those specifically take into account the coexistence of individuals with different ploidy levels in the same matrix (Samadi *et al.* 1999; Zhang *et al.* 1999; Espinosa and Noor 2002; Bruno *et al.* 2004;).

In the present study, multilocus genotypes were compared using an index of similarity based on shared allelic variants as in Samadi *et al.* (1999) and developed for

DNA fingerprinting (Lynch 1990). Statistic parameters were gathered from amplification variant frequencies and genotypic frequencies rather than allelic frequencies. Amplification variants were treated as an allele and scored as presence/absence. The resulting pairwise distance matrix was then used to analyse clonal diversity, population genetics parameters and genetic relationships between and among populations and individuals.

Analysis of genotypic diversity

Asparagopsis taxiformis reproduces both clonally and sexually. Identical genotypes can either be clone-mates or identical by chance. I performed tests to ascertain whether a) microsatellite markers have a good statistical power to discriminate clones, b) sexually produced genotypes share the same multilocus genotype and, c) putative clones are clustered spatially according to an expected vegetative spread (Epperson 1989; Berg and Hamrick 1994; Chung *et al.* 2004a).

To perform these tests I used both the number of alleles (amplification variants) and banding patterns. First, I considered the number of alleles encountered for each microsatellite locus and I calculate:

a) the theoretical number of distinct genotypes within the entire data set according to the following equation (Reusch *et al.* 1998):

$$Ng = \prod_{i=1}^L [a_i(a_i + 1)]/2 \quad (1)$$

where L is the number of loci and a_i is the number of alleles at the locus i .

b) the discriminating power of the microsatellite markers for each population as $1 - P_G$. The probability that two random, sexually produced genotypes are identical, (P_G) was calculated using the following formula:

$$P_G = \prod_n \sum_r (g_k)^2 \quad (2)$$

where g_k is k 's genotype frequency per locus, r is number of genotypes per locus, and n is the number of loci (Berg and Hamrick 1994; Chung *et al.* 2004a).

c) the probability of sampling without replacement two individuals that differ in multilocus genotypes in the population (D_G), assessed as a modification of the Simpson index (Pielou 1969; Gregorius 1987) according to the formula:

$$D_G = 1 - \sum \{ [n_i(n_i - 1)] / [N(N - 1)] \} \quad (3)$$

where n_i is the number of individuals of genotype i and N is the total number of individuals in the population.

Second, using the number and frequency of banding patterns for each microsatellite locus (e.g., genotype -/150/152/- in bp for locus AT-3 corresponds to the banding pattern 0110) I estimate:

d) the confusion probability (C_j) of the j -th assay unit (Tessier *et al.* 1999, Belaj *et al.* 2003) as:

$$C_j = \sum_{i=1}^I p_i [(N p_i - 1) / (N - 1)] \quad (4)$$

where p_i is the frequency of the i -th pattern; N , the sample size; i , total number of patterns generated by the j -th assay unit,

e) the Discriminating power (D_j , the probability that two randomly chosen individuals have different patterns, and thus are distinguishable from one another) of the j -th assay unit (Tessier *et al.* 1999; Belaj *et al.* 2003) as:

$$D_j = 1 - C_j \quad (5)$$

and,

f) the limit of D_j as N tends towards infinity:

$$D_L = \lim (D_j) = 1 - \sum_{i=1}^l p_i^2 \quad (6)$$

Analysis of genotypic diversity i.e. # of distinct genotypes, effective # of distinct genotypes, genotypic frequencies, pairwise Nei's genetic distances (Nei, 1987) and evenness of the genotypes, were computed using the GENOTYPE and GENODIVE computer programs (Meirmans and Van Tienderen 2004). Overall clonal diversity was calculated over the total number of samples and in each population as the proportion of distinct genotypes over the number of specimens sampled (G/N , Pleasant and Wendel 1989). A value of one indicates that each individual is a unique genotype. A value close to zero indicates that nearly all individuals exhibit the same genotype (clonally reproducing population). To assess whether the sampling intensity was sufficient to capture all the allelic diversity I plotted the mean number of alleles per locus per population adding sequentially all populations. The extra-Mediterranean populations were added as last. To assess whether enough genetic diversity was successfully captured from the eight loci utilized we plotted G/N values per locus adding loci sequentially and starting from the most polymorphic one. Each population was considered separately and overall values were also plotted in a separate graph.

Analysis of genetic structure

Molecular variance

Two different analyses of molecular variance (AMOVA) were carried out to study the partition of the genetic diversity among and within *Asparagopsis taxiformis* populations. The first analysis was performed considering only Mediterranean and extra-Mediterranean populations where 24 specimens were sampled. The second analysis included only the Mediterranean populations in two separate analyses (AMOVA and bayesian AMOVA independent from sample size). Microsatellite loci were considered as dominant markers and polymorphic amplification variants were scored directly as present/absent in the GenAlEx 6 (Peakall and Smouse 2005) software. Variation was expressed both as the proportion of the total variance and as Φ -Statistics, an F -Statistic analogues, where Φ_{rt} represents the correlation of individuals from the same geographic region relative to that of individuals from the whole data set, Φ_{pr} represents the correlation between individuals within a population relative to that of individuals from the same geographic region, and Φ_{pt} represents the correlation between individuals within a population relative to that of individuals from the whole data set. Estimates of Φ_{pt} values were also obtained for the assessment of gene flow within and between geographic regions.

Bayesian analysis of molecular variance

An alternative Bayesian analysis of molecular variance (Holsinger *et al.* 2002), independent from sample size, was also used to assess the partition of genetic variability among all the Mediterranean populations (a total of 13 populations and 271 specimens) as implemented in the HICKORY v1.0.4 software (Holsinger and Lewis 2003). Because

estimates of f derived from dominant markers data may be unreliable, especially when the sample size within populations is small, the estimate of θ^B , the bayesian analogue of population structure is based on the f -free and on the $\theta^B = 0$ models. Several runs with the default sampling parameters were performed (burn in = 50.000, sampling = 250.000, thin = 50) to ensure consistence of the results and independent estimates of $F_{ST}(\theta^B$ and $G_{st} - B$, the Bayesian analogues of F_{ST} and Nei's G_{st} respectively) were obtained. Finally, the Deviance Information Criterion (DIC; Spiegelhalter *et al.* 2002) was applied to estimate congruence between the data and a particular model and to choose among models (Holsinger and Wallace 2004).

Bayesian clustering

To understand if there is genetic structure within the Mediterranean populations, I performed a Bayesian-based clustering analysis as implemented in the STRUCTURE software version 2.1 (Pritchard *et al.* 2000). The analysis computes the appropriate number of clusters of genotypes or subpopulations (K) that fits the genetic variability encountered in the data set and interprets it without having prior information on the number of the populations sampled. Ten independent runs were performed of $K = 1$ to 13 at 50.000 MCMC (Markov chain-Monte Carlo) repetitions and a burn in period of 20.000 iterations without prior information under no admixture and assuming correlated allele frequencies. The posterior probability was then calculated for each value of K and the optimal number of clusters was chosen.

Principal coordinate analysis, spatial autocorrelation, Mantel tests, assignment tests

Amplification variants from all the Mediterranean populations were scored as binary markers and principal coordinate analysis was performed using the GenAlEx 6 software (Peakall and Smouse 2005). Mantel tests were conducted with the same software in order to assess the significance of the correlation between genetic and geographic distance and test for isolation by distance using linearized Nei's genetic distance values against minimum coastal distances among sampling sites. Assignment tests were performed in the WHICHRUN v3.2 software (Banks and Eichert 2000) in order to assess the directionality of the gene flow between recently and sub-recently invaded Mediterranean localities. The spatial pattern of the genetic variation was also evaluated using the SGS (Spatial Genetic Software) v1.0c (Degen *et al.* 2001), in order to assess the existence of spatial autocorrelation and isolation by distance. The SGS software uses the euclidean distance between pairs of data points and produces distograms based on the Tanimoto distance for dominant markers such as RAPD's and AFLP's (Gregorius 1978) and correlograms using Moran's Index for spatial autocorrelation of microsatellite data (Sokal and Wartenberg 1983). Permutation tests, (Monte Carlo simulations, $n = 1000$) were performed to evaluate deviation from a spatially random distribution against the null hypothesis of no spatial structure (Manly 1997).

Results

The eight microsatellite loci provided a total of 95 highly reproducible banding patterns of variable frequencies (Fig. 5.2a-h) and 47 allelic variants (AV) over a total of

319 specimens analysed (Table 5.2), that are distributed according to a stepwise production of alleles (Fig. 5.3a, Kimura and Ohta, 1975).

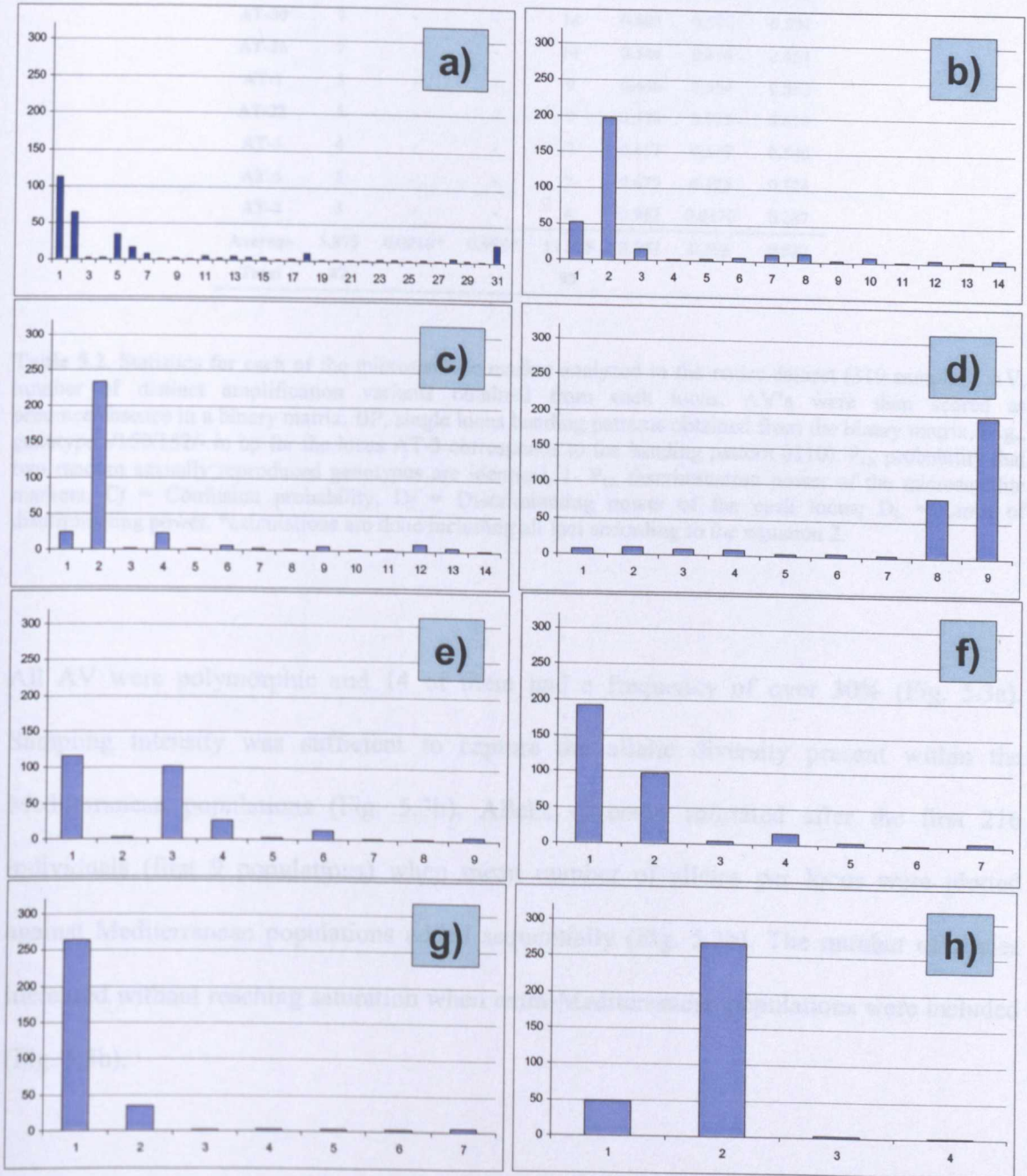


Fig.5.2. Banding pattern frequencies for each of the loci a) AT-7, b) AT-26, c) AT-30, d) AT-1, e) AT-23, f) AT-3, g) AT-5, h) AT-2. Y-Axis, number of individuals with identical banding pattern; X-axis, number of distinct banding patterns per locus.

Locus	# AV	P_G	$1 - P_G$	# BP	C_j	D_j	D_L
AT-7	11	-	-	31	0.175	0.825	0.815
AT-30	7	-	-	14	0.403	0.597	0.594
AT-26	7	-	-	14	0.544	0.456	0.454
AT-1	5	-	-	9	0.446	0.554	0.553
AT-23	5	-	-	9	0.378	0.622	0.610
AT-3	4	-	-	7	0.457	0.543	0.540
AT-5	5	-	-	7	0.675	0.325	0.324
AT-2	3	-	-	4	0.982	0.0170	0.287
Average	5.875	0.0016*	0.998*	11.875	0.507	0.493	0.522
Total	47			95			

Table 5.2. Statistics for each of the microsatellite marker analyzed in the entire dataset (319 samples). AV, number of distinct amplification variants obtained from each locus. AV's were then scored as presence/absence in a binary matrix. BP, single locus banding patterns obtained from the binary matrix, (e.g., genotype -/150/152/- in bp for the locus AT-3 corresponds to the banding pattern 0110). P_G , probability that two random sexually reproduced genotypes are identical, $1 - P_G$, discrimination power of the microsatellite markers, C_j = Confusion probability; D_j = Discriminating power of the each locus; D_L = Limit of discriminating power. *calculations are done including all loci according to the equation 2.

All AV were polymorphic and 14 of them had a frequency of over 30% (Fig. 5.3a). Sampling intensity was sufficient to capture the allelic diversity present within the Mediterranean populations (Fig. 5.3b). Allelic diversity saturated after the first 216 individuals (first 9 populations) when mean number of alleles per locus were plotted against Mediterranean populations added sequentially (Fig. 5.3b). The number of alleles increased without reaching saturation when extra-Mediterranean populations were included (Fig. 5.3b).

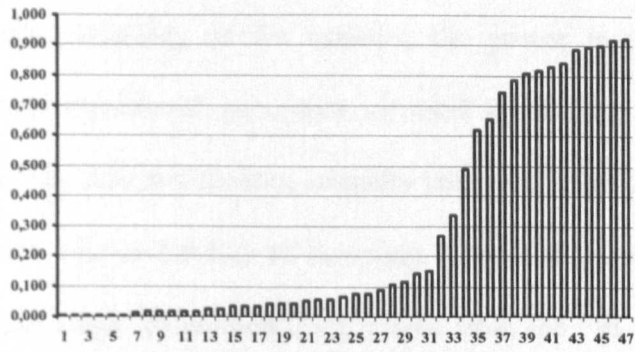


Fig. 5.3a. Distribution of amplification variant frequencies in the entire dataset.

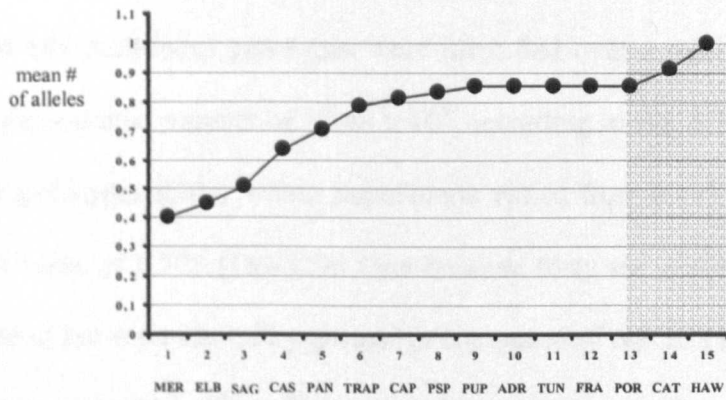


Fig. 5.3b. Mean number of alleles per locus by adding one after another all populations, starting from the Gulf of Naples.

Given the allelic variability of the markers, the power to discriminate clonal genotypes from sexually reproduced genotypes identical by chance was 0.998 ($1-P_G$), where the mean probability that two random, sexually produced genotypes are identical is $P_G = 0.0016$ (Table 5.2). The probability of sampling without replacement two different multilocus genotypes per single population (D_G) ranged from 0.67 to 1 (data not shown) with a mean value of 0.93; Table 5.2). The discriminating power of the markers was lower when calculated using the frequency of the banding patterns. The values of D_j , in fact, ranged from 0.017 for the locus AT-2 to 0.825 for the locus AT-7, with a mean value of 0.493 (Table 5.2). The mean value of the confusion probability index (C_j) was 0.507 (Table 5.2). A total of 184 multilocus genotypes were identified over a total of 319 individuals analysed out of a potential number of 10.48×10^9 , according to Eq. 5.1. The proportion of distinguishable genotypes (G/N) within populations varied from 0.417 to 1.0 (Table 5.3), with an overall value of 0.577 (184/319). One hundred forty six genotypes occurred only once and 38 genotypes were shared by groupings composed of two to 21 individuals among the remaining 173 individuals. The effective number of genotypes always differed from the observed number suggesting that genotype frequency within population is not homogeneous. G/N values vs number of loci employed per population and overall (Figs 5.3c and 5.3d) demonstrated that a minimum number of six loci was necessary to describe the uncovered clonal diversity.

Pop	# ind.	G	G/N	eff. G	Eff. G/N	div.	eve.	D _G
ADR	8	8	1.0	8.0	1.0	0.875	1.0	1.0
PAN	24	18	0.75	13.71	0.571	0.927	0.761	0.967
TRAP	24	22	0.917	20.57	0.857	0.951	0.935	0.992
TUN	12	12	1.0	12.0	1.0	0.916	1.0	1.0
MER	24	20	0.833	16.94	0.706	0.94	0.847	0.981
CAP	24	13	0.542	8.22	0.343	0.878	0.632	0.916
SAG	24	21	0.875	18.0	0.75	0.944	0.857	0.985
CAS	24	19	0.792	14.4	0.6	0.930	0.757	0.971
PUP	24	19	0.792	14.4	0.6	0.930	0.757	0.971
PSP	24	11	0.458	2.79	0.116	0.642	0.254	0.670
ELB	24	14	0.583	9.29	0.387	0.892	0.663	0.931
FRA	24	16	0.667	9.6	0.4	0.895	0.6	0.934
POR	11	8	0.727	6.36	0.578	0.842	0.796	0.927
CAT	24	10	0.417	4.64	0.193	0.784	0.464	0.865
HAW	24	19	0.792	14.4	0.6	0.930	0.757	0.854
Total	319	230	-	173.32	-	-	-	-
Average	-	15.33	0.743	-	0.580	0.885	0.685	0.930

Table 5.3. Statistics of clonal and multilocus genotype diversity within populations. G, number of distinct genotypes sampled in each population, G/N, distinct genotypes over the total number of individuals in a population, eff.G and eff. G/N, effective number of genotypes equivalent to the effective number of alleles, div, Nei's genetic diversity corrected for sample size, eve, evenness of the effective number of genotypes, D_G, the probability of sampling without replacement two individuals that differ in multilocus genotypes.

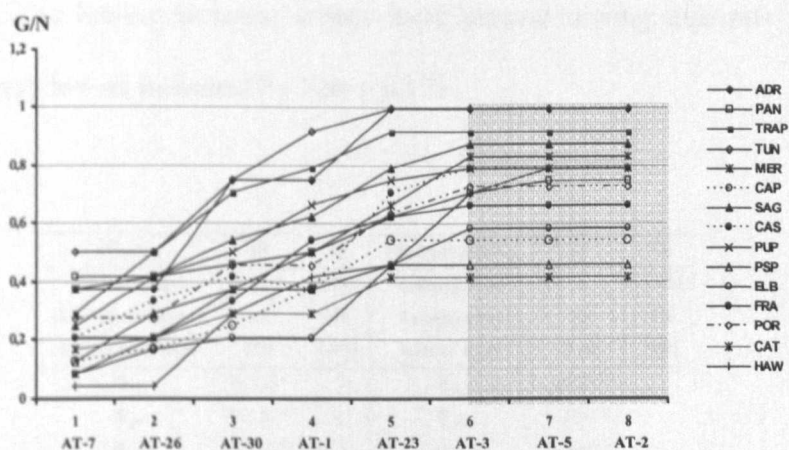


Fig. 5.3c. G/N for all populations by adding one after another all loci starting from the most polymorphic one.

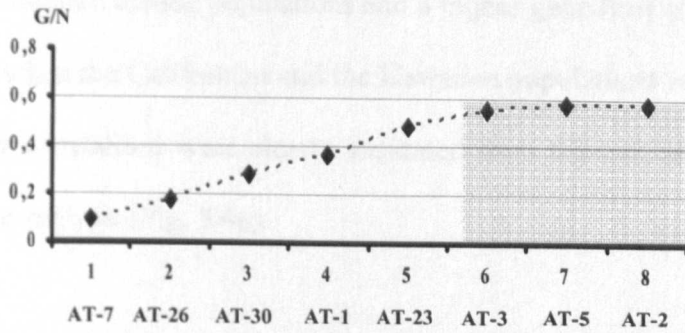


Fig. 5.3d. Effective G/N for all samples by adding one after another all loci starting from the most polymorphic one.

Analysis of genetic structure

Molecular variance

Higher differentiation among populations and within sites was detected by the AMOVA analysis when all populations were included in the dataset (Table 5.4). The highest variance was present among populations but high variance values were also present within quadrats. The lowest variance values were present among quadrats within sites. Gene flow was very low as indicated by $Nm = 0.171$.

all pops	df	%	Mediterranean	df	%
Among pops	11	53%	Among pops	9	16%
Among quadrats	24	6%	Among sites	20	9%
within quadrats	252	41%	within sites	210	75%
Φ_{st}	0.533*		Φ_{st}	0.159**	
Φ_{pr}	0.131*		Φ_{pr}	0.107**	
Φ_{pt}	0.595*		Φ_{pt}	0.249**	
Nm	0.171		Nm	0.754	

Table 5.4. Analysis of molecular variance (AMOVA) showing the percentage of variation apportioned among populations, among sites within populations and individuals within sites. AMOVA was performed in all populations of 24 specimens and in all 24 specimen populations from the Mediterranean Sea. Number of migrants, $Nm = 0.25(1 - \Phi_{pr}) / \Phi_{pt}$. * $p < 0.010$, ** $p < 0.0010$

A lower genetic distinction among populations and a higher gene flow ($N_m = 0.754$; Table 5.4) were detected when the Californian and the Hawaiian populations were excluded from the analysis. Those populations were clearly separated from the rest of the dataset in the principal coordinate analysis (Fig. 5.4a).

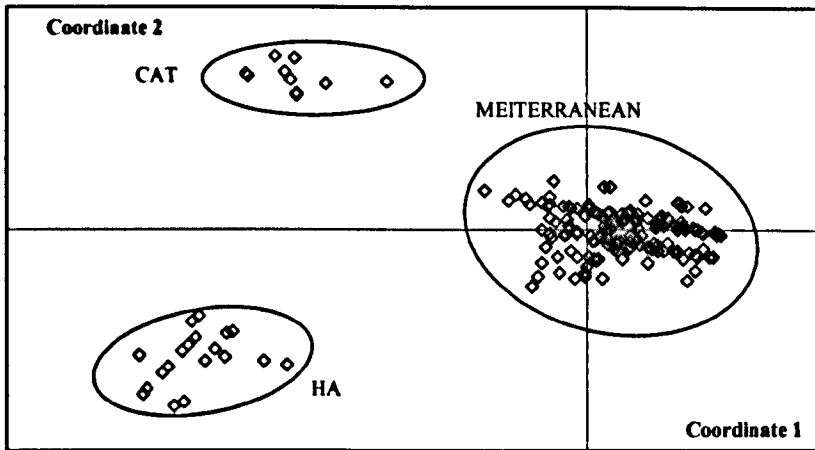


Fig. 5.4a. Principal coordinates analysis performed on all populations

The same analysis was also performed considering only the average Nei genetic distance per population and including only the Mediterranean sites. Adriatic and Tunisian populations were distinct from one another as well as from the remaining Mediterranean populations sampled (Fig. 5.4b, 5.4c). Populations from Portugal (outside the Mediterranean) and France, the other recently invaded locality included in the analysis (Verlaque personal communication) grouped together with the main Mediterranean group.

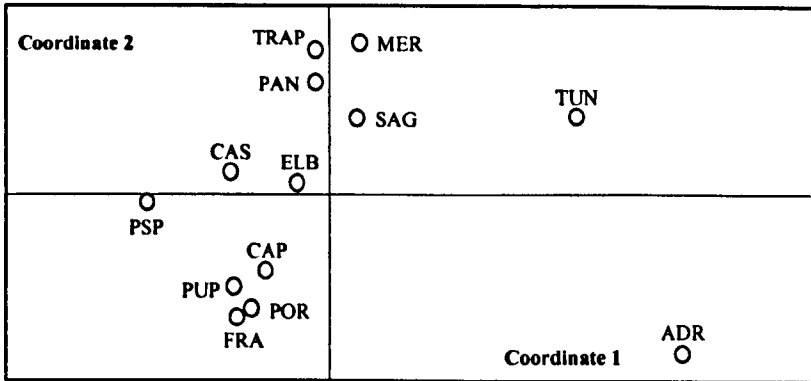


Fig. 5.4b. Principal coordinates analysis performed on Mediterranean populations only

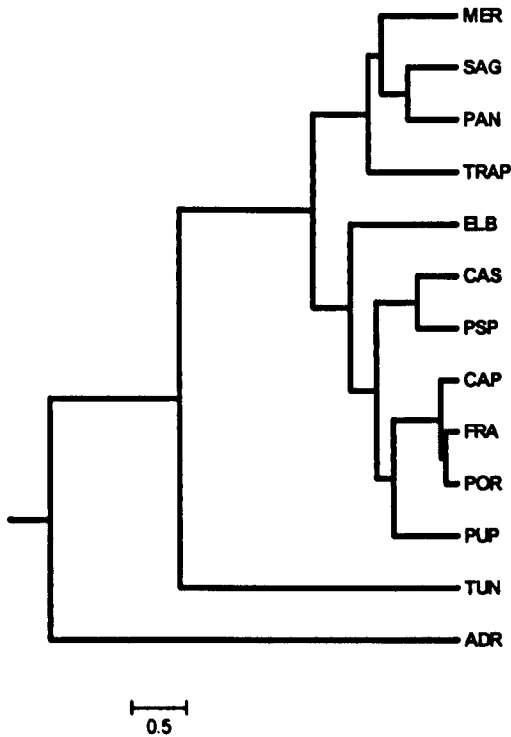


Fig. 5.4c. Dendrogram inferred from pair-wise Nei's genetic distances among populations

Results of the AMOVA analysis conducted for all the 13 Mediterranean populations against the f -free and the $\theta^B = 0$ models showed the following results. The $\theta^B = 0$ model (DIC = 1912.5345) was rejected by the deviance information criterion (Table 5.5).

Only Mediterranean populations				
Model	Dbar	Dhat	pD	DIC
f -free	805.0277	633.6557	171.3720	976.3997
$\theta^B = 0$	1876.6535	1840.7724	35.8810	1912.5345

Table 5.5. Bayesian analysis of molecular variance (AMOVA). (DIC), Deviance information criterion statistics of two models applied to the dataset from specimens of all the Mediterranean populations. Dbar, a measure of how well the model fits the data, Dhat, a measure of how well the best point estimate fits the data, pD, indicates the complexity of the model (approximate number of parameters being estimated)

The f -free model (DIC = 976.3997) generated a mean θ^B of 0.1047 ± 0.0061 (95% confidence intervals 0.0954 and 0.1190) and a $G_{st} - B$ of 0.2158 ± 0.0156 (95% confidence intervals 0.1846 and 0.2462). The statistical support of the f -free model suggest that there is no evidence of inbreeding among populations but there are differences in amplification variant frequencies among them and evidence of population genetic structure.

Bayesian clustering

The Bayesian-based clustering method applied on the 13 Mediterranean populations (total of 271 specimens), demonstrated that the only model able to explain sufficiently the distribution of genetic variability in the Mediterranean sea ($-\ln P(D) = 1821.9$) was the model with $K = 8$ clusters. The other models applied to the hypothetical 13 geographically distinct populations ($K = 1...7, 9...13$) failed to explain the data. According to these results, the 271 samples of *A. taxiformis* collected in the Mediterranean cluster in eight

groups. The membership frequency of each geographically predefined population (1 to 13) included in the analysis in each of the eight clusters is reported in Table 5.6. The predefined geographic populations do not belong completely to only one cluster but all of them are generally equally distributed with few exceptions in almost all of the eight clusters indicating lower population distinction among the Mediterranean populations and recent spread. Only the Adriatic and Tunisian populations have higher membership values for the seventh cluster.

	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8
ADR	-	0.141	-	0.008	-	-	0.836	-
PAN	0.087	0.132	0.087	0.142	0.211	0.086	0.125	0.132
TRAP	0.159	0.161	0.031	0.101	0.447	0.028	0.038	0.035
TUN	0.211	0.042	-	0.014	-	0.021	0.687	0.020
MER	0.521	0.078	0.058	0.136	0.097	0.035	0.026	0.049
CAP	0.038	0.055	0.177	0.099	-	0.313	-	0.303
SAG	0.116	0.136	0.066	0.391	0.032	0.088	0.072	0.098
CAS	-	0.117	0.236	0.237	0.159	0.113	0.018	0.114
PUP	-	0.118	0.061	0.266	0.011	0.348	0.012	0.182
PSP	0.044	0.064	0.555	0.027	0.125	0.080	-	0.103
ELB	0.129	0.044	0.109	0.048	0.050	0.216	0.011	0.393
FRA	-	0.112	0.196	0.099	0.014	0.316	0.022	0.240
POR	-	0.077	0.184	0.099	0.068	0.289	0.012	0.264
overall	0.108	0.099	0.147	0.142	0.105	0.156	0.085	0.158

Table 5.6. Proportion of membership of each predefined population in each of the eight clusters of genotypes inferred by the STRUCTURE software. In bold populations ADR and TUN are reported. The overall proportion of membership of the entire dataset in each of the eight clusters is reported in the last row.

Spatial analysis

Both spatial autocorrelation analysis and Mantel tests revealed significant decreases in genotypic similarity with increasing geographic distance. Mantel tests revealed that Hawaiian, Californian and Mediterranean populations are genetically isolated by distance ($r^2 = 0.022$, $P = 0.001$). The same was observed among only Mediterranean populations

($r^2 = 0.021$, $P = 0.001$), but not among populations from the Gulf of Naples ($r^2 = 0.004$, $P = 0.04$). Autocorrelation analyses showed isolation by distance at a world-wide scale up to 4000 km (Fig. 5.5a), within the Mediterranean basin up to 1000 km (Fig. 5.5b) but not over a small geographical scale (Fig. 5.5c), where genetic structure is not detected.

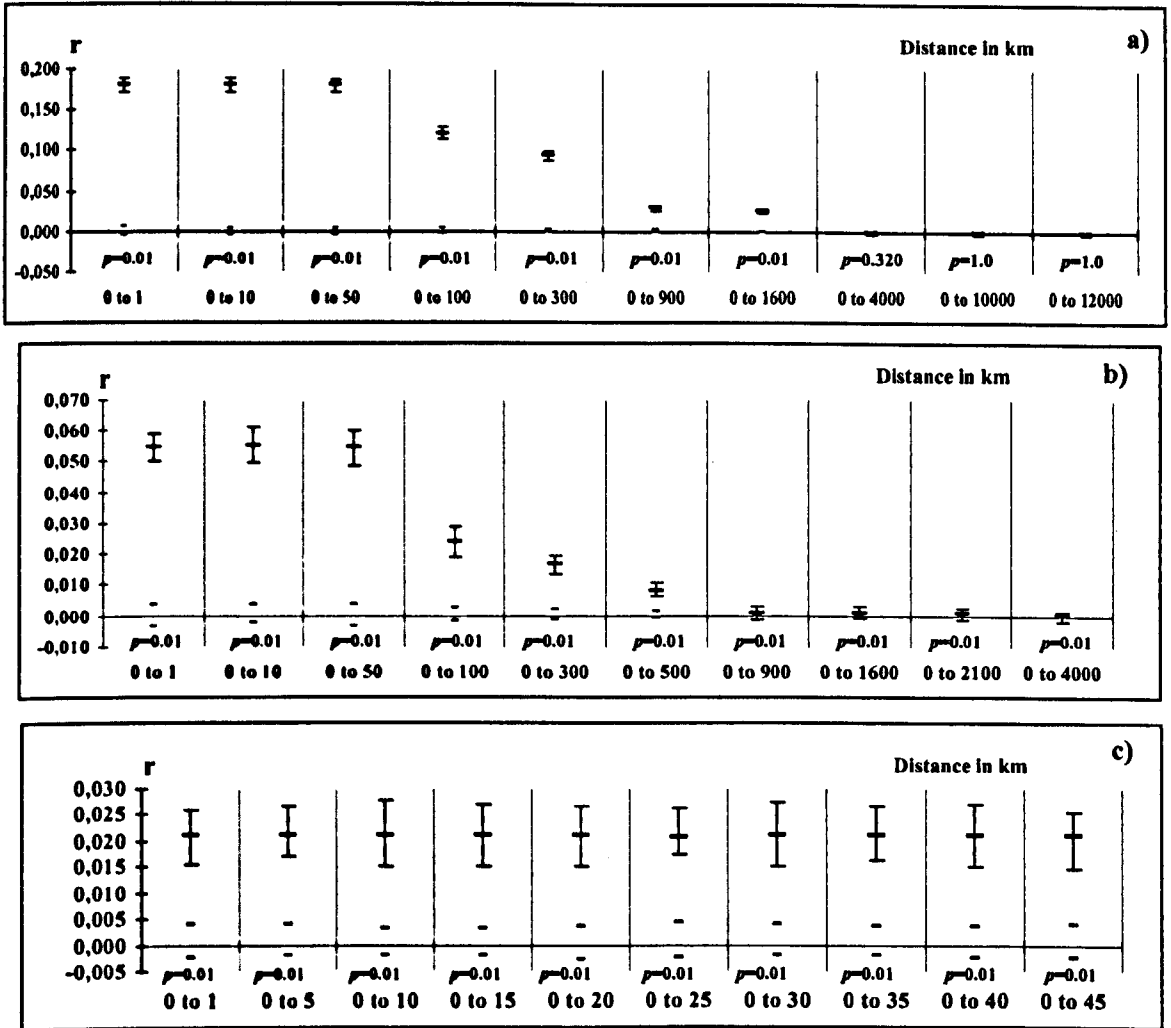


Fig. 5.5 Spatial autocorrelation analyses across a) all populations, b) all Mediterranean populations c) only populations from the Gulf of Naples. Autocorrelation (r) for a range of geographical distance classes (km), with 95% confidence error bars as determined by 1000 bootstrap replicates are reported. Gray bars delineate the upper and lower bounds of 95% confidence intervals associated with the null hypothesis of no spatial structure ($r = 0$). p values as determined by 999 permutations are given for each distance class.

Assignment tests performed in order to establish donor populations for the newly invaded areas of Marseille (France) and Faro (Portugal) revealed very similar values among the central Mediterranean populations. The most likely donor population to the French site (FRA) is the Capo Posillipo population, from the Gulf of Naples (CAP, log - likelihood = 16.588) followed by the one from the Island of Elba, Tuscany (ELB; log - likelihood = 17.143). The most likely donor population for the Portuguese population (POR) is again the CAP site followed by the French population (FRA, log - likelihood = 16.330 and 16.456 respectively).

Discussion

The eight microsatellite loci used in this study exhibited strong statistical resolution power. The peculiarity of utilizing microsatellites for population genetic studies in a polyploid species enticed me to perform a series of comparative test based on both the number of allelic variants and on differences in banding patterns in order to investigate the resolution power of loci. When microsatellites are utilized in polyploid taxa, in fact, statistics based on banding pattern frequencies and designed for dominant markers can be applied. My results always indicated that the assessment of clonal identity/diversity provided by the loci utilized is reliable. However, lower discrimination power was encountered when banding patterns were considered instead of allelic variants. This can be explained by the difference in frequencies of banding patterns generated by each locus (Fig. 5.2a-h). A specific locus, in fact, has a maximal discriminating power when it is characterized by banding patterns equally frequent (Tessier *et al.* 1999). In the analysis

none of the markers demonstrated homogeneity in the frequencies of the banding patterns generated (Fig. 5.2a-h).

Only six loci appeared to be sufficient to detect all the genetic diversity encountered within the dataset (Fig. 5.3c, 5.3d) even if the theoretical number of *Asparagopsis taxiformis*-lineage 2 distinct eight-locus genotypes is 10.48×10^9 . This is also shown when considering the number of alleles. The curve obtained plotting mean number of alleles against populations, in fact, reaches a plateau after nine populations confirming that the sampling effort at least within the Mediterranean was enough to capture the genetic diversity introduced in the basin (Fig. 5.3b). Such rapid saturation may be explained by the fact that rare alleles are not fixed yet due to the very recent expansion of the species along the Italian coasts and westward to the coasts of Portugal (Chapter III). Survival, colonization capability and expansion of populations are thus guaranteed by only a restricted number of genotypes that are favoured by the local environmental conditions and are able to reproduce successfully within the basin.

Invasive species often are bigger or exhibit higher fecundity rates in the introduced environment compared with the native one (Grosholz and Ruiz 2003; Brown and Eckert 2005). This enhanced vigour is believed to be a plastic response to more favourable or relaxed conditions encountered in the introduced range (increased availability of resources, absence of competitors, grazers and parasites, *etc.*). Under such benign conditions, invasive species reallocate a great part of their resources from defence and competition to overgrowth, sexual reproduction and dispersal. This strategy enhances the capability of an invader to out-compete natives in the habitat it is invading (Adler 1999; Brown and Eckert 2005).

The high multilocus genotypic diversity (mean $D_G = 0.93$; mean $G/N = 0.743$), the absence of inbreeding (see estimates of θ^B) and the high number of amplification variants encountered in this study seems to confirm the above mentioned data, suggesting sexual rather than clonal reproduction within populations. It is not clear if the California site included in the analysis has been recently invaded or could represent a native habitat of the species, but the low genetic diversity encountered in this population seems to support the second possibility.

Within a range of high genetic diversity, different levels of genetic variability were found in the different populations, suggesting the existence of high heterogeneity in the nature of introduction and spreading processes. The same was also suggested in a preliminary study in the brown seaweed *Undaria pinnatifida* (Daguin *et al.* 2005), a marine invasive species that spread to both hemispheres in the last 30 years (Silva *et al.* 2002). Discrepancies in genetic diversity between introduced populations were found. In this study, the high clonal diversity of sites (France and Portugal) where samples were collected few years after the first establishment of the species, can either indicate an active recruitment due to sexual reproduction or a massive and continuous introduction from different genotypes. High genetic diversity was also found in both haploid and diploid phases of *Cladophoropsis membranacea* together with lack of differences in allele frequencies indicating that the two phases reproduce sexually (Van der Strate *et al.* 2002). In the fresh water red alga *Batrachospermum helminthosum*, high genetic diversity within streams was observed and was attributed to sexual reproduction, which is known to be frequent in this genus (Hall and Vis 2002).

Genetic structure

The nuclear microsatellite markers utilized here were developed against the Indo-Pacific Mediterranean lineage 2 and cross-hybridised with lineage 1, indicating that the two *cox*-spacer haplogroups can be considered as one biological species. Microsatellites clearly resolved differences between lineages 1 and 2 and showed higher resolution than the mitochondrial *cox2-3* spacer (see Chapter IV) within lineage 2. The microsatellite markers utilized, in fact, clearly separate the Californian population from the Mediterranean ones whereas the sequence markers failed to do so. Previously, only inter-lineage genetic diversity was detected despite the fact that often specimens in lineage 2 were sampled from distant geographic locations (Chapter III). This result suggests that subgroups may exist within lineage 2 and eventually also within lineage 1 at a world-wide scale. Here I may have missed this information because the sampling effort was mostly concentrated in the Mediterranean Sea.

It was hypothesised that the high intraspecific variation among the LSU rDNA sequences in lineage 1+2 of *A. taxiformis* (Figs. 3.2a and 3.2b in Chapter III) is consistent with both recent hybridization events involving two LSU genotype lineages not yet homogenized by concerted evolution or with incomplete lineage sorting of ancestral polymorphisms through genetic drift, as documented in numerous other species (Takahashi *et al.* 2001). Because it is difficult to distinguish between hybridization and incomplete lineage sorting according to these data, I state that both possibilities should still be taken into consideration. In the future, crossing experiments between these two lineages and

genetic analysis of the progeny (in case the progeny is obtained) may give an ultimate answer.

Lineage 2 colonised the Mediterranean from the Indo-Pacific Ocean, most likely via the Suez Canal (Chapter III). Within a few years it extended its range to the coasts of France and has now reached the Atlantic Ocean (first observation near Faro in 2004; Mata and Santos personal communication). The central Mediterranean populations were poorly differentiated, making results of assignment not highly reliable, with low differences in log-likelihood values (Waser and Strobeck 1998). However, the assignment tests performed clearly indicate that one of the central Mediterranean lineage 2 populations has been the source for the French and Portuguese *Asparagopsis taxiformis*. The putative source population for the French *A. taxiformis* is CAP, from the Gulf of Naples (log - likelihood = 16.588) followed by the Elba population (log - likelihood = 17.143). The most likely donor site for the Portuguese *Asparagopsis* was again the CAP followed by the French population (log - likelihood = 16.330 and 16.456 respectively). The data collected in Chapter III and the present data seem to support the following scenario: *A. taxiformis*-lineage 2 invaded the Mediterranean from the Suez Canal within the last 150 years and slowly started to move westward through the Sicily Channel and Messina Strait (first record in Sicily in 2000; Barone *et al.* 2003). The species established along the Western Italian coasts recently and from there it is continuing its spread toward North and West colonizing France and Portugal. Further studies including *A. taxiformis* populations from the Mediterranean coasts of Spain are necessary to support this hypothesis.

The low population differentiation, low genetic structure and low variance found among the Mediterranean populations are consistent with the assumption that those

locations were colonized recently by *A. taxiformis* (Boudouresque and Verlaque 2002). Hence the genetic differentiation between distantly related populations (e.g., FRA vs MER) was not proportional to the geographic distance and the Bayesian analysis uncovered a panmictic structure among the geographically predefined populations. According to the bayesian analysis, in fact, specimens of *A. taxiformis* sampled within the Mediterranean are better structured in eight main divergent clusters or sub-populations that do not reflect the geographic position of sampled populations. Within each cluster, minor variations may have been created by mutations since the introduction of the species (Chapter III). Differences in allelic frequency among clusters are always low although cluster 7, characterized by the Adriatic and the Tunisian populations, shows higher divergence (Table 5.7). Clusters could represent either independent introductions or most likely they could reflect intensity of gene flow.

	CL1	CL2	CL3	CL4	CL5	CL6	CL7	CL8
CL1	-							
CL2	0.08	-						
CL3	0.12	0.07	-					
CL4	0.13	0.05	0.11	-				
CL5	0.12	0.05	0.13	0.10	-			
CL6	0.10	0.06	0.04	0.07	0.10	-		
CL7	0.14	0.07	0.31	0.19	0.18	0.22	-	
CL8	0.09	0.06	0.03	0.06	0.09	0.00	0.12	-

Table 5.7. Allele frequency divergence among the eight clusters or sub-populations uncovered by the bayesian clustering analysis.

It is generally known that dispersal capability and mating system rule the movement of genes between individuals within and among populations (Valero *et al.* 2001).

Most studies of direct dispersal of algal reproductive structures demonstrated that dispersal in the algae is limited to a few meters and viability of gametes is mainly restricted to few hours (reviewed in Van der Strate *et al.* 2002). In the fresh-water red algae *Batrachospermum helminthosum* it seems that long distance dispersal plays a role in the distribution observed along streams (Hall and Vis 2002). Gene flow in *A. taxiformis* is ensured from fluctuating fragmented thalli and dispersal of reproductive structures. The absence of genetic structure at local and basin scales suggests that an island model of gene flow can be assumed (Fig. 5.5) According to this model, sub-populations are able to exchange genes with the same probability in spite of their geographical distance (Wright 1931).

In conclusion, my analysis showed that the population from Hawaii (HAW; lineage 1) was distinct from populations belonging to the Indo-Pacific Mediterranean lineage 2. Within lineage 2, a clear distinction was present between geographically distant populations (Californian vs Mediterranean). Within the Mediterranean Sea, the 13 populations of the invasive Indo-Pacific Mediterranean lineage 2 exhibited high genotypic diversity, high gene flow and, as expected, low differentiation, which results from recent invasion of the species. Geographically predefined populations were found to be panmictic and structured in eight weak Bayesian clusters. Sexual reproduction is predominant and clonal reproduction does not seem to contribute significantly to determine genetic isolation at a small scale. Overall results confirm that *A. taxiformis* is a rapidly spreading species, expanding westward in the Mediterranean Sea.

CHAPTER VI – Summary, Concluding remarks

Summary

Algal systematics has been revolutionized in the last few decades by the use of innovative molecular methods. One of the highlights is the detection of considerable cryptic diversity, i.e., multiple morphologically similar cryptic and/or sibling species within what was previously believed to be a single species. The same tools were successfully applied in a phylogeographic context to detect biological invasions, assess the origin of invasive taxa and follow their progress in order to help forecasting their impact on recipient communities.

In this thesis, I studied the taxonomy, phylogeography, and population genetics of two species of the genus *Asparagopsis*; *A. armata* and *A. taxiformis*. Both species showed invasive behaviour along the Mediterranean and Atlantic coasts of Europe. I deployed a phylogeographic approach to assess if the European populations of each of these species originated from a single introduction or multiple cryptic ones. In addition, I tried to determine the region from where the introduced lineages derived by extending the phylogeographic study to a global scale. Some problems and questions encountered at the start of the PhD-thesis were: a) confusion in the literature between *A. taxiformis* and *A. armata* and hence, possible mistakes in the geographic distribution range of the two species resulting from erroneous reports, b) difficulties in assigning morphologically identical “*Falkenbergia*” stages of the two species to either one of them, c) and lack of knowledge about the donor population of the Mediterranean *Asparagopsis* spp.

Results indicated that *A. armata* and *A. taxiformis* are morphologically distinct species whose gametophytes can be distinguished from one another easily by the presence

– absence, respectively, of barbed spines on the rhizoids. In addition, I discovered at least three and possibly four cryptic species in what was considered the pan-tropical red algal species *A. taxiformis*. One of these cryptic species was found exclusively in the tropical Atlantic and in the eastern Mediterranean Sea, whereas the other three showed an Indo-Pacific distribution. One of these three Indo-Pacific ones currently invades coastlines in the central and western parts of the Mediterranean Sea and has now also been encountered on the southern coast of Portugal. The other species examined, *A. armata*, which is distributed along warm-temperate coasts in the northern and southern hemispheres, did not reveal any cryptic diversity. Results of population genetic investigations showed high genetic diversity indicating prodigious sexual reproduction. Lack of clear population genetic structure in the Mediterranean suggested active gene flow and absence of population differentiation since the invasion. Moreover, these lineages appeared to be polyploid. Finally, carpogonia (fertilized “fruiting bodies”) on the female gametophyte thalli affected the outcome of the microsatellite patterns, and had to be removed prior to DNA extraction.

In Chapter II, I examined the taxonomic units of the genus, levels and patterns of intra-generic and intra-specific genetic variation of the two species, as well as between gametophytes and tetrasporophytes of the two species. Thereto I used three DNA-markers, a nuclear, a plastid and a mitochondrial one. Although *Asparagopsis* species exhibit morphologically clearly distinct gametophytes, their life strategy is characterised by identical “*Falkenbergia*” stages, which was a main source of taxonomic confusion. This first part allowed defining species boundaries along the Mediterranean coasts. This study

also demonstrated cryptic diversity within *A. taxiformis*. The latter information prodded me to assess the phylogeography of the species on a global scale.

In **Chapter III**, the sampling of the two species was now done on a global scale. Molecular phylogenetic patterns gathered from nuclear, mitochondrial and plastid genomes uncovered three and probably four morphologically cryptic but genetically distinct lineages within *A. taxiformis*, whereas *A. armata* was found to consist of a single species worldwide. The central Mediterranean *A. taxiformis* populations result from Indo-Pacific founders whereas the specimens sampled from the eastern Mediterranean were recovered within the Atlantic lineage. Thus, Mediterranean populations of *A. taxiformis* result from multiple cryptic invasions. The level of resolution and the evolutionary speed of each marker was inferred using a molecular clock approach.

In **Chapter IV**, I identified eight microsatellite markers to be used in the lineage of *A. taxiformis* currently invading the central Mediterranean (lineage 2). The markers were needed to assess population genetic variation and the extent of gene flow among these populations in a nested set of geographic distances. The markers worked well with tetrasporophytes and gametophytes and functioned in thalli of lineages 1 and 2, but they failed with specimens of lineages 3 and 4 and with those of *A. armata*. In addition, I found out that the carpogonia present on many of the female thalli affected the microsatellite reading patterns; carpogonia result from fused female and male gametes. As expected, external (male) allelic contribution was detected in the carpogonia. Even after removal of the carpogonia, female gametophyte thalli exhibited multiple allelic patterns indicating polyploidy. In fact, this study is the first report of polyploidy in algae detected using microsatellite markers.

In **Chapter V**, the microsatellite markers were used to assess genetic structure within *Asparagopsis taxiformis*-lineage 2 and to establish levels of genetic diversity and structure in the Mediterranean populations of this invasive lineage. The differences in ploidy level encountered forced me to use statistics different from the ones developed for diploid organisms. Different strategies have been proposed for the statistic analysis of multilocus microsatellite genotypes in polyploid species that take into account the coexistence of individuals with different ploidy levels in the same matrix. In this Chapter, multilocus genotypes were compared using an index of similarity based on shared allelic variants and statistic parameters were gathered from amplification variant frequencies and genotypic frequencies rather than allelic frequencies. The analysis showed that the population belonging to lineage 1 (HAW) was distinct from populations belonging to the invasive indo-Pacific - Mediterranean lineage 2. In addition, geographically distant populations within lineage 2 (Californian vs Mediterranean) were genetically distinct as well. High genotypic diversity within each of the 13 Mediterranean populations, and high gene flow and low differentiation amongst them probably results from the recent invasion of this lineage into the Mediterranean basin. The sampled populations were found to be panmictic and structured in eight Bayesian clusters. Sexual reproduction is predominant and clonal reproduction does not seem to contribute significantly to population structure. Overall results confirm that *A. taxiformis* lineage 2 has rapidly expanded westward in the Mediterranean Sea. Its apparent expansion should be taken into consideration to design appropriate monitoring and management strategies.

Conclusive remarks and future prospects

The results presented in this thesis open new questions with respect to the fascinating biological features of the species. The three or four genetically distinct lineages in *A. taxiformis* need to be re-examined morphologically. Diagnostic morphological characters might exist. Moreover, the biological status of these genetically distinct lineages might be assessed by means of fertilization experiments between and within lineages.

In this thesis it has been proved that contrary to plant mitochondrial genes that accumulate synonymous substitutions almost three times slower than plastid genes, the red algal mitochondrial *cox 2-3* spacer accumulates mutations almost four times faster than the plastid RuBisCo spacer. In this context, estimates of nuclear, plastid and mitochondrial red algal mutation rates are needed to understand if and how life strategies, generation time and population size influence mutation rates of the three genomes.

The microsatellite markers developed against the Indo-Pacific Mediterranean lineage 2 of *A. taxiformis* demonstrated polyploidy in this species and or the existence of heterocaryotic cells that may alter the ploidy level of the species. These results corroborate findings by Kapraun *et al.* (2005). Flow cytometry experiments and DNA contents estimates may assess the actual ploidy levels of the two lineages analysed. The microsatellite markers successfully described levels and patterns of gene flow and population structuring among local populations of *A. taxiformis* lineage 2. In the future, further research is required in order to study: a) If populations accumulate genetic differences over different spatial and temporal scales, b) If clonal reproduction contributes to population genetic structure. c) If differences in genotypic frequencies between the two

distinct phases do exist and what the contribution is of gametophytes and tetrasporophytes to the expansion and persistence of the population.

Asparagopsis taxiformis is an invasive species with an interestingly elaborate life cycle. It seems to cope well with ecological and physiological conditions different from those in its native range. The invader affects the native Mediterranean marine ecosystems, e.g., the preservation and survival of endemic species. However, there is also a positive side to biological invasions. *Asparagopsis taxiformis* may increase biodiversity in the recipient area by occupying a previously empty niche or sharing a niche with locals, and in the end become a key component in the local marine biodiversity.

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Appendix 1

Programs and equations used for phylogenetic and population genetics analysis

Software

BIOEDIT v4.8.5	Sequence alignment
CEQ 2000 v3.0	Electropherograms analysis and allele detection
DNASTAR	Supplied with the Beckman sequencer for sequence evaluation and consensus reconstruction
GenALEx v6	Amplification variants analysis, Principal Coordinate Analysis, Mantel tests and hierarchical AMOVA for dominant markers. (Φ -Statistics, an F -Statistic analogues, for Φ_{rt} , Φ_{pr} , and Φ_{pt} estimates). Φ_{pt} values indicate gene flow levels within and between geographic regions
GENOTYPE-GENODIVE	# of distinct genotypes, effective # of distinct genotypes, genotypic frequencies, pairwise Nei's genetic distances and evenness of the genotypes
HICKORY v1.0.4	Bayesian analysis of molecular variance independent from sample size
LINTREE	Branch-length tests in order to eliminate sequences evolving significantly different from the average
MODELTEST v3.06	Hierarchical Likelihood Ratio Tests to find the best fitting parameters for the maximum likelihood analysis given the alignment

NETWORK v4.1.1.2	Median Joining haplotype networks reconstruction
PAUP* v4.0b10	Maximum parsimony, Maximum likelihood, Distance methods phylogenetic reconstruction, Bootstrapping. Binary matrixes reconstruction
STRUCTURE v2.1	Bayesian-based clustering analysis
RRTREE v1.1	Relative rate tests between groups of sequences.
SGS v1.0c	Spatial patterns of genetic variation
TCS v1.13	Haplotype networks reconstruction (gene genealogies).
TREE-PUZZLE v5.0	likelihood ratio tests between groups of sequences and Chi-square test between likelihood values of trees with and without the molecular clock enforced
WHICHRUN v3.2	Assignment tests

Equations

G/N	overall clonal diversity as the proportion of distinct genotypes over the total number of specimens sampled
$r = d/2t$	sequence divergence rate. d, the average (pair-wise) p-distance between the Atlantic lineage 3 and the Indo-Pacific lineage 4, t, the hypothesized time elapsed since their divergence
$Ng = \prod_{i=1}^L [a_i(a_i + 1)]/2$	theoretical number of distinct genotypes

$1 - P_G$ discriminating power of the microsatellite markers

$P_G = \prod_n \sum_r (g_k)^2$ probability that two random, sexually produced genotypes are identical

$D_G = 1 - \sum \{[n_i(n_i - 1)]/[N(N - 1)]\}$ probability of sampling without replacement two individuals that differ in multilocus genotypes in the population

$C_j = \sum_{i=1}^I p_i [(N p_i - 1)/(N - 1)]$ confusion probability

$D_j = 1 - C_j$ discriminating power

$D_L = \lim (D_j) = 1 - \sum_{i=1}^I p_i^2$ limit of D_j as N (sample size) tends towards infinity.

Appendix 2

Nei's genetic distance among populations calculated in Chapter V

	MER	ELB	SAG	CAS	PAN	TRAP	CAP	PSP	PUP	CAT	FRA	TUN	ADR	POR	HAW
MER	-														
ELB	0,019	-													
SAG	0,016	0,024	-												
CAS	0,022	0,019	0,010	-											
PAN	0,016	0,017	0,011	0,011	-										
TRAP	0,020	0,032	0,020	0,018	0,015	-									
CAP	0,026	0,009	0,022	0,012	0,021	0,035	-								
PSP	0,035	0,031	0,029	0,008	0,028	0,029	0,019	-							
PUP	0,045	0,029	0,024	0,013	0,030	0,038	0,016	0,019	-						
CAT	0,271	0,314	0,224	0,264	0,263	0,250	0,324	0,306	0,296	-					
FRA	0,039	0,018	0,028	0,015	0,029	0,043	0,004	0,017	0,012	0,330	-				
TUN	0,038	0,047	0,036	0,059	0,042	0,057	0,054	0,084	0,063	0,316	0,061	-			
ADR	0,079	0,079	0,064	0,083	0,079	0,082	0,071	0,108	0,083	0,296	0,078	0,057	-		
POR	0,035	0,017	0,027	0,014	0,027	0,037	0,004	0,018	0,012	0,318	0,004	0,060	0,072	-	
HAW	0,379	0,454	0,342	0,381	0,374	0,344	0,436	0,404	0,413	0,315	0,439	0,387	0,352	0,428	-

Appendix 3

Technical remarks

Sampling strategy

For the population genetics analysis, *Asparagopsis taxiformis* specimens were collected according to a sampling design agreed between partners of the “ALIENS” project. Samples were collected at different spatial scales. The basic sampling unit was 8 individuals from a quadrat size suited to the size of the thalli. Three replicate sets were collected 5 to 10 m from each other representing a locality. Each locality is thus accompanied by 24 silica dried single individuals and 1 herbarium specimen. Samples and herbarium specimens of *Asparagopsis* spp. to be used for a phylogeographic study and sets of 24 specimens sampled according to the same sampling protocol have been obtained from several research groups elsewhere.

Preservation protocol

DNA isolation from red algae it is not an easy task mostly due to the co-isolation of hydrocolloids (agars, carrageenans) and polysaccharides. These compounds produce highly viscous solutions that may inhibit endonuclease and DNA polymerase activities (Wattier *et al.* 2000). In order to obtain the best quality and quantity of DNA, first, the optimal preservation of algal specimens was evaluated after testing two preservation media: ca. 3 cm³ of *Asparagopsis taxiformis* fresh tissue was preserved in 12 mL Falcon tubes in the presence of a) alcohol 70% and b) silica gel for a period of almost three months. DNA was then extracted from the above specimens according to a) the Porebski *et al.* DNA extraction protocol (1997) based on a modified CTAB extraction method for DNA extraction from

leaf material containing large quantities of polyphenols, tannins and polysaccharides; and b) a less time-consuming modified version of the CTAB based Porebski *et al.* (1997) DNA extraction procedure. The later method is described in details in the section “material and methods” of Chapter II. Finally, quality and quantity of the DNA, extracted according to the two extraction procedures against the effect of the two preservation methods, was evaluated by: a) means of 1% agarose TAE buffer gel electrophoresis against known standards and ethidium bromide staining, b) a spectrophotometer, by reading absorbance at wavelengths of 260 nm and 280 nm and c) by PCR amplification of the DNA markers used in this thesis for the phylogeographic analysis in order to assess whether DNA was suitable for PCR amplification or not. The above experiments demonstrated that fresh *Asparagopsis* spp. tissue results better preserved and, the DNA is less degraded when silica gel is used as a preservation medium and is changed 24 hours after initial preservation to permit absolutely dry storage. Moreover, the second DNA extraction method resulted to be the one less time consuming and the most convenient giving high quality and quantity of DNA suitable for PCR amplification. Once preservation method of the fresh tissue was established, algal specimens were asked from several research groups and colleagues world-wide. Final processing of the specimens involved a quick washing in sea water in order to eliminate epiphytic diatoms and a Whatman 3MM filter paper step in order to eliminate the excess of water. Specimens were finally dried in silica gel for transport and storage. Silica gel was changed 24 hours after initial preservation in order to obtain absolutely dry specimens. In this way, samples were sent safely via regular mail to the laboratory where DNA extraction and genetic analysis were carried out.

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