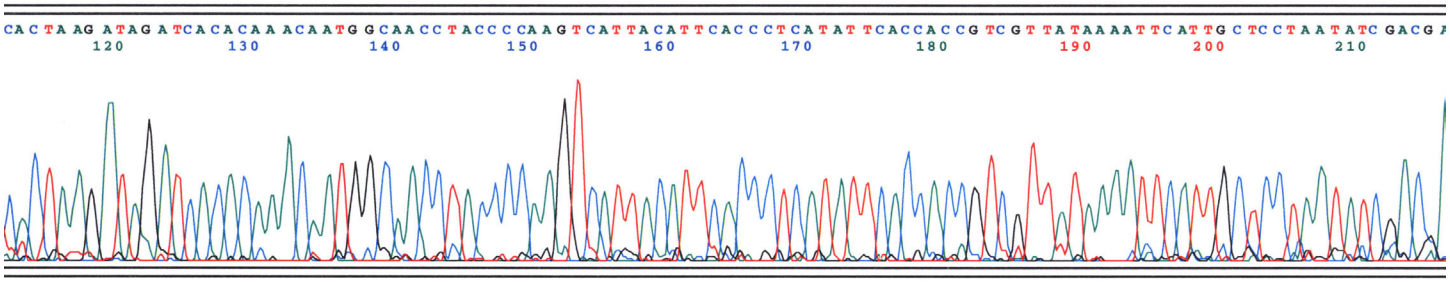

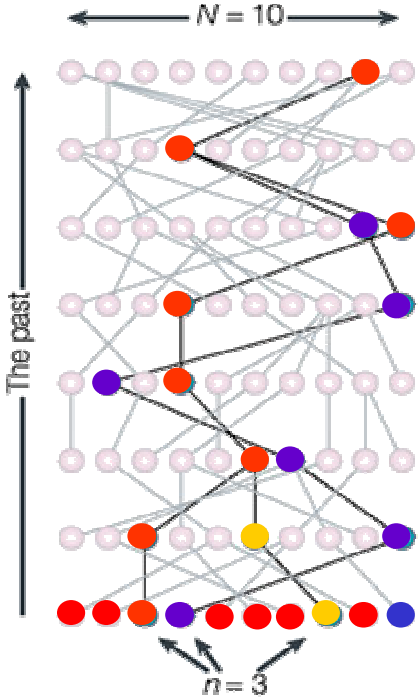

Phylogeography, gene Flow and Population Structure of *Crambe crambe* (Porifera: Poecilosclerida)

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PHYLOGEOGRAPHY, GENE FLOW AND POPULATION STRUCTURE OF *Crambe crambe* (PORIFERA: POECILOSCLERIDA)

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PHYLOGEOGRAPHY, GENE FLOW AND POPULATION STRUCTURE OF *Crambe crambe* (PORIFERA: POECILOSCLERIDA)

Memòria presentada per Sandra Duran i Alarcon per accedir al Títol de Doctor en Ciències Biològiques al Departament de Biologia Animal (Unitat d'Invertebrats) de la Facultat de Biologia, Universitat de Barcelona, sota la direcció dels doctors Xavier Turon i Barrera i Marta Pascual Berniola

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Barcelona, Setembre de 2003

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Agraïments

No sé per on començar…...(que típic, oi?).

A veure, la primera casualitat o fet que va dur-me on sóc (a part del dia en que em van concebre) va ser quan a segon de carrera vaig dirigir-me al departament de Biologia Animal a la caça d'algun científic simpàtic que em volgués adoptar com a "ajudant" en les meves hores lliures. Vaig trucar a la primera porta que vaig trobar al entrar pel passadís, i un entusiasmat Dr. Juli Pujade em va dir: pots començar quan vulguis! I així vaig entrar a col.laborar en aquest departament. Aquells primers anys de milers i milers de mosques i himenòpters a la lupa, van ser els meus primers pinitos en la ciència i els recordo amb molt de carinyo, especialment pels bons moments passats amb en Juli, la Palmira, en Gerard i la resta del grup... D'aquests anys de col.laboració en van sortir les meves dues primeres publicacions. Tota una aventura! Juli, gràcies de tot cor per l'oportunitat que em vas donar.

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Durant el primer any, no hagués avançat al laboratori sense l'ajuda de la gent d'antro: Toni, Emili, Marc, Meritxell, I sense els consells de la gent dels Serveis Cientificotècnics encara estaria encallada amb la primera tongada de seqüències: Amaia, Ramón i Albert, gràcies per la vostra ajuda!

Les estades en centres estrangers han estat molt importants en l'elaboració d'aquest treball. La primera estada a Montpellier amb L'Arnaud Estoup va ser un xoc amb la realitat, que titularia "sola entre els clons", aquella temporada a França em va ensenyar molt, tant a nivell professional com a nivell personal, i en tinc molts bons records. Vull agrair a la gent del Centre de Biologie et Gestion des Populations la seva ajuda al laboratori, i el treure'm de tant en tant a banyar-me al riu l'Herauld. Merci per tout.

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Gràcies al tiramissú de "Casa Victoria" a North End (Boston), (el millor Tiramissú ever), vaig veure la llum i vaig entendre les claus d'inferència del Nested Clade Anàlisi del Templeton (1998). Gràcies Mikel per haver compartit el moment de la il·luminació Tiramissuniana. I per haver-te passat les "vacances" a -10ºC, fent-me costat i animant-me mentre seqüenciava esponges i em *cagava* en els customs de Wildlife que em van retenir la població de Sicília a New Jersey fins a l'última setmana, arruinant unes magnífiques vacances al tròpic...

Els Àngels de Xavi: Isa, Su, Meritxell Codina, Raquel, i la darrera en arribar, Rocío, sempre han estat més que companyes de despatx o de poiata i han fet del dia a dia una festa.

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Dedico aquesta tesi a les dones de la meva familia, totes dones fortes que han lluitat per ser felices, i ho han aconseguit. Sé que elles, amb orgull, han vist en mi tot el que no han pogut fer. Jo he vist en elles tot el que vull ser.

Agost del 2003.

Sandra

If we count backwards this multiplication of individuals in each species, in the same way as we have multiplied forward, the series ends up in one single parent"

Carolus Linnaeus, 1758

"All evolutionary changes start with changes within populations."

Wen-Hsiung Li

Table of contents

Objectives and structure of the present work

The main goal of this thesis has been to study the population structure of a marine sponge species (*Crambe crambe)* at different geographic levels using different molecular markers.

In many organisms, particularly terrestrials, molecular markers have been used to answer questions about the structure and connectivity of populations, the potential of dispersal or the history of species, and these data were of crucial help in conservation and management of species. Most of these studies used allozymes and, more recently, mitochondrial or nuclear sequences with success. Also, microsatellite markers are being developed and applied with terrific success in many species. Unfortunately, application of molecular markers in marine environments lags much behind, especially for invertebrate organisms, where population genetic studies are still scarce and most of them involve only allozymes. Thus, this study attempts to contribute to the development and application of molecular markers for ecological and population genetic studies in marine invertebrates.

Most marine invertebrate organisms are benthic, and many of them sessile, with larvae as the main form of dispersal. Because of the intrinsic difficulty on following larval movements in natural environments, molecular markers appear as the perfect tool to track and characterize populations, and even individuals.

Hence, in this thesis several molecular markers have been applied in a sponge species, and with the complementary help of previous ecological and biological knowledge of the species, the present-day population structure of this sponge has been studied, trying to uncover which factors are affecting and might have affected in the past the structure found, while comparing the resolution and the "snapshot" information recovered with each of the markers used.

The first chapter corresponds to the introduction to the "molecular ecology philosophy" with especial emphasis on the history and facts of the markers used, a quick look at the works published in this field for marine invertebrate organisms as well as to what we do know about the biology and ecology of the sponge studied.

The second chapter includes the use of mtDNA sequences of the gene Cytochrome Oxidase subunit I to characterize 7 populations from the western

Mediterranean and one population from the Canaries Islands, 86 individuals in total. This study represents the first survey of sequence variation of mtDNA genes among populations of any sponge species and has been accepted for publication in Marine Biology.

In the third chapter the ribosomal Internal Transcribed Spacer-1 (ITS-1), 5.8S rRNA, and the Internal Transcribed Spacer-2 (ITS-2) have been analyzed for the same populations and individuals used in the mtDNA study plus a few other populations, reaching a total of 11 populations and 121 individuals. This study has been submitted for publication.

The fourth chapter describes the development and characterization of polymorphic microsatellite loci from a partial genomic DNA enriched library; allelic variation of the loci described is assessed and compared for two distant populations in order to weigh up the utility of microsatellites as high-resolution genetic markers for this species. These are the first microsatellite loci reported from any species in the phylum. This study has been published in Molecular Ecology Notes (2002, 2:478-480).

In the fifth chapter, the formerly developed microsatellite loci are genotyped for the same 11 populations as in chapter 3, the number of individuals analyzed has been increased to 286. This is the first study to use microsatellites in ascertaining population structure of a sponge. This study has been submitted for publication.

The sixth and last chapter corresponds to the general conclusions of the thesis.

CHAPTER 1: General introduction

"I will take a shot of Genes with Ecology … and mix it well please!"

1-1. Generalities

1-1-1. Molecular ecology and phylogeography

Molecular ecology is the interface between molecular biology, ecology, and population biology. As such it includes genetic studies on population structure, effective population size, gene flow, population history, and demography. In addition to population genetic approaches using molecular markers, molecular ecology also uses gene and species phylogenies to study processes with effects at the population level.

Phylogeography seeks to interpret the mode by which historical processes in population demography may have left evolutionary footprints on the contemporary geographic distributions of gene-based organismal traits. This analysis and interpretation of lineage distributions usually requires extensive input from molecular genetics, population genetics, ethology, demography, evolutive biology, paleontology, geology, and historical geography (Avise 2000).

Understanding the molecular basis of genes has transformed biological research since 1953, and ecologists have participated of the fruits of this revolution. The use of molecular markers, beginning with restriction fragment length polymorphisms (RFLPs) to today's single nucleotide polymorphisms (SNPs), has had a significant impact on our ability to trace parentage and kinship, to measure gene flow and migration patterns and to reconstruct the demographic histories of populations and species. The advent of modern genetic technology has provided an unparalleled ability to examine evolutionary and ecological forces in nature (Purugganan & Gibson 2003).

1-1-2. Population structure

The populations of most, if not all, species show some level of genetic structuring, which may be due to a variety of non-mutually exclusive causes. Even the European eel (*Anguilla anguilla*), often considered as the classical example of a random mating population because all individuals are thought to migrate to the Sargasso Sea for reproduction, has been shown to be geographically structured (Wirth & Bernatchez 2001). Environmental barriers, historical processes and life histories (e.g. mating system) may all, to some extent, shape the genetic structure of populations (e.g. Gerlach & Musolf 2000; Kyle & Boulding 2000; Tiedemann *et al.* 2000; Goldson *et al.* 2001). In addition, as the geographical distribution of a species is typically more extended than an individual's dispersal capability, populations are often genetically differentiated through isolation by distance (i.e. populations in close proximity are genetically more similar than more distant populations).

The number of alleles exchanged between populations is indicative of the genetic structure of a species, so understanding gene flow and its effects is central to many fields of research including population genetics, population ecology, and conservation biology… Migration homogenizes allele frequencies between populations and determines the relative effects of selection and genetic drift. High gene flow precludes local adaptation (i.e. fixation of alleles, which are favoured under local conditions), and will therefore impede also the process of speciation (Barton & Hewitt 1985). On the other hand, gene flow introduces new polymorphisms in the populations, and increases local effective population size (the ability to resist random changes in allele frequencies), thereby opposing random genetic drift, generating new gene combinations on which selection can potentially act. Reliable estimates of population differentiation are also crucial in conservation biology, where it is often necessary to understand whether populations are genetically isolated from each other, and if so, to what extent. The knowledge of population structuring may therefore provide valuable guidelines for conservation strategies and management (e.g. Avise 1992; Avise 1998; Grant & Bowen 1998; Holland 2000; Sweijd *et al.* 2000; Eding & Meuwissen 2001; Lockwood *et al.* 2002; Ruggiero *et al.* 2002).

We can measure genetic structure at different levels, that is:

-The individual level: heterozygosity/homozygosity

-The local population: allelic and genotypic diversity (Fis)

-Among populations: distribution of allele frequencies across the local subpopulations (Fst)

All these F-statistics collectively describe the population structure. We can explain this population structure as rising from recurrent forces that occur in each generation (gene flow, mating system, drift, potential of dispersal…). However, there are occasional processes that have very important consequences for evolution: historical events. For instance, habitat fragmentation may decrease gene flow and increase drift and inbreeding, while a range expansion may increase gene flow and decrease drift and inbreeding. Other important non-recurrent changes in population size include bottlenecks, founder events, and population growth.

1-1-3. Population structure vs. population history

When measuring a pattern of genetic variation, the main concern is to interpret the causes that drew the pattern found: Is the pattern due to recurrent evolutionary forces? Or it is due to historical factors? Unfortunately, these two forces are completely confounded in many measures we make. The reason is that historical events change the recurrent evolutionary forces (such as gene flow), but the response is not immediate. Equilibrium takes a long time to achieve, in fact, it is probably rarely/never achieved in benthic invertebrates due to their reproductive strategies (mating structure, dispersal abilities). Population genetic equations are generally written to describe what the conditions will be at this equilibrium state, at the *ideal* state.

What we measure on real populations is the result of both recurrent processes and historical processes that are not necessarily in equilibrium. Hence, one must take great care in interpreting what genetic survey data mean, especially parameters such as F-Statistics that are unable to distinguish between both sources of genetic structure. For instance, suppose a population has been fragmented into two or more subpopulations that experience no gene flow at all. If they had a recent shared

ancestry, the populations could still display some genetic similarity that would yield a estimate of F_{st}<1, erroneously implying non-zero gene flow. The same may occur if a population expands its range over a large area from some smaller ancestral range. The new populations may keep for some time a genetic similarity with the original ones as a result of recent shared ancestry, not actual gene flow. Particular care should be exercised when Fst values are used to infer number of migrants (Nm) between populations and, hence, actual gene flow values. This inference is based on stringent assumptions, such as that the island model applies to the populations studied and that they are at equilibrium. These assumptions are rarely met in benthic invertebrate populations.

Starting with Wahlund (1928), population geneticists have realized that genetic survey data can reveal information about population subdivision. Wright (1931, 1943) introduced F-Statistics as a way of utilizing allele frequency data gathered in different geographical locations to quantify population subdivision and estimate amount of gene flow. However, modern genetic surveys using restriction site or DNA sequence data also provide information on the evolutionary relationships of the genetic variation being scored, which is often presented as an allele or haplotype tree. Consequently, information is now available about the allele changes through evolutionary time as well as geographic space. This new temporal information can be used to understand the spatial distribution of current allelic variation. The relationship of population structure with population history can be investigated by using the gene trees.

1-1-4. Gene trees and gene networks (coalescent theory)

Given a sample of genes, the relationships among them can be traced back in time to a common ancestral gene. The genealogical pathways interconnecting the current sample to the common ancestor constitute a gene tree or gene genealogy (Posada & Crandall 2001). Gene genealogies are approximated by the estimated haplotype or allele trees.

Phylogeny *sensu stricto* studies the hierarchical relationships between genes from different species. By contrast, relationships between genes sampled from

individuals within a species are tokogenic and not hierarchical (Hennig 1966) because they are the result of sexual reproduction. (Fig.1).

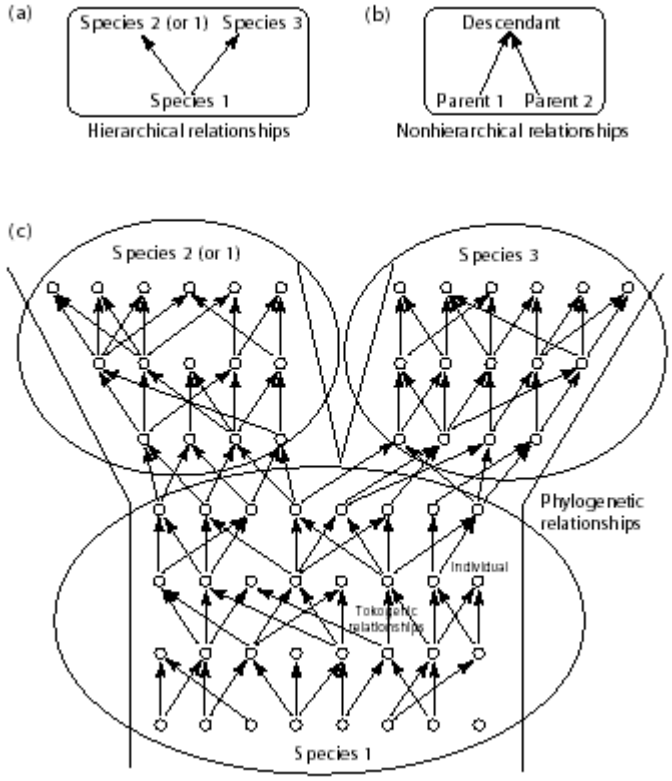

Figure 1. Tokogeny versus phylogeny. (a) Processes occurring among sexual species (phylogenetic processes) are hierarchical. That is, an ancestral species gives rise to two descendant species. (b) Processes occurring within sexual species (tokogenetic processes) are nonhierarchical. That is, two parentals combine their genes to give rise to the offspring. (c) The split of two species defines a phylogenetic relationship among species (thick lines) but, at the same time, relationships among individuals within the ancestral species (species 1) and within the descendant species (species 2 and 3) are tokogenetic (arrows). From Posada & Crandall (2001).

There are a lot of different methods to analyse sequences for phylogenetic estimation; traditional methods were developed to estimate interspecific relationships, such as maximum likelihood, maximum parsimony and minimum evolution. These methods may not take into account properly the fact that, at an intraspecific and population level, several phenomena violate some of their assumptions leading to poor resolution or inadequate portraits of genealogical relationships (Posada & Crandall 2001).

It is obvious that individuals within a species diverged later than individuals from different species. Then, within species data sets have fewer characters for phylogenetic analysis, diminishing the statistical power of traditional phylogenetic methods. Typically intraspecific studies involve many individuals for comparison, whereas interspecific phylogenetic studies tend to be based on one representative individual per species. Because of the density of sampling, intraspecific data sets

reach considerable sample sizes, regularly more than 100 individuals. This would require excessive computational time for most methods that have been developed for interspecific comparisons. So, new phylogenetic methods taking into account population evolutionary patterns are needed. Network methods appear as an answer to these needs. Networks can account effectively for processes acting at the species and population level and they are able to incorporate predictions from population genetics theory (Posada & Crandall 2001). These are the main advantages of networks over strictly bifurcating trees for estimating within-species relationships. Most network methods are distance methods, with the common idea of minimizing (with some specific restrictions) the distances (number of mutations) among haplotypes.

1-1-5. Findings in marine invertebrate species

Most marine invertebrates spend at least part of their life cycle in open water as free-moving gametes, larvae, or adults. Thus, even in sessile species, opportunities for moderate to high gene flow would seem to be the norm except where strong ecological or biogeographic impediments to dispersal exists. Nevertheless, a wide variety of phylogeographic outcomes have been observed. The expected pattern would be that species with high dispersal potential (i.e. with planktotrophic larvae) will have smooth genetic structure and high gene flow (see examples in Palumbi & Wilson 1990; Lacson 1992; Ovenden *et al.* 1992; Russo *et al.* 1994) and species with low dispersal potential (i.e. with lecitotrophic larvae) will have stronger genetic structure (see examples in (Janson & Ward 1984; Day & Bayne 1988; McMillan *et al.* 1992; Duffy 1993; Hunt 1993), but there are examples showing that expectations are sometimes surprisingly wrong (see examples in Solé-Cava *et al.* 1994; Grant & da Silva-Tatley 1997; Uthicke & Benzie 2000; Lazoski *et al.* 2001), and a big bunch of factors (biological, physical, ecological….) might act together and contributing to shape the population structure of marine invertebrate species through time.

In the last decade, population genetic and phylogeographic studies in marine invertebrates have grown considerably, beginning with allozyme studies, continuing

through sequence data and in the last years incorporating microsatellites as one of the "star" tools. The questions investigated in these studies have been wide-ranging; the major topics are numbered below:

1-Contrasting patterns of dispersal and reproduction
2-Examining population genetic structure and gene flow
3- Studying speciation and hybridisation
4- Scanning colonisations/invasions
5-Uncovering the phylogeography of species
6-Investigating clonal structure
7-Distinguishing cryptic species

Although most of the studies can be considered "basic science" the knowledge acquired can be applied, especially in conservation matters, a field expanding more and more in the present days (Avise 1998; Sweijd *et al.* 2000). An ecological trouble developed mostly in this century is the anthropogenic transport of marine organisms, often worldwide, via ballast waters and ship hulls (Carlton & Geller 1993; Lodge 1993). Some of the invasions involve sibling species and, thus may go undetected by morphological appraisals alone. Molecular phylogeographic studies can also help document invasion events and pinpoint sources of the introductions (i.e. Holland 2000; Tarjuelo *et al.* 2001; Turon *et al.* 2003).

A sample of molecular ecological and phylogeographic works published up to now for marine invertebrates is presented in Table 1, indicating the central topic studied and the organism studied. Note that a single study may cover more than one topic. The list does not pretend to be exhaustive, but representative of the taxonomic and conceptual extent of the main lines of research undertaken in this field.

Table 1. Summary of some molecular, ecological and phylogeographic works published by now for marine invertebrates. 1- Dispersal and reproduction, 2-Genetic structure, 3-Speciation and hibridisation, 4-Colonisations/invasions, 5-Phylogeography, 6- Clonal structure, 7-Cryptic species/morphotypes.

Organism	Topics (numbered as above)						
	1	2	3	4	5	6	7
Sponges		Benzie *et al.* 1994; Duran *et al.* 2002; Wörheide *et al.* 2002; Duran *et al. in press;* Wörheide *et al.* in press			Wörheide *et al.* 2002; Wörheide *et al.* in press		Solé-Cava & Thorpe 1986; Solé-Cava *et al.* 1991; Boury-Esnault *et al.* 1992; Muricy *et al.* 1996; Klautau *et al.* 1999; Lazoski *et al.* 2001
Anemones		Billingham & Ayre 1996				Bronson *et al.* 1997	
Corals	Russo *et al.* 1994; Hellberg 1996	Hellberg 1994; Benzie *et al.* 1995; Bastidas *et al.* 2001; Rodrigiez-Lanetty & Hoegh-Guldberg 2002	Diekmann *et al.* 2001; Vollmer & Palumbi 2002		King *et al.* 1999; Rodrigiez-Lanetty & Hoegh-Guldberg 2002	Coffroth *et al.* 1992; McFadden 1997; Adjeroud & Tsuchiya 1999	
Clams		Benzie & Williams 1992; King *et al.* 1999					
Periwinkles		De Wolf *et al.* 2000					

Table 1. Continued

Organism	Topics numbered as above						
	1	2	3	4	5	6	7
Gastropods	Kyle & Boulding 2000; Wilke & Davis 2000	Grant & da Silva-Tatley 1997; Quattro *et al.* 2001					
Oysters		Reeb & Avise 1989; Launey *et al.* 2002					
Abalones		Huang *et al.* 2000					
Nudibranchs	Todd *et al.* 1998						
Cuttlefishes		Pérez-Losada *et al.* 2002					
Squids		Shaw *et al.* 1999					
Holothurias		Uthicke & Benzie 2000					
Starfishes	Hunt 1993	Waters & Roy 2003			Waters & Roy 2003		
Ophiuras							Baric & Sturmbauer 1999
Sea Urchins		Palumbi & Wilson 1990; McMillan *et al.* 1992; Palumbi *et al.* 1997	Palumbi *et al.* 1997				
Ascidians	Ayre *et al.* 1997			Tarjuelo *et al.* 2001; Stoner *et al.* 2002; Turon *et al.* 2003			Tarjuelo *et al.* 2001
Polychaetes				Patti & Gambi 2001	Patti & Gambi 2001		

Table 1. Continued

Organism	Topics numbered as above						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Bryozoans	Goldson *et al.* 2001						
Mudcarbs				Gopurenko *et al.* 1999			
Shrimps	Duffy 1993	Bohonak 1998; Aubert & Lightner 2000					Gusmao *et al.* 2000
Prawns		Brooker *et al.* 2000					
Brachiopods		Endo *et al.* 2001					

1-2. The species choice

In order to choose the organism of study, we sought a sessile marine invertebrate with lecitotrophic/philopatric larvae. That was because the limited dispersal potential of the philopatric larvae would theoretically lead to a strong population structure (at least at a large geographic scale) that would help to check out the resolution of each marker used. To choose an organism ecologically well known was also a key factor in order to facilitate interpretation of the data obtained. For all these reasons, and because the Benthic Ecology group of the Department of Animal Biology at UB has a powerful inescapable fascination towards "the red sponge", the common Mediterranean sponge *Crambe crambe* was selected for the study. At that moment, the biological studies performed on the species (mainly by the group at UB and the people of the Center of Advanced Studies at Blanes) had led to ideas about how the species should disperse and how populations should be established and structured. These ideas were untestable without the help of molecular tools. An extra motivation was that at that moment no molecular markers (except allozymes) had been applied for genetic population studies in any sponges.

1-2-1. The genus Crambe and its distribution

The genus *Crambe* (Vosmaer) contains five living species distributed mostly along the Mediterranean and East Atlantic coasts. In addition, a fossil species from New Zealand originally described under the name of *Vetulina* oamaruensi (Hinde & Holmes 1892) has recently been claimed as belonging to the genus *Crambe* (Uriz & Maldonado 1995). Living species and their distribution are listed below.

- *Crambe tuberosa* (Maldonado & Benito 1991)

Species only known by a unique, small encrusting specimen collected from the coralligenous facies of Alboran Island (western Mediterranean).

- *Crambe tailliezi* (Vacelet & Boury-Esnault 1982)

Species formerly discovered off the Mediterranean coasts of France and subsequently found in other western Mediterranean locations (Uriz *et al.* 1992; Maldonado 1993).

- *Crambe acuata* (Lévi 1958)

Species known from the Atlantic and East coasts of Africa, the coasts of Madagascar, and the Red sea.

- *Crambe crambe* (Schmidt 1868)

It is one of the most abundant sponges in the infralittoral western Mediterranean assemblages. Its geographic distribution covers the whole western Mediterranean from the Gibraltar strait to the Adriatic Sea (Schmidt 1862), even reaching Atlantic zones such as the Canary and Madeira Islands and the Saint Vincent Cape (Portugal). It has also been cited twice in the eastern Mediterranean coast in Egypt and Turkey (Burton 1936; Saritas 1972), although these specimens seem to be valid reports of *C.crambe* (Uriz, pers. com.) we believe that its abundance must be much lower than in the western area as it has not been reported again.

- *Crambe erecta* (Pulitzer-Finali 1992)

Species known from just one specimen collected from North Kenya Banks (east African coast).

Finally, it should be kept in mind that there are potential confusions between *Crambe crambe* and other sponge taxa, such as *Batzella inops, Stylinus brevicuspis* or *Hymeniacidon sanguinea* (M.J. Uriz, pers. com.).

1-2-2. The target species: *Crambe crambe*

Crambe crambe (Schmidt 1862) is a Poecilosclerid red encrusting sponge (Fig. 2). It reaches a maximum surface area of 0.5 m^2 and it is always found free of macro-epibionts. This species has been reported to have strong bioactivity that appears to perform multiple ecological roles such as space competition or predator avoidance (Becerro *et al.* 1997). Furthermore, it is practically free of symbionts (Galera *et al.* 2000), a very convenient characteristic taking into account the important potential problems that DNA contaminations from prokaryote symbionts pose to molecular studies on sponges (Lopez *et al.* 2002). Regarding its ecological distribution, *C. crambe* is one of the most abundant species in the Mediterranean littoral waters. It inhabits both well-illuminated and dark habitats at depths ranging from 1 to 60 meters and from hard substrata to *Posidonia oceanica* sea grass beds; it is one of the few species able to grow even among the invasive algae *Caulerpa taxifolia* in the western Mediterranean.

Figure 2. C*rambe crambe*

The distribution range of the sponge goes from the east Atlantic Ocean (Canary and Madeira Islands) to the western Mediterranean reaching the Adriatic Sea (Thiele 1889) and the coasts of Egypt and Turkey (Burton 1936; Saritas 1972). The sponge attains its maximum abundance in the western Mediterranean Sea (Fig.3) where it can reach a abundances of ca. 67±2.7 individuals per m^2 and 47%±1.9% cover (Uriz *et al.* 1998) The species grows on a seasonal basis, being the warm season the period of active growth. However, growth rates were remarkably low in *C. crambe* (Turon *et al.* 1998).

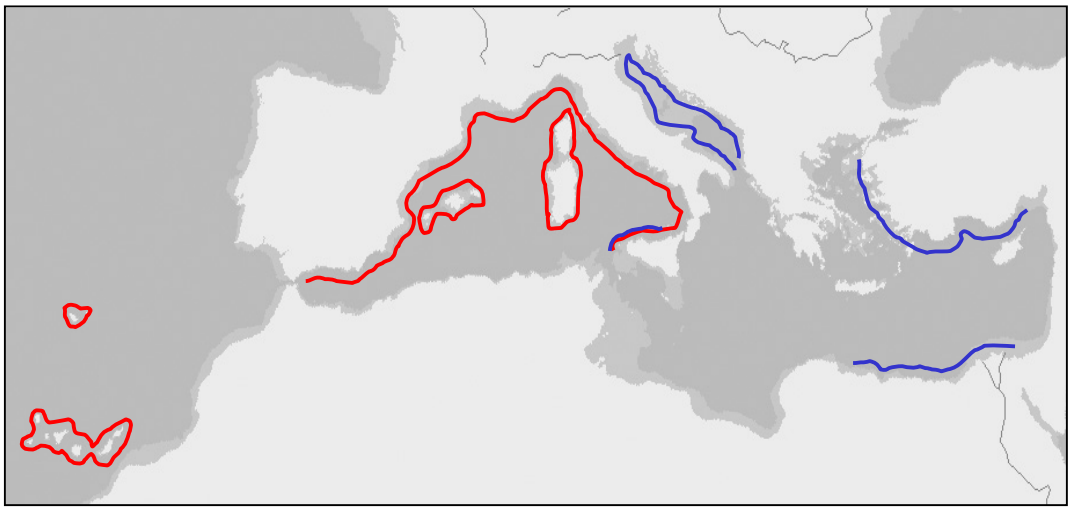

Figure 3. *Crambe crambe* distribution area. In red are areas where it has been found in abundance. In blue, areas where its abundance is unknown or supposed to be scarce. In blue+red are areas where it is known to be at low abundance.

Studies on *C. crambe* growth dynamics (Turon *et al.* 1998) show that this sponge is able to reproduce asexually by means of fission and that fusion of sponges also occur, although, the importance of these asexual processes was not as relevant to its demography as in other encrusting invertebrates.

C.crambe is hermaphrodite and reproduces sexually by releasing sperm to the water via the oscula. Sperms are captured by other individuals via the inhalant pores and fertilize the mature oocytes. Embryos are incubated and larvae are released through oscula when ready for dispersal (from the end of July to the end of August in

north western Mediterranean). Embryos are found in high numbers from April onwards, the density of embryos is 76.2±12.5 embryos cm^{-2}, although some specimens can brood an extremely high density of embryos (more than 200 embryos cm^{-2}) (Uriz *et al.* 1998). Once released, larvae swim in a corkscrew motion in a wavy, irregular way and are dispersed several meters away from the parental sponge immediately after release. Larvae have a 24-72h free-living stage (laboratory assays), after that, they settle down with a tendency to settle within spaces surrounded by conspecifics (Uriz *et al.* 1998).

Predation on *C.crambe* larvae and settlers has been documented by small fish (Uriz *et al.* 1996), a cnidarian (*Eudendrium ramosum* L.) (Uriz *et al.* 1998) and copepods feeding on young sponges (rhagons) (Mariani & Uriz 2001). Predation on *C.crambe* adults has not been reported.

In summary, we can say that *C.crambe* is one of the ecologically best-known sponge species in the Mediterranean Sea. Its symbiont free nature together with its high abundance and its distribution makes this sponge the best choice for a population genetics survey. Given the phylopatric behaviour of its larvae due to its short life-span, we expect that *Crambe crambe* populations will be closed enough to show high levels of differentiation even at a medium scale (tens of kilometres) and that this sponge will follow an Isolation by Distance pattern of dispersal.

1-3. The marker choice

Due to the remarkable developments in the field of molecular genetics, a variety of different techniques to analyse genetic variation have emerged during the last few decades. These genetic markers may differ with respect to important features, such as genomic abundance, levels of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. Therefore, none of the available techniques is superior to all others for a wide range of applications; rather, the key-question is which marker to use in which situation. The choice of the most appropriate genetic marker will depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical

facilities and know-how, time constraints, and financial limitations (Brown 1996; Parker *et al.* 1998; Ross *et al.* 1999).

1-3-1. PCR-Sequencing

PCR-sequencing involves determination of the nucleotide sequence within a DNA fragment amplified by the Polymerase Chain Reaction (PCR) using specific primers (15-35 bp) for a particular genomic site. The method most commonly used to determine nucleotide sequences is based on PCR reactions with labelled dideoxy terminators. PCR-sequencing provides the ultimate measurement of genetic variation. Universal primer pairs to target specific sequences in a wide range of species are currently available for mitochondrial and nuclear genomes. Because sequencing is costly and time-consuming, most studies have focused on only one or a few loci. Due to this restricted genome coverage and the fact that different genes may evolve at different rates, the extent to which the gene diversity estimated reflects overall genetic diversity is questionable. Because it is in general difficult to target a gene or area with enough nucleotide variation below the species level, PCR-sequencing is particularly useful to address questions at higher taxonomic levels, e.g. phylogeny reconstruction (Sanger *et al.* 1977). Nevertheless, some mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) genes/areas are known to be high-resolution markers for intraspecific studies in most of the phyla.

1-3-1-1. MtDNA sequences

Approximately 70 percent of phylogeographic studies conducted to date involved analyses of animal mitochondrial (mt) DNA either primarily or exclusively (Fig. 4).

In 1975, Brown & Wright published the first significant analysis of mtDNA variation in nature in a brief paper on parthenogenetic lizards. This paper pioneered a series of studies spanning two decades that documented the power of mtDNA analysis in deciphering the evolutionary origins and ages of numerous vertebrate taxa, reviewed in Avise (1992).

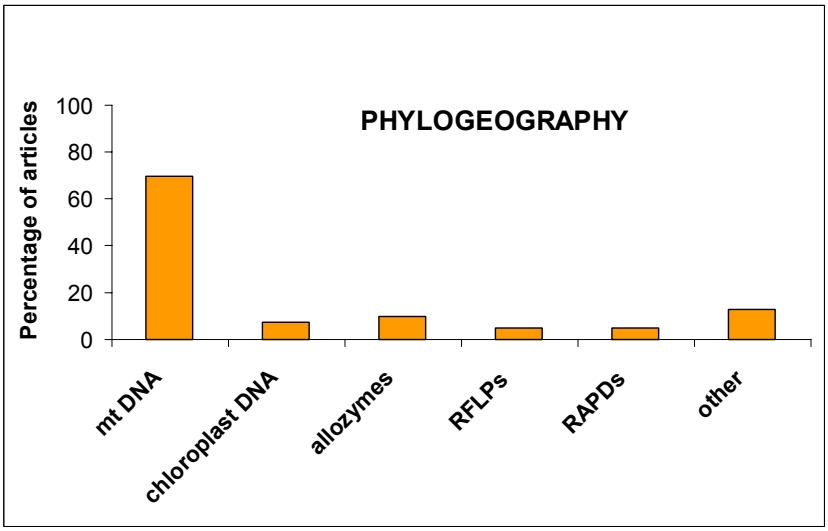

Figure 4. Breakdown of the phylogeographic articles according to the molecular assay procedure employed (from 1987 to 1998). From Avise (2000)

Several early studies used Random Fragment Length Polymorphism (RFLP) markers to document at the molecular level what had been suspected from cytological and genetic investigations early in the century: mtDNA is transmitted maternally in higher animals (for an exception see Zouros *et al.* 1994). Because of the uniparental transmission of mtDNA in the majority of animals, recombination was thought not to be occurring, but recent studies have found examples not only in animals with doubly uniparental inheritance of mtDNA (i.e. *Mytilus galloprovincialis)* but also in a few animals with standard maternal mtDNA inheritance: an amphibian, a crustacean, a mammal and a fish (Ladoukakis & Zouros 2001; Hoarau *et al.* 2002).

In 1979, Brown *et al.* published another influential article announcing an unexpected fast pace of mtDNA sequence evolution as gauged by interspecies comparisons of higher primates. This led to a widely used “clock” calibration for animal mtDNA: about 2 percent sequence divergence between pairs of lineages per million years (or 1 percent sequence evolution per lineage per 10^6 years) (Avise 2000). The finding of a high evolutionary rate for mtDNA came as a complete surprise. At face value, it appeared to violate a fundamental principle of molecular evolution: that constraint on function implies constraint on macromolecular structure.

Several hypotheses have been put forward to account for the rapid evolution of animal mtDNA (Wilson *et al.* 1985; Gillespie 1986; Richter 1992; Li 1997; Nebdal & Flynn 1998):

- Relaxation of functional constraint, because mtDNA does not code for proteins involved directly in its own replication or transcription, and because a molecule that produces only 13 kinds of polypeptides might tolerate less accuracy in translation.
- A high mutation rate, due to an inefficiency of DNA repair systems, high exposure to mutagenic free radicals in the oxidative mitochondrial environment, or fast replicative turnover within cell lineages.
- The nakedness of mtDNA. This molecule is not complexed with histone proteins that are evolutionarily conserved and might constrain rates of nuclear DNA evolution.

Regardless of the cause, rapid sequence evolution is a prerequisite for a marker to be used as a microevolutionary phylogenetic tool, thus, high mutation rates in mtDNA (for exceptions see (Gillespie 1986; Shearer *et al.* 2002; Duran *et al. in press*) assure a good resolution of this marker for this kind of studies.

A very important aspect of the early mtDNA studies was the introduction of explicit phylogenetic concepts to intraspecific evolution. Before these studies, a fixed idea was that phylogeny had no meaning at the intraspecific level because, for sexually reproducing organisms, conspecific lineages are anastomosing rather than hierarchically branched (Fig.1). Hennig (1966) characterized biological speciation as the demarcation between the realms of tokogenetic associations (genetic relationships among individuals, where phylogenetic concepts supposedly did not apply) and phylogenetic associations among species. However, due to the uniparental mode of DNA transmission and general lack of recombination, mtDNA gene trees are non-anastomosing and hierarchically branched even within sexually reproducing species. Thus, the extended matrilineal component of organismal history can be assessed using the algorithms and perspectives of phylogenetic biology.

Regarding mtDNA, we have to keep in mind that we inherit our mtDNA from just one of our sixteen great-great grandparents, yet this maternal ancestor has only contributed one-sixteenth of our nuclear DNA (Wainscoat 1987). Thus, although the information recorded in mtDNA represents only one of many molecular tracings in the evolutionary histories of organisms, it is nonetheless a specified genealogical history, that of the females (Avise *et al.* 1987).

Table 2 summarizes the major empirical findings on animal mtDNA and two of the unorthodox perspectives (Avise 1991) they entail for phylogenetic appraisals at microevolutionary scales.

Table 2. Molecular and transmission genetics of animal mtDNA: unanticipated discoveries and unorthodox conceptual orientations for microevolutionary analysis. From Avise (2000)

Observations
1) Animal mtDNA displays extensive intraspecific polymorphism and often evolves faster than typical single-copy nuclear DNA.
2) Most mtDNA variants involve nucleotide substitutions or small length changes; gene order is highly stable over short evolutionary time.
3) Populations of mtDNA molecules inhabit somatic-cell and germ-cell lineages.
4) Most individuals typically are nearly homoplasmic for a single prevalent mtDNA sequence; genetic sorting from heteroplasmy is relatively rapid.
5) MtDNA inheritance is asexual, maternal (almost exclusively), and normally apparently without intermolecular genetic recombination.
Immediate phylogeographic outcomes:
1) Individual animals can be viewed as OTUs in phylogenetic appraisals.
2) MtDNA genotypes record matrilineal relationships within and among species.

1-3-1-2. rDNA sequences: The Internal Transcribed Spacers

rDNA codes for the RNA component of the ribosome. The rDNA is a multigene family with nuclear copies in eukaryotes arranged in tandem arrays (Figure 5). They are organised in nucleolus organiser regions (NORs), potentially at more than one chromosomal location. Each unit within a single array consists of the genes coding for the small and large rRNA subunits (18S and 28S). The 5.8S nuclear rDNA gene lies embedded between these genes but separated by two internal transcribed spacers: ITS1 and ITS2. The external transcribed spacer (ETS) and the intergenic spacer (IGS) separate the large and small subunit rDNAs (Fig.5).

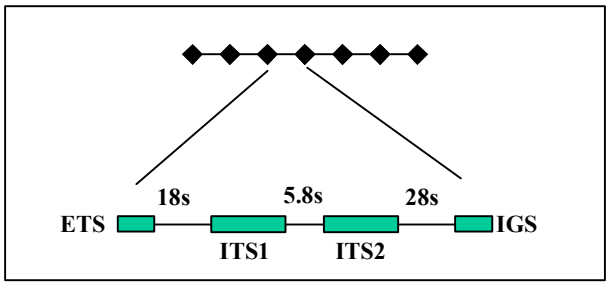

Figure 5. Organisation of one rDNA array. Single repeat units (black dots) are tandemly organised. Each of them consists of the rRNA genes: 18S, 5.8S and 28S. Spacers separate these genes, namely the external transcribed spacer (ETS), the internal transcribed spacers (ITS 1 and ITS 2) and the intergenic spacer (IGS).

Different selective forces are acting on the rDNA region with the consequence of varying degrees of sequence conservation across single repeat units. Therefore, each part can be employed for specific phylogenetic questions across a broad taxonomic spectrum (Hillis & Dixon 1991). The small subunit is highly conserved and has been used to shed light on deep evolutionary branches, *e.g*. for relationships between *Archaebacteria* and *Eubacteria,* and to uncover evolution within Metazoans, while the more conserved domains within the 28S region have been used to cover evolutionary time through the Palaeozoic and Mesozoic eras. The faster evolving ITS regions, however, have been employed for populations and congeneric phylogenies *(Wörheide et al. 2002)*. The smallest rDNA gene of the cluster, the 5.8S, is too short to provide a robust phylogenetic signal.

PCR amplification of the ITS region has become a popular choice for phylogenetic analysis of closely related species and phylogeography studies within species, some recent examples of their use in marine invertebrates are: van Oppen

et al. (2000); McCormack *et al.* (2002) or Thacker & Starnes (2003). This popularity stems from the derivation of universal primers located in the coding regions flanking the ITS. Both direct sequencing and cloning of PCR products can be used for ITS analysis. Their deployment depends on the hierarchical scale of the question. Direct sequencing generates a consensus sequence for phylogenetic analysis. For population questions, if within individual polymorphism is found, additional profit is gained from information on variation at the level of single repeat units. Hence, PCR products need to be cloned and sequenced.

Ribosomal DNA genes evolve cohesively within a single species and exhibit only limited sequence divergence between rDNA copies within single individuals (Arnheim *et al.* 1980). In contrast, comparisons between species show normal levels of sequence divergence. The combination of these two observations is referred to as concerted evolution (Dover 1982). The mechanisms driving concerted evolution are unequal crossing over and gene conversion. Irrespective of the precise mechanism, the degree of within-individual homogenisation is a result of the interplay between homogenisation mechanisms and mutation processes (Schlötterer & Tautz 1994) Since rDNA clusters are frequently distributed on several chromosomes, a potential problem relates to what extent concerted evolution is hindered by the different chromosomal location of the arrays.

1-3-3. The Microsatellites

Within the past decade microsatellites (Variable number of tandem repeats: VNTR, Simple sequence length polymorphism: SSLP, or Single sequence repeats: SSR) have become one of the most popular genetic markers for molecular ecologic studies.

Microsatellites consist of tandem repeats of sequence units, each generally less than 5 base pairs in length, such as $(TG)_n$ or $(CGA)_n$. They are widely dispersed in eukaryotic genomes and in the chloroplast genome of plants, and are often highly polymorphic due to variation in the number of repeat units (Bruford & Wayne 1993), furthermore they are generally considered neutral because they are generally in non-coding regions, so that selection and environmental pressure do not influence their

expression directly. However, microsatellites might be linked to selected genes and thus may not be strictly neutral (Estoup & Angers 1998).

The mean density of microsatellites within species varies widely among taxonomic groups and sometimes among species within a taxonomic group (Estoup & Angers 1998). Regarding the nature of the motif array sequence, microsatellites can be classified as perfect, imperfect or compound sequences (Weber 1990). Perfect microsatellites are composed of uninterrupted stretches of repeat units, while in imperfect microsatellites; one to several bases interrupts the perfect array. Compound microsatellites consists of neighbouring repeated sequences composed of different repeat types.

In 1989, three papers independently reported the isolation and characterisation of allelic variability at microsatellite loci using PCR (Litt & Luty 1989; Tautz 1989; Weber & May 1989). In these studies, microsatellites in several species were either cloned and sequenced, or identified in sequence databases. PCR primers were designed from sequences flanking the tandem repeat, and the polymorphic amplified products were separated on polyacrylamide gels allowing the resolution of alleles differing by as little as 1 base pair. Nowadays automated sequencers allow the sizing of alleles in a faster and more reliable way.

Since those initial studies, microsatellite loci have been widely used and have proved to be highly polymorphic. Furthermore, their abundance and ubiquitous distributions have rendered them very popular and valuable markers. Microsatellites have been established as a marker of choice for the identification of individuals and paternity testing, and even more, the high sensitivity of PCR-based microsatellite analysis opened completely new research areas, such as the analysis of samples with limited DNA amounts, or degraded DNA such as faeces or museum material (Schlötterer 2000). Microsatellite analysis has also been employed in population genetics, molecular systematics and ecology (Estoup & Angers 1998; Goldstein & Schlötterer 1999). However, the great potential of microsatellite markers is actually limited by the time consuming and technically demanding development step, the poor knowledge of their mutational processes and, consequently the lack of specific methods for analysing the data.

One of the major drawbacks of PCR-based microsatellites is that when no single locus sequence is available in the literature they must be first isolated from the genome of the species in question. For isolation of microsatellite loci, there are highly detailed protocols available at the World Wide Web (see for instance http://www.inapg.inra.fr/dsa/microsat/microsat.htm). Another strategy consists in testing primers described for microsatellite loci in close species (cross-priming strategy), but rates of success vary greatly depending on taxa and loci. Generally, when cross-priming strategy works and PCR amplifications are obtained with non-specific primers, the result tend to be lower levels of polymorphism and lower PCR pattern quality with an increase on the frequency of null alleles (alleles with no PCR amplification) (Estoup & Angers 1998).

Intra-allelic processes, such as replication slippage and unequal crossing over, can explain mutational events in microsatellite loci. Although several mutation models have been proposed for microsatellites (i.e. Estoup *et al.* 2002; Li *et al.* 2002), two main models will be considered here: the infinite alleles model (IAM, Kimura & Crow 1964) and the stepwise mutation model (SMM, Kimura & Ohta 1978). The SMM describes the gain or loss of a single tandem repeat and hence mutation can drive to allelic states already present in the population. Under IAM model, a mutation involves any number of tandem repeats and always results in an allelic state not previously encountered in the species. Nevertheless, it seems that none of the models are strictly followed by microsatellite loci, and therefore more complex models might be involved in the polymorphism in length observed within loci. For a detailed review of the factors affecting microsatellite evolution see Estoup & Cornuet (1998).

The high polymorphism of microsatellites allows studying population structure at different levels, from a biogeographic range to a local or intrapopulation level. Microsatellites may allow the detection of genetic differences that less polymorphic markers could not reveal (Estoup *et al.* 1998). Their variability is so large that, even with a small number of loci and a large number of individuals, all individuals may have unique multilocus genotypes permitting analyses at the individual level. Most of their applications in ecology had been in uncovering population differentiation, effective population size; establishing mating structures, estimating genetic

relatedness between groups or pairs of individuals, parentage assignment, and detecting clone sizes. However its application in phylogenetic relationships among populations has not yet been substantial (Estoup & Angers 1998).

CHAPTER 2: Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida)

2-1. Abstract

Crambe crambe is a common encrusting sponge found in the Mediterranean and Atlantic littoral. An analysis of a partial sequence (535 bp) of the mitochondrial DNA (mtDNA) gene cytochrome oxidase subunit I (COI) was conducted in an attempt to determine population structure in this species. This is the first study of population genetics using this kind of marker in the phylum. Samples (N=86) were taken in eight populations separated by distances from 20 to 3000 Km spanning from the western Mediterranean to the Atlantic. Low variability of this gene was found as only two haplotypes were identified along with low nucleotide diversity (π=0.0006). However, the different frequencies found among populations revealed genetic structure and low gene flow between close populations as expected from the dispersal biology of the species. The low variability found in sponges is in agreement with reports on cnidarians and points to a high conservation of mtDNA in diploblastic phyla.

2-2. Introduction

In the last years, molecular techniques have allowed the study of population structure and dynamics in benthic invertebrates. These techniques provide powerful tools for analysing dispersal, colonisation patterns and gene flow between populations over varying geographic scales (Palumbi 1995).

Sponges are a group whose larvae probably disperse over short distances (Borojevic 1970; Sarà & Vacelet 1973; Maldonado & Uriz 1999; for an exception see Vacelet 1999). They are one of the dominating benthic groups in terms of biomass and species diversity, yet studies on the population structure and gene flow among conspecific populations are still scarce. Up to now, allozymes have been the most commonly applied markers in those studies (reviewed in Solé-Cava & Boury-Esnault 1999; Borchiellini *et al.* 2000). Results showed a prevalence of cryptic species that had gone undetected with previous morphological studies (Solé-Cava & Thorpe 1986; Boury-Esnault *et al.* 1992; Benzie *et al.* 1994; Klautau *et al.* 1994; Muricy *et al.* 1996; Boury-Esnault *et al.* 1999; Klautau *et al.* 1999) and that can potentially confound studies of genetic connectedness among populations. Once valid units (species) have been detected and investigated, the results tend to show high levels of genetic variation and population structure (even over small geographic scale), correlated with the allegedly low dispersal capability of sponge larvae or/and its high philopatry (Benzie *et al.* 1994; Klautau *et al.* 1999; Solé-Cava & Boury-Esnault 1999; Lazoski *et al.* 2001).

There is a need for expanding the panoply of molecular tools available in the context of population genetics of sponges, especially considering that; in general, allozymes evolve at a slower rate than mtDNA and nuclear DNA such as microsatellites (Bossart & Prowell 1998). Recently Wörheide et al. (2002), have shown the utility of Internal Transcribed Spacers rDNA sequences in sponge phylogeography. On the other hand, analysis of mtDNA sequence data has proved to be a powerful tool for tracing recent evolutionary history, such as founder events, populations bottlenecks and population range fluctuation in marine invertebrates (Gopurenko *et al.* 1999; King *et al.* 1999; Wilke & Davis 2000; Tarjuelo *et al.* 2001). This is mainly because of its maternal inheritance without recombination, higher

mutational rate, shorter coalescence times and more sensitiveness than nuclear genes in reflecting the genetic impact of population subdivision over large geographical scales (Avise et al. 1987; Palumbi et al. 2001). MtDNA has become a method of choice for intraspecific phylogeographic studies (Avise 2000).

The objective of this study was to use mtDNA sequence variation for detecting intraspecific genetic structure among populations of a sponge species and to assess the utility of this marker. Erpenbeck et al. (2002) and Schröder et al. (2003), have explored the usefulness of COI sequence data in unravelling interspecies relationships in sponges but, to our knowledge, this study represents the first survey of sequence variation of this gene among populations of any sponge species.

Crambe crambe (Schmidt 1862) is a common encrusting sponge widespread along the western Mediterranean sub-littoral (Boury-Esnault 1971; Pulitzer-Finali 1983; Uriz et al. 1992) and recently found in the Canary and Madeira Islands in the Atlantic ocean (pers. obs.). It was also cited once in the Adriatic sea (Schmidt 1862), and in the eastern Mediterranean coast in Egypt and Turkey (Burton 1936; Saritas 1972) respectively. The scarcity of reports from the eastern Mediterranean (even acknowledging that there are much less studies in this area) suggests that the species is far less abundant than in the western Mediterranean basin. *C. crambe* is one of the best-known sponge species from the point of view of its biology and ecology (Becerro et al. 1997; Turon et al. 1998; Uriz et al. 1998). In addition, *Crambe crambe* is virtually free of microsymbionts (Becerro 1994; Galera et al. 2000), which avoids one of the most problematic aspects of sponge population studies, that of determining whether symbiont-derived markers or true sponge markers are being investigated (Lopez et al. 2002).

Field and laboratory studies on the swimming behaviour and dispersal abilities of its larvae (Uriz et al. 1998) indicate that *C. crambe* populations are likely to show restricted gene flow at scales of tens of Km (hundreds at most). Moreover microsatellite analysis of one Mediterranean and one Atlantic populations of *C. crambe* indicated a marked genetic structure between these populations (Duran et al. 2002). Considering this previous biological and genetic knowledge, we will investigate which kind of population structure can be recovered from comparisons of mtDNA sequences in this species.

2-3. Materials and methods

2-3-1. Sampling

86 individuals of *Crambe crambe* were collected from six locations along the western Mediterranean coast and from two locations on the Atlantic Ocean, spanning about 3000 Km (Fig.6). Sampling was undertaken by scuba diving. In order to avoid sampling the same clone, sponges were collected at least five meters apart from each other.

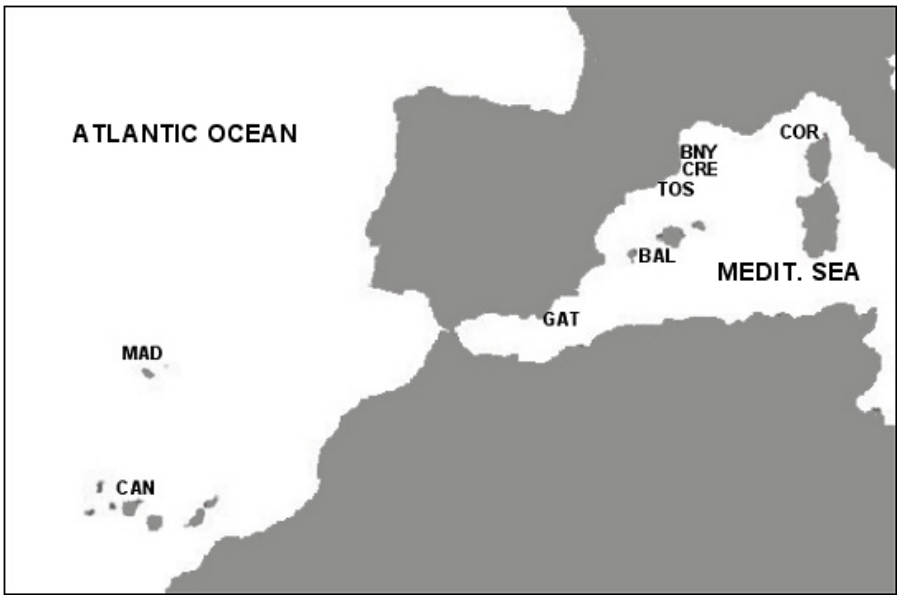

Figure 6. Map showing the localities sampled. MAD: Madeira, CAN: Gran Canaria, GAT: Cabo de Gata, BAL: Eivissa, TOS: Tossa de Mar, CRE: Cap de Creus, BNY: Banyuls Sur Mer, COR: Corsica

2-3-2. DNA extraction and amplification

We extracted total DNA from sponge tissue using the 'QUIamp Mini Kit' (Quiagen). We used the Universal Primers HCO2198 and LCO1490, described in

Folmer et al. (1994), for the amplification of a 710 bp fragment of the cytochrome oxidase subunit I gene (COI).

PCR amplification was performed in a 20µl total reaction volume with 0.4µl of each primer (25µM), 0.5µl dNTP's (10 mM), 2µl 10X buffer containing 15mM $MgCl_2$ (Promega), 1 U Taq Polymerase (Promega) and 0.5µl template DNA. An initial denaturation at 94ºC for 2 min was followed by 35 cycles (94ºC for 50 s, 40ºC for 55 s, and 72ºC for 1 min) and a final extension at 72ºC for 7 min.

2-3-3. DNA sequencing

The sequencing reaction was carried out with the 'ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer) on an ABI Prism 377XL automated sequencer using the same primers used for the amplification step. Two sequences per individual (forward and reverse) were obtained. In 6 individuals of the minoritary haplotype (see below), we performed a second independent PCR to ensure that the result obtained was not an artefact.

The nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database with accession numbers AF526297 and AF526298.

2-3-4. Data analysis

Sequences were aligned in a multiple alignment with the Clustalx program (Thompson et al. 1997). After alignment and trimming, the final sequence length used was 535 bp. Nucleotide diversity and haplotype diversity (Nei 1987) were calculated for each location. We calculated pairwise F_{ST} values and their significance for all populations. We used the exact test of population differentiation (Raymond & Rousset 1995a) to test the null hypothesis that observed haplotype distribution is random with respect to sampling location. We also run the Ewens-Watterson neutrality test with 10000 permutations. Analysis of molecular variance (AMOVA) was conducted to estimate the fraction of variability attributable to differences among and within populations. These analyses where performed using ARLEQUIN version

2000 (Schneider et al. 2000) and DnaSP version 3 (Rozas & Rozas 1999). To test for isolation by distance (Rousset 1997) we used the Mantel test implemented in Genepop (version 3.3) (Raymond & Rousset 1995b)

2-4. Results

Nucleotide sequences 535 bp in length were obtained from the COI gene of the 86 *Crambe crambe* specimens, revealing one polymorphic position resulting in two haplotypes (H1 and H2) and low nucleotide diversity (π=0.0006). The observed change was a transition (T-C) resulting in a synonymous substitution. Although there are virtually no prokaryote symbionts in this species, there is always a slight possibility to amplify contaminations from small DNA-templates of water column (bacteria, algae, etc.). To confirm the Metazoan nature of our sequences we performed a BLAST search in GenBank. The best match we obtained was with another sponge (*Placospongia*), followed by cnidarians (*Montastrea, Agaricina*) and molluscs *(Diodora, Tryonia*). This result, as well as the constancy obtained in the sequences (only two haplotypes), makes us confident that our sequences were not of symbiont origin or contamination.

Both haplotypes were found throughout the geographical range sampled along the Mediterranean coast although different populations exhibited distinct haplotype frequencies (Table 3). Only haplotype I (H1), the most frequent in the Mediterranean region, was found in the Atlantic populations (Fig.6, Table 3). H1 had a frequency greater than 60% in all Mediterranean populations except for Banyuls-sur-Mer, where H2 was the most frequent, with a frequency of 90%. Estimates of haplotype diversity (*h*), a measure of within-population haplotype variation (Nei 1987), ranged from 0 to 0.533 (Table 3).

Table 3. Populations studied (Code as in Figure 6), geographical location, haplotype frequencies, total sample size (N), haplotype diversity (*h*) and its standard error (SE) are listed.

Population	Location	H1	H2	N	*h* (SE)
MAD	32°45'N 17°00'W	1.00	0.00	8	0.00
CAN	27°48'N 15°47'W	1.00	0.00	11	0.00
GAT	36°48'N 2°14'W	0.82	0.18	11	0.327 (0.153)
TOS	41°43'N 2°56'E	1.00	0.00	15	0.00
CRE	42°19'N 3°19'E	0.60	0.40	10	0.533 (0.094)
BNY	42°29'N 3°08'E	0.09	0.91	11	0.182 (0.143)
BAL	38°54'N 1°26'E	0.89	0.11	9	0.222 (0.166)
COR	42°41'N 9°26'E	1.00	0.00	11	0.00

The results of the exact test for population differentiation based on haplotype frequencies revealed significant heterogeneity in the distribution of haplotypes across the samples ($P<0.05$). Pairwise tests for genetic differentiation among populations revealed significant values in comparisons involving Banyuls-sur-Mer with the rest of populations sampled (Table 4). There was no evidence for isolation by distance (Mantel test, $P=0.802$). The AMOVA showed that there was a significant genetic variance among populations ($\phi_{ST}=0.565$). The allele distribution was found to be non-neutral by the Ewens-Watterson test for either Banyuls-sur-Mer or Eivissa, whereas the Cabo de Gata and Cap de Creus populations were found to have neutral distributions. In the remaining populations this test could not be run, as only one haplotype was present.

Table 4. Pairwise *F*st values between populations (Codes as in Figure 6). Significant values at *P*<0.05 are indicated with an asterisk.

	MAD	CAN	GAT	TOS	CRE	BNY	BAL
CAN	0.000						
GAT	0.059	0.100					
TOS	0.000	0.000	0.140				
CRE	0.296	0.349*	0.016	0.405*			
BNY	0.883*	0.900*	0.665*	0.915*	0.393*		
BAL	-0.014	0.023	-0.091	0.060	0.099	0.755*	
COR	0.000	0.000	0.100	0.000	0.349*	0.900*	0.023

2-4. Discussion

Our results revealed high genetic similarity among the western Mediterranean and Atlantic populations of *C.crambe* separated by distances up to 3000 km, as a result of low levels of genetic diversity (π=0.0006) found in the COI gene studied. This degree of diversity is much lower than that found among populations of other invertebrates with lecitotrophic larvae (e.g. ascidians, π=0.0018-0.0032, Tarjuelo et al. 2001). High genetic similarity over long distances (8000 km) was already reported for another sponge species (*Chondrosia* sp.*,* Lazoski et al. 2001), although using allozymes.

It is hard to believe that a species whose larvae live from 24 to 72 hours (Uriz et al. 1998) is able to actively disperse more than a few hundreds of kilometers, so the genetic similarity found in *C. crambe* is unlikely to result from natural gene flow between localities. Yet, larvae may be transported in ballast water and released several hundred km away (Carlton & Geller 1993). Considering the low levels of variability found in our study, we have no resolution to ascertain whether or not there is an influence of anthropogenic dispersal in *C.crambe.*

The single change found between the two haplotypes allowed us to detect low levels of gene flow (based on frequency of both haplotypes) at distances of tens of kilometres in the North of Spain. The ecological hypothesis of low dispersal leading to strong genetic structure is, therefore, tenable in spite of the homogeneity found with this molecular marker.

A recent origin of the species or genetic drift associated to historical events such as a population bottleneck due to selective sweep or to a founder event from a source population might explain the lack of variability found. A recent bottleneck effect seems rather dubious, as we have sampled an important part of the distributional range of the species. On the other hand, a founder event in the Mediterranean is plausible, as colonisation of this sea should have been posterior to the Messinian desiccation crisis ca. five million years ago (Maldonado 1985). However, Atlantic populations (presumptive source) did not show a higher genetic variability than the Mediterranean ones. Since *C.crambe* distribution range spans to the east, analyzing specimens from the eastern Mediterranean would be interesting to elucidate possible colonization patterns. Unless one considers a very recent origin of this species, the most likely explanation for the pattern found is that the mitochondrial gene studied is unusually conserved in this species. Preliminary results with the genes Cytochrome oxidase subunit II and Cytochrome *b* in a subset of the specimens here analysed also showed a lack of variability in mtDNA (authors, unpubl. res.). Also, the genetic similarity found with COI gene between Tossa de Mar and Gran Canaria populations (F_{ST}=0) contrasts with the differentiation found in a microsatellite study in the same populations (F_{ST}=0.29, (Duran et al. 2002), which strongly suggests that mtDNA is very conserved at least in this species.

In a study of a partial sequence of a sponge mitochondrial genome (not including the COI gene), Watkins & Beckenbach (1999) found high aminoacid identity with cnidarians. These authors took this as indicative of an unusual level of conservation of mtDNA in diploblastic phyla, much higher than that found in other Metazoans. McFadden *et al.* (2000) and France & Hoover (2002) also found slow rates of evolution in COI in cnidarians.

Whether mtDNA conservation is a common feature of diploblastic phyla with respect to triploblastic ones and the evolutionary implications of the contrasting

degrees of variation in nuclear and mitochondrial DNA in these groups is undoubtedly an exciting field that deserves further investigation. Biogeographical studies with COI and other mtDNA genes in several sponge species could allow more consistent generalizations about the evolutionary traits of this kind of genes in sponges. More variable DNA markers (e.g., Internal Transcribed Spacers or microsatellites) should be used to assess phylogeographic patterns and population structure in this and possibly other sponge species.

2-5. Acknowledgments

I am grateful to M.Zabala, C.Palacin, S.López-Legentil, P.Wirtz and E.Cebrian for providing *Crambe crambe* specimens from Corsica, Cabo de Gata, Banyuls-sur-Mer, Madeira and Gran Canaria respectively. I also want to thank I.Tarjuelo for her advise in the laboratory work.

CHAPTER 3: Phylogeographic history of the sponge *Crambe crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macaronesian islands from the Mediterranean Sea

3-1. Abstract

We studied sequence variation in the nuclear ribosomal internal transcribed spacers (ITS-1 and ITS-2) in 114 individuals from 11 populations/localities of the sponge *Crambe crambe* across the core species range in the western Mediterranean Sea and Atlantic Ocean, reporting intragenomic variability for the first time in sponges. Phylogeographic, nested clade and population genetic analyses were used to elucidate the species evolutionary history. The study revealed highly structured populations affected by restricted gene flow and isolation by distance. A contiguous range expansion in the whole distribution area of the sponge was inferred. Phylogenetic analyses indicate a recent origin of most sequence types that could be explained by a recent origin of the species or a by recent bottleneck event in the studied area. A recent expansion of the distribution range to the Macaronesian region from the Mediterranean Sea was also detected, suggesting that *C. crambe* was recently introduced from the Mediterranean Sea to the Atlantic Ocean via human mediated transport, and that the pattern observed is not the result of a natural biogeographic relationship between these zones

3-2. Introduction

Understanding the distribution of alleles throughout a species range is fundamental to molecular ecologists, allowing inferences about how history influenced the spatial distribution of these particular genes. Phylogeography is seen as the bridge between population genetics and phylogenetic systematics (Avise *et al.* 1987; Avise 2000). Phylogenetic methods can be used to infer haplotype trees and estimate patterns of relatedness among haplotypes focusing on the historical relationships of gene lineages. By comparing the phylogenetic tree to the geographic structure of the data, we can infer historical patterns of population subdivision and understand current distribution patterns of the studied species.

Population genetic parameters, analyses of population structure, as well as analysis of demographic history (reviewed in Emerson *et al.* 2001), can provide information about processes driving observed patterns of genetic variation. For instance, comparisons of haplotype and nucleotide diversity provide insights into the historical demography of a population (Grant & Bowen 1998). Analyses partitioning molecular variation among populations and groups of populations are useful to examine patterns of geographic structure. The challenge then is to determine which is the best method to answer the questions at hand. The use of multiple approaches including phylogenetic inference, nested clade analysis, and genetic diversity measures seems to be the most appropriate way for elucidating not only geographic structure but also the evolutionary history that produced such structure (i.e. Bernatchez 2001; Tarjuelo *et al.* 2001; Althoff & Pellmyr 2002).

Sponges constitute a group of marine invertebrates whose larvae disperse over short distances (Borojevic 1970; Vacelet 1999); for an exception see (Vacelet 1999). They are one of the dominating benthic groups in terms of biomass and species diversity, yet studies on the structure and gene flow among conspecific populations are scarce. Until now, allozymes have been the most commonly applied markers in those studies (reviewed in Solé-Cava & Boury-Esnault 1999). Molecular data have shown a prevalence of cryptic species that had gone undetected with previous morphological studies (Borchiellini *et al.* 2000), with the risk of potentially confounding studies of genetic connectedness among populations. A few recent

phylogeographic studies have used sequence data from the nuclear ribosomal internal transcribed spacers ITS-1 and ITS-2 (van Oppen *et al.* 2000; Lopez *et al.* 2002; Wörheide *et al.* 2002; Wörheide *et al.* in press) demonstrating the utility of ITS to resolve phylogeographic relationships at large spatial scales in sponges.

Crambe crambe (Schmidt 1862) is a common encrusting sponge widespread along the sub-littoral of the Western Mediterranean Sea (Boury-Esnault 1971; Pulitzer-Finali 1983; Uriz *et al.* 1992), and recently found in the Canaries (Maldonado & Uriz 1996) and Madeira (P. Wirtz, pers. comm. 2002) archipelagos in the Eastern Atlantic Ocean (these archipelagos are often referred to as Macaronesian islands). It was also cited once in the Adriatic Sea (Schmidt 1862), and in the Eastern Mediterranean coast of Egypt and Turkey (Burton 1936; Saritas 1972). Its low abundance in the Central Mediterranean (i.e. Sicily, E. Ballesteros, pers. comm. 2002) and the scarcity of reports from the Eastern Mediterranean suggest that the species is less abundant in those regions than in the Western Mediterranean.

Crambe crambe is one of the best-known sponges from a biological and ecological viewpoints (Becerro *et al.* 1997; Turon *et al.* 1998; Uriz *et al.* 1998) and a promising organism for producing numerous bioactive metabolites important from a pharmacological standpoint (Jares-Erijman *et al.* 1991; Berlinck *et al.* 1992). In addition, *C. crambe* is virtually free of microsymbionts (Becerro 1994; Galera *et al.* 2000), minimizing the effect of exogenous DNA in genetic studies (Lopez *et al.* 2002).

Field and laboratory studies on the swimming behaviour and dispersal abilities of its larvae (Uriz *et al.* 1998) indicate that the pelagic phase before settlement is short (ca. 48-72 hours). This suggests a small dispersal potential for this species, at least between areas separated by open sea without a continuum of rocky littoral habitats. A study based on DNA sequence data of the mitochondrial gene cytochrome *c* oxidase subunit I showed homogeneity across sponge populations (Duran *et al. in press*) and no geographic resolution, while a microsatellite study of one Atlantic and one Mediterranean populations showed significant differentiation between them (Duran *et al.* 2002). In the present study we expanded our genetic sampling to include nuclear markers of the region comprising the ribosomal Internal Transcribed Spacer-1 (ITS-1 hereafter), 5.8S rRNA, and the Internal Transcribed

Spacer-2 (ITS-2 hereafter) for the same individuals analyzed in our previous studies, with the addition of individuals from other populations. In all, we included populations covering most of the species range, allowing us to investigate the role of evolutionary and ecological processes, such as restricted gene flow, and population history in shaping the distribution of alleles from this locus throughout the species range.

In this article we provide evidence that *C. crambe* has experienced a relatively recent demographic expansion of its distribution area and it has recently invaded the Canaries and Madeira archipelagos in the Atlantic Ocean, a colonization most probably mediated by human-related activities.

3-3. Materials and methods

3-3-1. Study area and sample collections

Individuals of *Crambe crambe* were sampled from 9 populations from the Western Mediterranean and 2 populations from the Macaronesian archipelagos in the Atlantic Ocean (Fig.7, Table 5). Asexual fissiparous reproduction is known to occur in this sponge but both fission rates and individual growth rates have been found to be very low (Turon *et al.* 1998). Although the real size of clones is not known we sampled individuals at least five meters apart to minimize the chance of sampling the same clone. We collected a minimum sample of 10 individuals per population to increase the probability of finding low frequency variants. Sponge tissue was collected by SCUBA, and kept in absolute ethanol at -20 °C until processed.

3-3-2. DNA extraction

Fragments of the sponge individuals were meticulously cleaned of exogenous tissues with the aid of sterile forceps under a stereo-microscope to avoid contaminating the DNA extractions. Total genomic DNA was extracted using the DNeasy® Tissue Kit (QIAGEN).

3-3-3. PCR amplification and sequencing

The full ITS region, including the 5.8S rRNA gene, was amplified using the primers 9F: 5'-GTA GGT GAA CCT GCG GAA GG-3' (Carranza 1997) and 28SRev: 5'-GTT AGT TTC TTT TCC TCC GCT T-3' (Lobo Hajdu, pers comm. 2002). Amplifications were carried out in a 50 µL volume reaction, with 1.25 units of AmpliTaq® DNA Polymerase (Perkin Elmer), 200 µM of dNTP's and 1 µM of each primer. The PCR program consisted of an initial denaturing step at 94 ºC for 60 seconds, 35 amplification cycles (94 ºC for 15 sec, 45 ºC for 15 sec, 72 ºC for 15 sec), and a final step at 72 ºC for 6 minutes. Amplifications were carried out in a GeneAmp® PCR System 9700 (Perkin Elmer).

PCR amplified samples were purified with the GENECLEAN® III kit (BIO 101 Inc.). Cycle-sequencing with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer) using dye-labeled terminators (ABI PRISM™ BigDye™ v. 3.0 Terminator Cycle Sequencing Ready Reaction Kit) was performed in a GeneAmp® PCR System 9700 (Perkin Elmer). The sequencing reaction was carried out in a 10 µL volume reaction: 2 µL of Terminator Ready Reaction Mix, 2 µL of HalfTerm, 10-30 ng/mL of PCR product, 5 pmoles of primer and dH_20 to 10 µL. The cycle-sequencing program consisted of an initial step at 94 ºC for 3 minutes, 25 sequencing cycles (94 ºC for 10 sec, 50 ºC for 5 sec, 60 ºC for 4 min) and a rapid thermal ramp to 4 ºC. The BigDye-labeled PCR products were cleaned with AGTC® Gel Filtration Cartridges (Edge BioSystems) and directly sequenced using an automated ABI PRISM® 3100 Genetic Analyzer.

3-3-4. DNA editing

Chromatograms obtained from the automated sequencer were read and contigs assembled using the sequence editing software Sequencher™ 4.0. Complete sequences were then edited in GDE (Smith *et al.* 1994), and trivial alignments (no indel events needed to be postulated) generated.

3-3-5. Cloning

The ITS-1 and ITS-2 regions form part of the ribosomal nuclear array, and therefore several copies of each exist per nuclear genome (Long & Dawid 1980). The multiple copies of this cluster appear to be nearly identical within a given organism due to the process of concerted evolution (Hillis & Dixon 1991). Exceptions to this rule exist among metazoans (see Carranza *et al.* 1996 for a review), but Porifera seem to feature intragenomic homogeneity for these genes (Wörheide *et al.* 2002). Our DNA amplifications resulted always in a single discrete band, but a few intraindividual polymorphisms were detected by direct sequencing of the amplified products. These polymorphisms were detected by the presence of two base calls of similar intensity for certain positions, although no length variation was detected.

Given the presence of polymorphisms, PCR products from individuals with more than one polymorphic site (18 individuals in total) were cloned into pCR®2.1-TOPO® (Invitrogen™) following the manufacturer's protocol. Positive clones were grown overnight in LB media; minipreps were prepared with FastPlasmid Mini (Eppendorf) and were sequenced with M13 primers. A total of 116 clones were sequenced.

In order to avoid the term 'haplotype' which represents an haploid component of a given sequence, we will use the term 'sequence type' to refer to every distinct type of ITS detected in the individuals studied, as proposed by (Wörheide *et al.* 2002).

3-3-6. Phylogenetic analysis

We estimated the maximum likelihood model that best fits the data under the hierarchical likelihood ratio test (hLRT) criterion as implemented in Modeltest v. 3.06 (Posada & Crandall 1998). Once the model was selected, the number of sequences was reduced to represent unique sequence types. Using the model estimated under the hLRT, we performed a heuristic search consisting of 1,000 random addition replicates (RAS) followed by tree bisection and reconnection (TBR) branch swapping

in PAUP* (Swofford 1998). Nodal support was estimated using the bootstrap approach (Felsenstein 1985) with 1,000 replicates of simple addition and TBR.

3-3-7. Network estimation and nested analysis

The null hypotheses of no genetic differentiation among populations of the whole area studied, and between populations within the Mediterranean Sea and the Atlantic Ocean, respectively, were tested by permutation tests (10,000 replicates) on χ^2 contingency tables (Hudson *et al.* 1992) using Chiperm v.1.2 (D. Posada, unpublished software available at http://inbio.byu.edu/Faculty/kac/crandall_lab/programs.htm). A sequence type cladogram was estimated with the program TCS 1.12 (Clement *et al.* 2000) using the statistical parsimony procedure (Templeton *et al.* 1992; Crandall *et al.* 1994). This method estimates the unrooted tree and provides a 95% plausible set for all sequence type linkages within the unrooted tree. The resulting network was then used to construct the nested clade design following the inference procedure given in Templeton *et al.* (1987); Templeton & Sing (1993); and Templeton *et al.* (1995). Once the nested design was determined, an exact permutation contingency analysis of categorical variation was implemented (a) for each step level, (b) for the associations between clades and (c) for geographic locations. The contingency test was performed using the software GEODIS v. 2.0 (Posada *et al.* 2000) on the clades with more than one sequence type and more than one sample location, following the algorithm given by Templeton & Sing (1993). This software detects significant genetic and geographic associations within the sequence type cladogram and incorporates the geographic distances as clade distance (Dc) and nested clade distance (Dn). Dc measures how geographically widespread are the individuals that bear sequence types from a specific given clade. Dn measures how far the individuals bearing sequence types from a given clade are from all other individuals that bear sequence types included in the immediate higher step clade. The statistical significance of these two measures was estimated by recalculating them in 10,000 random permutations. This randomization procedure allowed testing the null hypotheses of no geographic association within the nested clade design (Templeton *et al.* 1995).

Phylogeographic interpretations of significant values for Dc and Dn were inferred using the inference key available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm

3-3-8. Solving cladogram ambiguities

To solve any ambiguities before constructing the nesting, we used the empirical predictions derived from coalescent theory (Crandall & Templeton 1993; Templeton & Sing 1993; Crandall *et al.* 1994; Posada & Crandall 2001). These predictions can be summarized in three criteria (Pfenninger & Posada 2002): (1) *Frequency criterion*, as high frequency sequence types might have been present in the population for a long time, they had more chances of originating new sequence types than did younger sequence types; so low frequency sequence types are more likely to be connected to sequence types with high frequency; (2) *Topological criterion*, sequence types are more likely to be connected to interior sequence types than to tip sequence types; and (3) *Geographic criterion*, sequence types are more likely to be connected to sequence types from the same population or region than to sequence types occurring in distant populations.

3-3-9. Population genetics parameters and analyses of population structure

The population genetics analyses were performed using Arlequin 2.0 (Schneider *et al.* 2000). We calculated sequence type and nucleotide diversity for all populations. Sequence type frequencies per population were calculated and represented in frequency plots. We used analysis of molecular variance (AMOVA) to examine hierarchical population structure, performing 16,000 permutations to guarantee having less than 1% difference with the exact probability in 99% of cases (Guo & Thompson 1992). We used our *a priori* expectation of a genetic division between the Mediterranean Sea and the Atlantic Ocean to group populations into regions.

3-4. Results

In total 647 bp comprising the complete ITS-1, 5.8S rRNA, and ITS-2 regions were sequenced for 114 individuals (plus 116 clones), detecting 16 different rRNA types (accession numbers AY319369-AY319411) defined by eight variable sites, including two sites in the ITS-1 and six in the ITS-2 (Table 6).

Table 5. Population code, sample size (N), number of sequence types (Ns) nucleotide (π) and sequence type (h) diversity for each population studied.

Population	code	N	Ns	π (SD)	h (SD)
Madeira	1	10	3	0.000726 (0.000764)	0.4394 (0.1581)
Canaries	2	12	2	0.000778 (0.000774)	0.5033 (0.0639)
Cabo de Gata	3	11	6	0.002089 (0.001493)	0.8007 (0.0497)
Balearic Is.	4	10	6	0.002473 (0.001740)	0.8190 (0.0636)
Tossa de Mar	5	10	9	0.002664 (0.001805)	0.8952 (0.0376)
Cap de Creus	6	11	6	0.001682 (0.001300)	0.6912 (0.1025)
Banyuls	7	10	6	0.002310 (0.001662)	0.7912 (0.0894)
Marseille	8	10	4	0.001823 (0.001404)	0.6154 (0.1358)
Corsica	9	8	5	0.002219 (0.001622)	0.8205 (0.0769)
Naples	10	12	9	0.002251 (0.001578)	0.8333 (0.0600)
Sicily	11	10	7	0.002364 (0.001662)	0.8889 (0.0361)

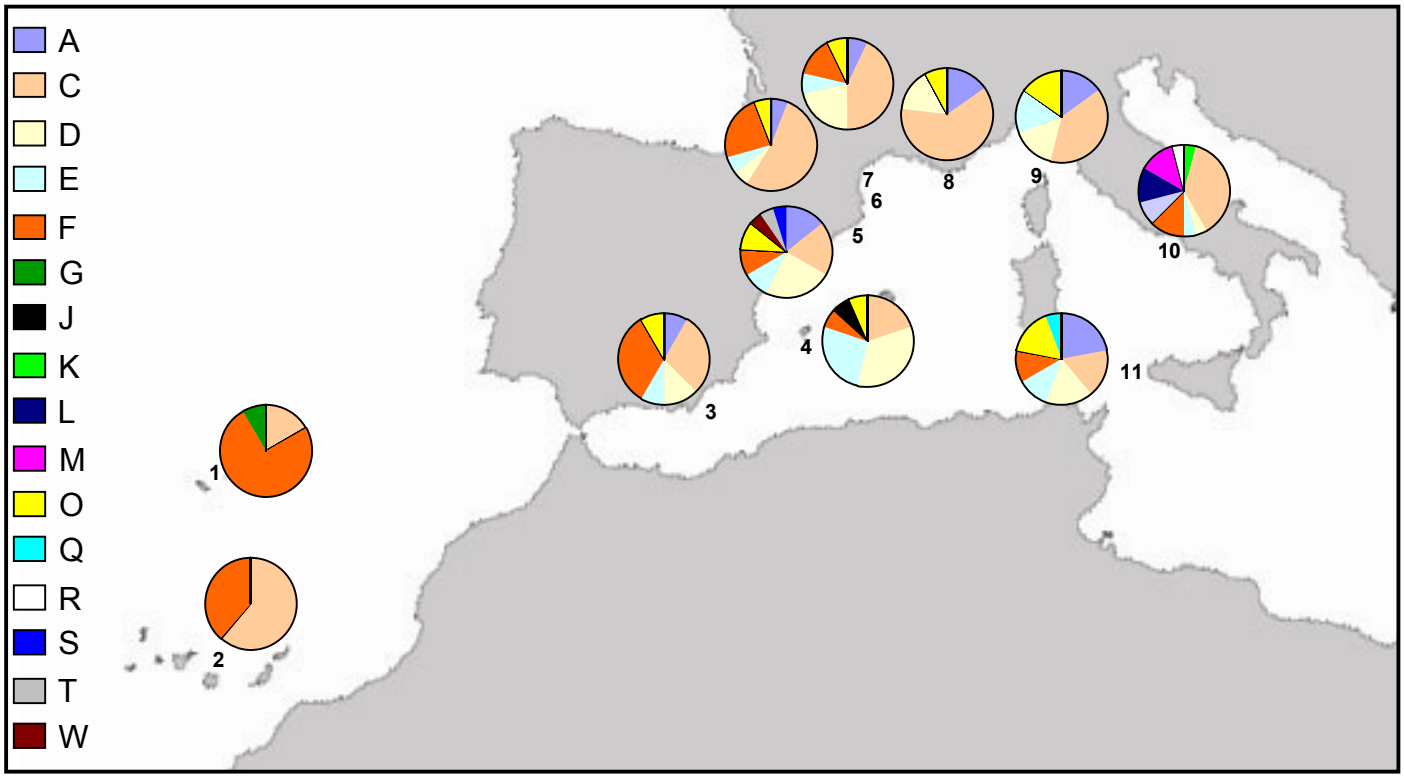

Figure 7. Map showing the localities sampled (see Table 5 for details) with the geographic distribution of the sequence types and sequence type frequencies per population.

Table 6. Sequence types (SeqT) and their frequencies per population.

	ITS1		ITS2						POPULATIONS										
SeqT	**2**	**81**	**384**	**440**	**482**	**492**	**516**	**569**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A	T	C	A	T	C	T	G	T			0,08		0,14	0,06	0,07	0,15	0,15	0,04	0,22
C	A	C	A	T	C	T	G	T	0,17	0,61	0,29	0,20	0,19	0,53	0,43	0,62	0,39	0,38	0,17
D	T	T	A	C	C	T	G	T			0,13	0,33	0,24	0,06	0,21	0,15	0,15	0,04	0,17
E	T	T	A	T	C	T	G	T			0,08	0,27	0,10	0,06	0,07		0,15	0,04	0,11
F	A	T	A	T	C	T	G	T	0,75	0,39	0,33	0,07	0,10	0,24	0,14			0,13	0,11
G	A	T	G	T	C	T	G	A	0,08										
J	A	C	A	T	T	T	G	T				0,07							
K	A	T	A	T	C	T	G	A										0,08	
L	A	C	A	T	C	T	G	A										0,13	
M	A	C	A	T	C	T	A	T										0,13	
O	T	C	A	C	C	T	G	T			0,08	0,07	0,10	0,06	0,07	0,08	0,15		0,17
Q	T	T	A	T	C	C	G	T											0,06
R	A	T	A	T	C	T	A	A										0,04	
S	T	T	A	C	C	T	A	T					0,05						
T	T	C	A	C	C	T	A	T					0,05						
W	T	T	A	T	C	T	A	T					0,05						

The 5.8S region appeared invariable in all sequenced individuals. The length of the individual spacers was 219 bp for ITS-1, and 275 for ITS-2. Uncorrected p-distances ranged from 0.46% to 1.7% (average 1.2%). We found intragenomic variation attributable to lack of homogenization in the variable sites of some individuals, a fact that has been neglected or not observed in other ITS studies. In these individuals, the number of sequence types per individual ranged from one to seven (average 1.6). The number of sequence types per sampling site ranged from two to nine, with a tendency to decrease towards the western and northern range of the distribution (Fig.7, Table 5). Total nucleotide composition was A = 0.12, C = 0.30, G = 0.30, T = 0.28 for ITS-1; A=0.24, C=0.28, G=0.26, T=0.22 for 5.8S; and A=0.11, C=0.30, G=0.31, T=0.28 for ITS-2. The greatest number of differences among sequence types was six.

3-4-1. Patterns of phylogenetic relatedness of the sequence types

For the phylogenetic estimation of the sequence data, under the hLRT criterion, the best fit model of nucleotide substitution corresponds to a Hasegawa-Kishino-Yano (1985) model of nucleotide substitution with a proportion of invariable sites (%I) and a gamma distribution (Γ) of among-site rate variation (HKY85 + θ + Γ hereafter) (Hasegawa *et al.* 1985) (Ti/Tv ratio = 6.8435; θ = 0.9715; α = 0.5804). For the Maximum-likelihood analysis under the best-fit model, the search strategy yielded optimal trees at –ln L = 968.39652 in 79.5 % of replicates, and resulted on two islands of one tree each. These two trees and the unrooted strict consensus are shown in Figure 8, and they differ in the branching pattern of several alleles. Due to the low level of variation among sequences, bootstrap values were low, only two nodes being supported slightly above the 50% threshold (Fig. 8 c).

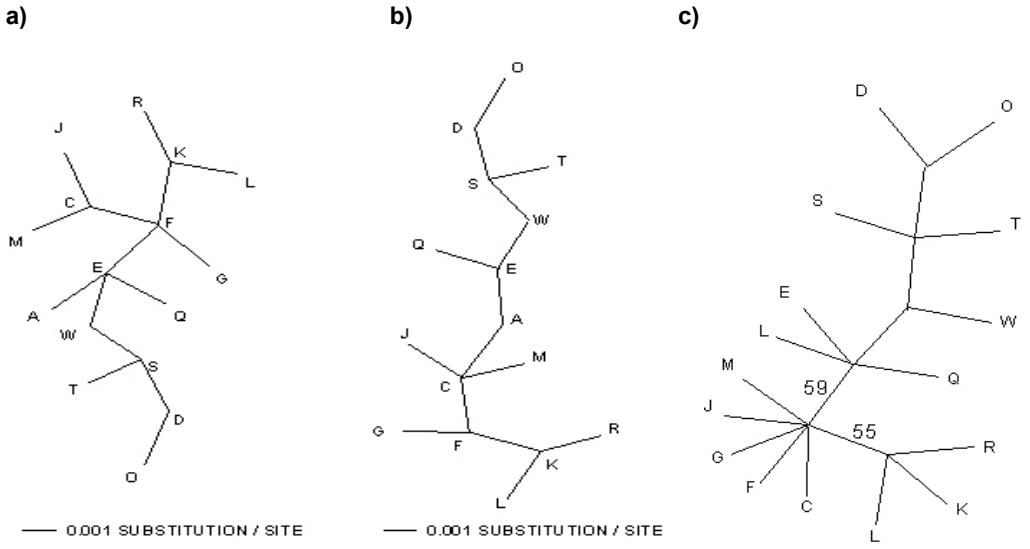

Figure 8. Maximum likelihood estimates of sequence type phylogeny. (a), (b) The two equally likely trees generated under model of sequence evolution HKY85 + θ + Γ (–ln L = 968.39652) unrooted and with branch length information. (c) Strict consensus of the two maximum likelihood trees. The tree is shown unrooted, and no branch length information is provided. Bootstrap values above 50% are shown.

In the statistical parsimony analysis (Fig.9), the network revealed a central, interior position for the most frequent sequence type C. Five sequence types out of the 16 are one mutational step from sequence type C. The most parsimonious cladogram also revealed five closed loops among sequence types, three of them exterior and two interior. To resolve the ambiguous loops in the cladogram, we suggest to break the connections indicated by dotted lines in Figure 3.

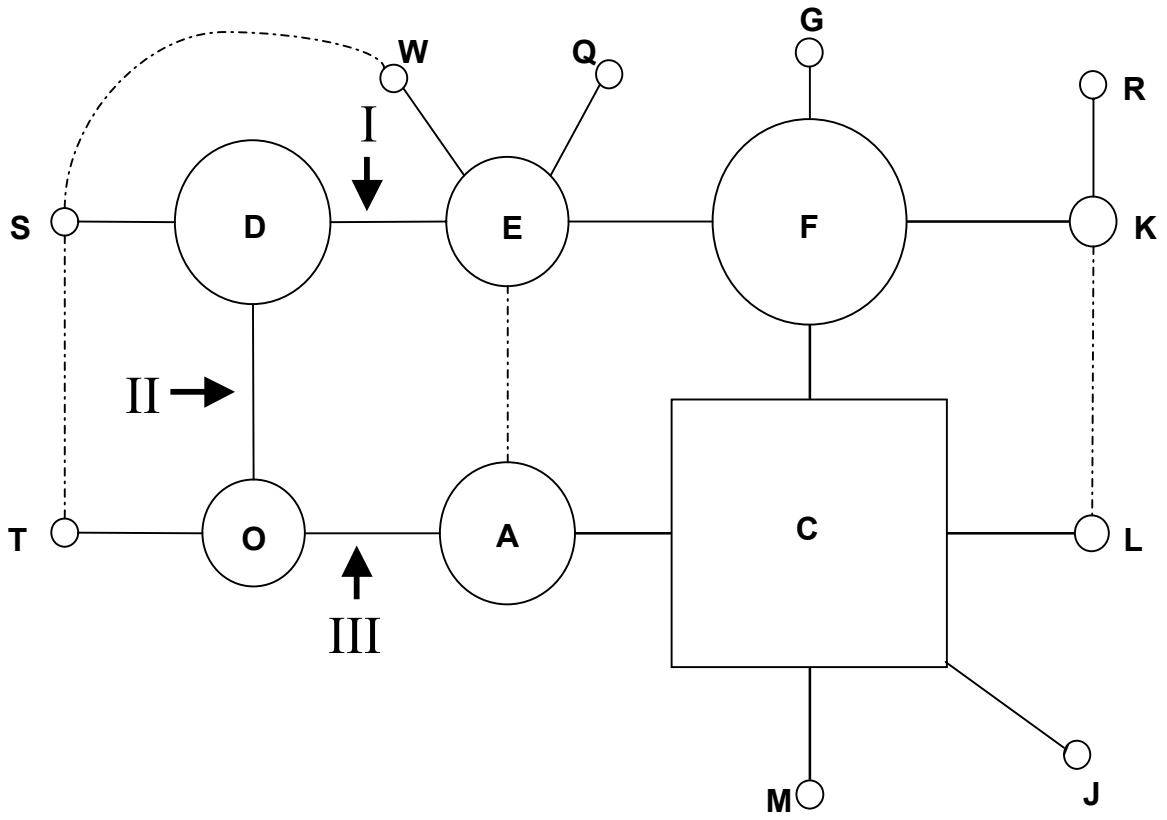

Figure 9. Statistical-parsimony cladogram and proposed loop solutions. Lines in the statistical-parsimony cladogram represent one mutational step between sequence types; dashed lines represent connections removed to resolve loops. Arrows indicate the three favoured possibilities to solve the interior loop. The area of the circles is proportional to the frequency of sequence types; the square denotes the inferred ancestral sequence type.

In the loop formed by connecting sequence types C—L—K—F, the connection between sequence types L and K seems less likely according to the topology and frequency criteria, even though they are found in the same population. The ambiguous loop connecting sequence types S—T—O—D can be solved in a similar way, and the same two criteria easily solve the loop connecting D—E—S—W. For the remaining two interior loops, the A—C—E—F can be solved breaking the connection between E and A appealing to the frequency criterion because sequence type A is more likely linked to sequence type C than to sequence type E. But the remaining interior loop (D—E—F—C—A—O) could be broken at three different places (I, II, III, see Fig.9) with similar probabilities according to the different criteria. So we decided to explore the three alternative ways and proceeded with the analyses. The three resulting cladograms led to different nesting designs and, thus,

potentially to different inferences about population history. We explored the inferences drawn from all possible solutions of the loop, and despite the differences in the resulting nesting designs, the interpretation of the population history was essentially identical for options I and III and slightly different for option II. Therefore the two nesting solutions corresponding to the removal of connection I and II are presented (Fig. 10).

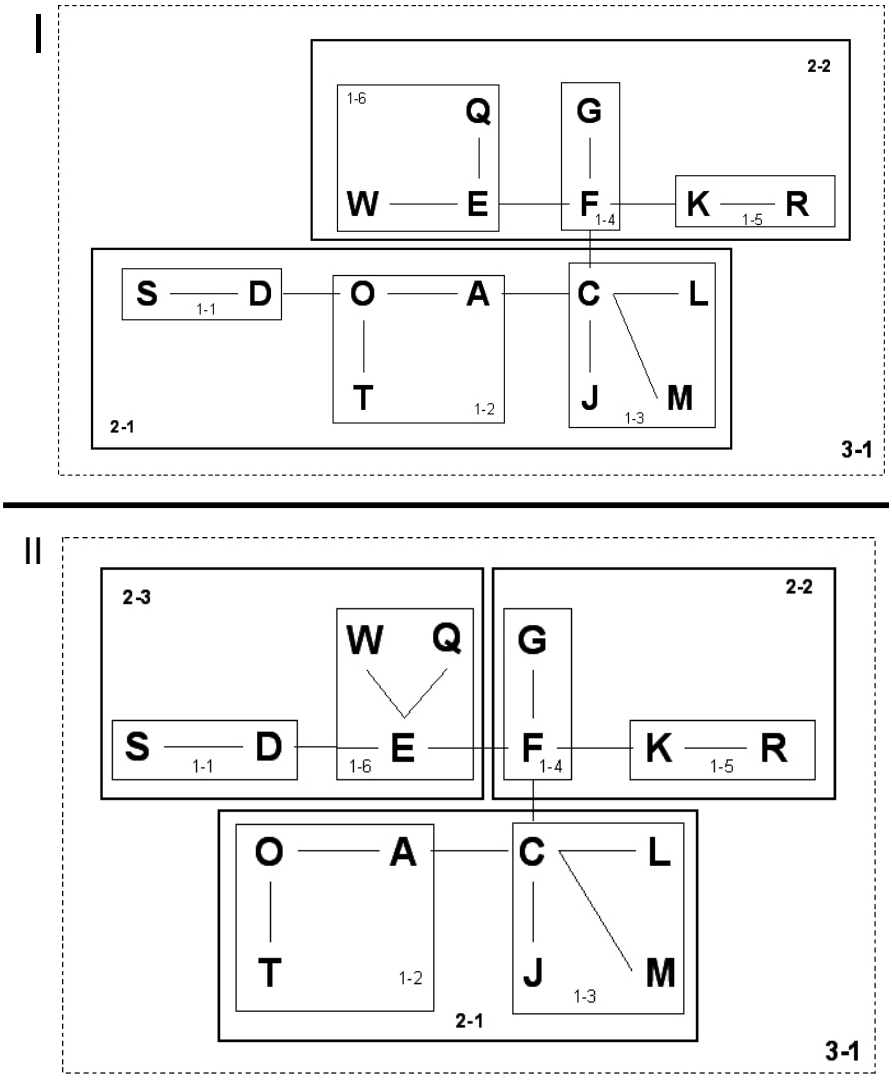

Figure 10. Two options of the clade nesting of the parsimony network, depending on the resolution of the interior loop. Sequence type names as in Table X. Lines represent one mutational step connecting two sequence types. Boxes show the sequence types nested together into one-, two- and three- step clades. All sequence types are nested into clade 3-1.

3-4-2. Population history

The results of the nested clade analysis (NCA) for options I and II (Fig. 10) can be followed in tables 7 and 8.

Table 7. Above, nested contingency analysis for the sequence type-geography association for the nested design given in Fig 10-I. Below, the same for the nested design given in Figure 10-II. Columns show increasing nesting levels, from sequence types to 3-step clades. Dc and Dn distances are given for each clade. Detection of significant differences between the observed and expected distances under a situation of random geographic distribution of sequence types is indicated with a superscript capital S (significantly small) or L (significantly large). I-T are the interior-tip distances. Tip clades are shown in bold.

Option I

Sequence types	Dc	Dn	1-step clades	Dc	Dn	2-step clades	Dc	Dn	3-step clades
S	**0**	**376**							
D	588	579	**1-1**	**575^{S}**	**763^{S}**				
I-T	588	202							
O	676	630							
T	**0**	**473**	1-2	655^{S}	801^{S}				
A	674	637				**2-1**	**975^{S}**	**1045^{S}**	
I-T	675	161							
C	1213	1208							
J	**0**	**883**							
L	**0^{S}**	**1139**	1-3	1213^{L}	1080^{L}				
M	**0^{S}**	**1139**	I-T	476^{L}	236^{L}				
I-T	1213^{L}	105							3-1
F	1415	1410							
G	**0**	**1254**	1-4	1415	1415^{L}				
I-T	1415	156							
K			**1-5**	**0^{S}**	**1509**				
R						**2-2**	**1358^{L}**	**1242^{L}**	
E	659	653							
W	**0**	**479**	**1-6**	**660^{S}**	**1132^{S}**				
Q	0	777	I-T	854^{L}	226^{L}				
I-T	659	25							

Table 7. Option II

sequencetypes	Dc	Dn	1-step clades	Dc	Dn	2-step clades	Dc	Dn	3-step clades
O	676	630							
T	**0**	**473**	**1-2**	**655^{S}**	**858^{S}**				
A	674	637							
I-T	675	161							
						2-1	**1066**	**1080**	
C	1213	1208							
J	**0**	**883**	1-3	1213^{L}	1128^{L}				
L	**0^{S}**	**1139**	I-T	557^{L}	270^{L}				
M	**0^{S}**	**1139**							
I-T	1213^{L}	105							
F	1415	1410							
G	**0**	**1254**	1-4	1415^{S}	1449^{S}				
I-T	1415	156							3-1
						2-2	1483^{L}	1349^{L}	
K			**1-5**	**0^{S}**	**1798^{L}**				
R			I-T	1415^{L}	348^{S}				
E	659	653							
W	**0**	**479**	**1-6**	**660**	**600**				
Q	0	777							
I-T	659	25				**2-3**	**604^{S}**	**878^{S}**	
						I-T	552^{L}	358^{L}	
S	**0**	**376**	1-1	575	571				
D	588	579	I-T	-84	-28				
I-T	588	202							

The two options analyzed yielded significant associations between sequence type clades and geographic distribution at all levels (Table 7). In both options the inference at the 1-step clade level was the same. Within the clade 1-3 a restricted gene flow (RGF) with isolation by distance (IbD) was found. For the two-step clades different inferences were found between the two alternative nestings. In option I, restricted gene flow with isolation by distance was found for both clades 2-1 and 2-2, and the overall inference was a continuous range expansion of the species. For option II, restricted gene flow with isolation by distance was also inferred for the clade 2-2, while for the clade 2-1, restricted gene flow with some long distance colonisation was detected. The overall inference for option II was restricted gene flow with isolation by distance.

Table 8. χ^2 test of geographic association of clades and biological inference from the NCA analysis of the two nested clade options favoured (I and II). Probability *P* is the probability of obtaining a χ^2-statistic larger or equal to the observed statistic based on 10,000 re-samples. Abbreviations for the inferences are: CRE, contiguous range expansion; IbD, isolation by distance; LDC, long distance colonization; RGF, restricted gene flow.

Option I

Clades nested with	χ^2-statistic	*P*	Chain of inference	Inference
1-1	3.4667	1	No significant clade distances	-
1-2	6.6037	0.9924	No significant clade distances	-
1-3	43.2955	0.0972	1-2-3-4 NO	RGF with IbD
1-4	2.9763	1	No significant clade distances	-
1-6	9.8222	0.7770	No significant clade distances	-
2-1	40.9016	0.0021	1-2-3-4 NO	RGF with IbD-
2-2	47.4882	0.0014	1-2-3-4 NO	RGF with IbD
Entire Cladogram	25.6757	0.0037	1-2-11 YES-12 NO	CRE

Table 8. Option II

Clades nested with	χ^2-statistic	*P*	Chain of inference	Inference
1-1	3.4667	1	No significant clade distances	-
1-2	6.6037	0.9925	No significant clade distances	-
1-3	43.2955	0.0965	1-2-3-4 NO	RGF with IbD
1-4	2.9763	1	No significant clade distances	-
1-6	9.8222	0.7812	No significant clade distances	-
2-1	24.4714	0.0039	1-2-3-4 NO	RGF with IbD
2-2	19.3846	0.0412	1-2-3-5-6-7 YES	RGF with LDC
2-3	2.5386	0.9681	No significant clade distances	-
Entire Cladogram	67.4164	0.0000	1-2-3-4 NO	RGF with IbD

3-4-3. Patterns of recent population structure

We found large sequence type diversity (Mean 0.736191 ± 0.154313) and low values of nucleotide diversity (Mean 0.001944 ± 0.00065) per population (Table 5). A significant genetic differentiation between localities, based on a χ^2 test, (Hudson *et al.* 1992) was found between Mediterranean and Atlantic populations ($P<0.005$), among Mediterranean populations ($P<0.05$), and among Atlantic populations ($P<0.05$). Pooling all the populations together the significance was also high ($P<0.0001$).

Incorporating both sequence divergence and sequence type frequencies per populations, the AMOVA detected significant structure between Mediterranean and Atlantic groups, among populations within groups, and within populations, the latter with the highest percentage of the differentiation found (Table 9).

Table 9. Analysis of molecular variance (AMOVA) among populations of *Crambe crambe*. Groups correspond to Mediterranean Sea and Atlantic Ocean. The significance tests were based on 16,000 permutations. An asterisk indicates significant values at *P*<0.05.

Source of variation	d.f.	Sum of squares	Variance components		Percentage of variation
Among Groups	1	2.744	0.04235 Va	*	9.78
Among Populations within groups	9	5.596	0.01426 Vb	*	3.29
Within populations	178	67.015	0.37649Vc	*	86.93
Total	188	75.354	0.43311		

3-5. Discussion

The level of intraspecific variation (1.2%) detected in the ITS's of *Crambe crambe* is six times higher than that found in the mitochondrial gene cytochrome *c* oxidase subunit I (COI), as determined by a study at a similar geographic scale with the same sponge individuals (Duran *et al. in press*). Other sponge COI data included in (Shearer *et al.* 2002) also reveal a low variability in this gene. The amount of variation in the ITS region of *C. crambe* is in the range of that found in the sponge *Leucetta chagonensis* from the Pacific Ocean (0.1-1.6%, Wörheide *et al.* 2002), and it clearly differs from that of *Astrosclera willeyana* from the Indo-Pacific Ocean, which shows length variation in the ITS region (Wörheide *et al.* in press). The GC content found in *C. crambe* (59%) is slightly higher than that from others sponge species (Wörheide *et al.* 2002; Wörheide *et al.* in press). When the intraspecific variation of ITS in this sponge is compared with the range of variation found in other marine species it is found to be relatively low. Examples can be found in the algae (1.7%, Connell 2000), corallimorpharians (2.5%, Chen *et al.* 1996), and scleractinian corals (3-29%, Odorico & Miller 1997; Diekmann *et al.* 2001; Rodriguez-Lanetty & Hoegh-Guldberg 2002). Due to the multicopy nature of the ribosomal array, we have found

intraindividual polymorphism in the ITS regions, which had not been previously reported in sponges.

Inspection of sequence type frequencies and their distribution among populations (Fig.7) suggest a population differentiation that can be explained by different phylogeographic patterns. First, following coalescent theory predictions (Crandall 1996), sequence type C is likely to be the most ancestral type because it is present in all populations studied, is the most frequent, and has the highest number of mutational connections. Second, the Atlantic populations have only three sequence types, one of them unique (G), suggesting a potential invasion from the Mediterranean Sea with a founder effect event. To our knowledge, no other Atlantic populations of *C. crambe* have been reported, and the limited Atlantic distribution of the species may also be partially responsible for the pattern found in the phylogeographic study. Finally, the distribution of sequence types in the Mediterranean suggests a subdivision with at least three different zones, the first one in the Western part of the range (Cabo de Gata, Balears, Tossa de Mar, Cap de Creus, and Banyuls sur Mer), the second one in the central area (Marseille, and Corsica) and the third one in the eastern part of the range (Naples), this later population being the most diverse genetically, with 9 sequence types present. Although Sicily is also rich in sequence types and geographically located in the central part of the range, its sequence type composition and frequency fit better with the western group, as it has more sequence types in common with this group than with Naples. All these patterns might have been caused by large or medium-scale hydrodynamic processes that strongly influence larval dispersal and constitute potential barriers to gene flow among populations. Water circulation within the different basins in the Western Mediterranean (Hopkins 1985) could limit larval transport and favour genetic differentiation among populations in agreement with the patterns found. A similar result suggesting a strong influence of Mediterranean currents in larval dispersal has been found in a phylogeographic study of the polychaete *Sabella spallanzanii* (Patti & Gambi 2001).

3-5-1. Inferring intraspecific sequence evolution

The Maximum likelihood (ML) estimate of sequence type phylogeny and the statistical parsimony (SP) cladograms exhibited somewhat compatible topologies. However, ML failed to resolve most of the relationships among sequences and among higher-level clades due to the low sequence divergence observed. This low degree of nucleotide variation between sequence types results in a poorly resolved consensus tree with low support for almost all branches. Intraspecific phylogenies generally resolve lineages that have been separated for long periods of time, where accumulation of genetic divergence translates into a signal that may correspond well with geographic separation. In our case, we find little phylogenetic signal even among geographically structured sequence types. This can be taken as suggestive of a recent origin of most sequence types. The SP cladogram, although it includes five loops, is better resolved. This result highlights how network approaches may be more effective than phylogenetic approaches at detecting intraspecific evolution, as previously suggested by Posada & Crandall (2001).

Three hypotheses might explain the shallow divergence among sequence types. First, *C. crambe* might be a relatively young species that has recently spread across its range and, accordingly, it has had no time to generate high ITS sequence divergence. An alternative hypothesis regarding the high levels of sequence type diversity relative to nucleotide diversity is that *C. crambe* is an old species that has experienced changes in its population demography, i.e. a strong recent bottleneck that has reduced its former genetic diversity followed by a new expansion and accumulation of new mutations. Lastly, these results could be the consequence of low mutation rates at the locus studied, or even at the genomic level.

3-5-2. Inferring Population history

The population history of *C. crambe,* as inferred by the NCA, involved historical events as well as recurrent gene flow. For the one step level clades, both nesting designs gave the same results. Restricted gene flow with isolation by distance was inferred for the clade 1-3, which includes the most frequent and

widespread sequence type (sequence type C) and the unique sequence types M and L from Naples and J from the Balearic Islands. Because restricted gene flow implies only limited movement by individuals during any given generation, it takes time for a newly arisen sequence type to spread geographically. Keeping in mind that the ancestral sequence type (probably C) is expected to be frequent near its site of geographic origin and that most mutational derivates of the ancestral sequence type will also occur near the ancestral site of origin (Templeton 1998), it seems that the Western Mediterranean is the region where the oldest populations of the sponge are found nowadays. This area has thus acted as the centre of radiation to other zones, specifically to the Macaronesian archipelagos.

On the two-step clade level, two different inferences were made depending on the nested clade design. For the nesting option I restricted gene flow with isolation by distance was inferred for all the clades. Isolation by distance with some long distance colonization was found in nesting option II for clade 2-2 (Sequence types F, G and K, R) suggesting the potential recent invasion of the Atlantic Ocean, possibly linked to one or more sporadic long dispersal events. It is also evident that for sequence type F there is a break in its distribution in Corsica and Marseille but it appears again in Naples. Atlantic populations have high frequencies of the two most frequent sequence types (C and F) suggesting a founder effect. Only one new sequence type (G) unique to Madeira is found in the Atlantic, indicating that the colonization may have been recent and that there has not been enough time to accumulate more changes. The fact that the abundance of this sponge in the Atlantic populations studied is high (S. Duran pers. obs. 2003) suggests that the sponge has found conditions for spreading in this new area. Even if Atlantic waters are different from the Mediterranean ones in both physical and biological conditions, it is known that the Macaronesian region (Canaries, Madeira and Azores Archipelagos) has a strong Mediterranean component in its faunal composition (Wirtz & Martins 1993; Wirtz 1998), of which our results provide further evidence.

Crambe crambe is a sponge with high levels of bioactive metabolites; these substances avoid both predation and competition and have powerful antimicrobial and antiviral properties (Becerro *et al.* 1994). In the Mediterranean, it is one of the most efficient sponges in terms of space competition and lacks known predators.

These characteristics may confer to this sponge invasive capabilities and high potential for colonizing new areas where physical conditions are within its tolerated range.

A colonization of the Canaries and Madeira by larvae arriving via oceanic currents seems unlikely if we take into account that larvae of *C. crambe* stay in the water column for just a few hours or a couple of days at most (Uriz *et al.* 1998). The Canary Current, that runs southwards from the Iberian Peninsula, reaches maximal velocity in the order or 30 cm/s (Batten *et al.* 2000; Zhou *et al.* 2000), which would imply a dispersal of only 50 Km for a larva passively drifting during 2 days. This dispersal range is far too short to cover about 1,300 Km that separate the Macaronesian archipelagos from the Mediterranean Sea. Besides, *C. crambe* apparently has not been able to enter the Atlantic Iberian waters further away than Cape San Vicente (M.J. Uriz, pers. comm. 2002) and has not been reported from the Atlantic shores of North Africa, neither is it present in the Azores (J. Xavier, pers. comm. 2002). This discontinuous distribution, together with the low genetic diversity of ITS's, indicates that a human-mediated invasion is likely in this case (although rafting on natural debris cannot be completely discarded). The high differentiation between sequence type frequencies in the two Atlantic populations is indicative of low gene flow between them, and strongly suggests two independent colonisations of these archipelagos. The introduction of *C. crambe* to the Atlantic might have happened by the transport of larvae in ballast water, or via fouling on ship hulls or rafting in debris. Transport of marine invertebrates via ballast water has been documented for many species with a planktonic phase in their life cycle, in many marine habitats as well as trophic groups (Carlton & Geller 1993).

Other biogeograhic studies of sponge communities have reported Western Mediterranean sponge assemblages originating from Atlantic assemblages (Maldonado & Uriz 1996; Carballo *et al.* 1997). Our results, however, suggest that this particular species has a colonisation history from the Mediterranean to the Atlantic. While this is consistent with other observations of a similarity between the Mediterranean and the Macaronesian faunas, the influence of Mediterranean fauna has never been evaluated from a historical perspective. In this respect, the Macaronesian archipelagos have undergone intense maritime trading with Spain and

Portugal for historical reasons. The case of *C. crambe* may hence constitute the first evidence of recent introduction of Mediterranean fauna in the Macaronesia archipelagos due to human transport, and not due to a biogeographic relationship of both zones.

Despite the high sequence type diversity in some populations, the amount of sequence divergence remains low between all sequence types, so populations of *C. crambe* might be relatively young. If we take into account that the genus *Crambe* is represented by five species, three of them found in the Mediterranean Sea (Maldonado & Uriz 1996), it seems plausible that an origin and radiation of the genus occurred in the Mediterranean Sea after the Messinian crisis 5-6 Mya (Duggen *et al.* 2003) from an unknown phylogenetic lineage (Maldonado 1985).

Regarding the entire cladogram, Option I showed a contiguous range expansion. This expansion in the whole area of distribution of the sponge is consistent with the loss of sequence type variation to the North, and to the West of the geographic area of the distribution. Also, those sequence types found in the ancestral population(s) that were the source of the range expansion (C and F) became geographically widespread. Some of the sequence types found in the expanding populations (D in the Mediterranean) became more frequent than some of the older sequence types from which they have originated (E or O). The inference for the Option II of the NCA for the entire cladogram is isolation by distance with restricted gene flow. This option would be in agreement with the dispersal features of the species.

Even though the two inferences are slightly different, both options detect an invasion of this sponge from the Mediterranean to the Atlantic. Thus, even if our results contrast with other previous studies that describe the colonization history of marine invertebrates species between the Mediterranean Sea and the Atlantic Ocean, we strongly believe that the colonization pattern suggested by our data could be more general in marine invertebrates than previously recognized.

3-6. Acknowledgments

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CHAPTER 4: Polymorphic microsatellite loci in the sponge Crambe crambe (Porifera: Poecilosclerida) and their variation in two distant populations

4-1. Abstract

Seven polymorphic microsatellite loci were characterized in the marine encrusting sponge *Crambe crambe* from a partial genomic DNA enriched library. Preliminary data on allelic variation of these loci in two distant populations of C. crambe are presented to assess their potential utility as high-resolution genetic markers for this species. The number of alleles per locus ranged between 3 and 16 and the distributions of allele frequencies differed considerably between the two populations, indicating a marked genetic differentiation between them. These are the first microsatellite loci reported from any species in the phylum.

4-2. Introduction

Sponges are one of the most important marine invertebrate groups both ecologically, because of their widespread presence in marine benthic communities, and phylogenetically, because of their basal position among Metazoa. Furthermore, sponges are also a source of pharmacologically interesting molecules (Faulkner 2002) whose production shows high intraspecies variability (Becerro *et al.* 1997). Sponges combine dispersal through short-lived lecitotrophic larvae with asexual reproduction in many species (Maldonado & Bergquist 2002). They are therefore a suitable model for the study of dispersal and genetic differentiation. Most studies on population genetics of this group have been performed with allozyme markers (reviewed in (Borchiellini *et al.* 2000). In this study, we assessed the potential utility of microsatellite loci as high-resolution genetic markers for this group.

The sponge *Crambe crambe* (Schmidt 1862) is a common encrusting species widespread along sublittoral rocky bottoms in Western Mediterranean, Canary Islands, and Madeira Islands (Uriz *et al.* 1992 and pers. obs.). Crambe crambe reproduces mainly sexually releasing short life-span larvae (Uriz *et al.* 1998) and asexually by fission of individuals (Turon *et al.* 1998). It produces highly active secondary metabolites and it is possibly one of the best known sponge species from the point of view of its biology and chemical ecology (Becerro *et al.* 1997). However, we lack data on its population structure and dynamics.

We have started a population genetic study of *Crambe crambe* in both the Mediterranean sea and Atlantic ocean in order to assess the genetic structure of this species associated with dispersal features, and the relative importance of sexual versus asexual reproduction at a fine spatial scale. Here we report the first microsatellites isolated from any species in the phylum.

4-3. Materials and methods

Genomic DNA obtained with QIAamp® DNA minikit columns (Quiagen) from a single specimen of C. crambe collected from Blanes (41°40.4'N, 2°48.2'E) was used for the construction of a partial genomic library, following the enrichment protocol from (Kijas *et al.* 1994) based on streptavidin-coated magnetic particles

(Magnesphere, Promega, Madison, WI). Two 5'-biotinylated, 3'-aminated (CT)10 and (GT)10 oligonucleotides were used as probes. The detection step of the clones including microsatellites followed the protocol described in (Estoup *et al.* 1993). (Detailed protocol available at http://www.inapg.inra.fr/dsa/microsat/microsat.htm).

Approximately 1800 bacterial colonies were isolated from the library and screened with (CT)10 and (GT)10 oligonucleotide repeats biotine-labelled. We sequenced 35 out of 65 clones with the strongest positive screening signal and longest insert size (>400 bp). Thirteen out of these 35 clones with sufficiently long flanking sequence were used for primer design and tested for allele size variation.

This was done by analysing 60 sponge specimens, 30 from Tossa de Mar (Northwestern Mediterranean, 41°43.2'N, 2°56.0'E) and 30 from Gran Canaria (Eastern Atlantic, Canary Islands, 27º48'N, 15º47'W).

PCR reactions were performed with a Perkin Elmer 480 thermocycler in 20µl reactions containing 2µl Buffer B (20mM Tris-HCl (pH 8.0), 100mM KCl, 0.1Mm EDTA, 1Mm DTT, 50% glycerol, 0.5% Tween®20 and 0.5% Nonidet®-P40) 1.6µl MgCl2 (25mM), 0.5µl dNTP (10mM), 0.5µl of each primer (10mM), 1U Taq and approximately 10-50ng of template DNA. Cycle parameters were 2 min at 95ºC, followed by 40 cycles of 1 min at 95ºC, 30 sec at the appropriate annealing temperature (see Table 1), and 30 sec at 72ºC, followed by an extension cycle of 5 min at 72ºC. The forward primer for each locus was fluorescently labeled with 6-Fam, Hex or Ned to estimate the allele sizes with an automated sequencer (ABI 3700). Alleles were sized relative to an internal standard (EcoGen 70-500).

4-4. Results and discussion

We obtained a total of seven functional primer pairs (Table 4) since the other six loci were either monomorphic or failed to amplify. Analyses of Hardy-Weinberg equilibrium, linkage disequilibrium, Fst and Fisher's exact test of population differentiation were conducted using GENEPOP version 3.3 (Raymond & Rousset 1995b).

Allele number, heterozygosity and Fst between the two populations studied are summarized in Table 4. The number of alleles per locus ranged from 3 to 16 (mean

8). No significant linkage disequilibrium was detected between loci in either population. Most loci were at Hardy-Weinberg equilibrium except for A-CR1 and E-CR43 in both populations and J-CR91 in Gran Canaria, which showed a heterozygote deficiency ($P<0.005$). It is not clear with the present data set whether this deficiency results from the presence of null alleles and/or particular population structure such as metapopulation or isolation by distance within sampling unit. At the C-CR28 locus, 13% of individuals failed to amplify favouring the null allele hypothesis for this locus. The distributions of allele frequencies in the two populations (Fig.11) differed significantly at every locus ($P<0.001$), and the Fst between these populations for our seven loci was 0.29, indicating a high degree of genetic isolation.

Interestingly, three individuals from Canary Islands had identical multilocus genotypes (7 loci). It is unexpected that they are issued from clonal reproduction as they were sampled in locations separated by a few kilometers. Those individuals are more likely to have identical genotypes by chance as most alleles scored for those individuals were found at high frequency in this population.

Our data suggest that the described microsatellite loci may serve as a sensitive tool for estimating population differentiation both in studies at large and fine-scale in *Crambe crambe* as they were able to detect appreciable structure within and between the two populations studied.

Table 10. Microsatellite loci from the sponge C. crambe. Ta, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; N, number of chromosomes analysed. The final column shows the F_{ST} measure of population structure for the two populations

Locus (Accession no.)	Primer sequence (5'-3')	T_a	Repeat motif	Size (bp)	Tossa de Mar			Gran Canaria			F_{ST}
					N	No. of alleles	H_O/H_E	N	No. of alleles	H_O/H_E	
A-CR1 (AY117403)	F:GTGGGTGCATCTCCTCTTGT R:TATCCAGCTTGTATTCACCA	40	$(TC)_{25}$	206	60	11	0.26/0.44	60	12	0.32/0.43	0.062
E-CR43 (AY117408)	F:GTGGGGAATTAGCCCTCAG R:GCAGCTGTAACATCAGTCCAA	45	$(GT)_3ATTC(GT)_3ATTC(GT)_7$	114	60	7	0.12/0.32	60	6	0.10/0.20	0.112
J-CR91 (AY117407)	F:TGTAAAAAGCTAATAACGTGTCATAAA R:TCATCCCATATCCCATGTCA	45	$(CA)_{12}$	256	60	5	0.37/0.38	60	5	0.18/0.34	0.061
I-CR83 (AY117405)	F:AGCAATCACAAACCCAGGAT R:GGACTGATGGCATGGTGTTA	50	$(TG)_3CA(TG)_6$	276	60	5	0.38/0.38	60	3	0.06/0.06	0.328
H-CR59 (AY117404)	F:CCTCACTACCATGGGTGACA R:TGCGCAAGATATGTTTGTGG	55	$(TG)_7$	161	60	2	0.20/0.20	60	2	0.05/0.08	0.633
K-CR54 (AY117409)	F:TTGCCTAATTCCTAGCCTGA R:AACACTGGTGCTAGAGGCTGT	55	$(AATT)_4$	210	60	7	0.32/0.35	60	4	0.13/0.16	0.454
C-CR28 (AY117406)	F:CAGAGCCAGCAGTCTAATCG R:CCCTCTGATTGGCTTTTCAA	55	$(GA)_{10}$	313	52	6	0.35/0.37	52	2	0.01/0.01	0.408

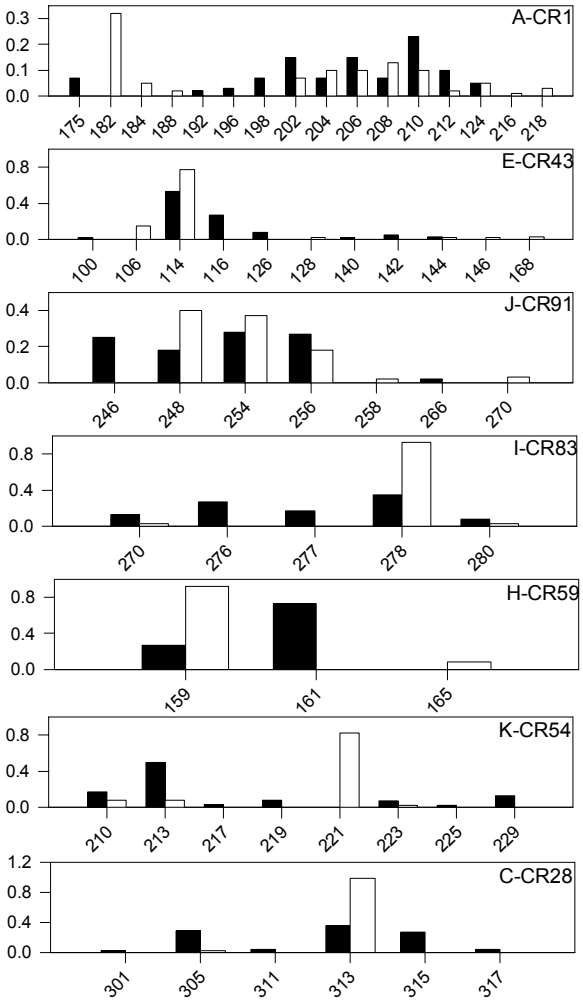

Figure 11. Allele frequency histograms for the Tossa de Mar (solid bars) and Gran Canaria (open bars) populations of *C.crambe*. Allele names represent the sizes of PCR fragments. For each locus, the distribution of allele frequencies differs significantly between populations (P<0.001)

4-5. Acknowledgments

I wish to thank Emma Cebrian for collecting samples from the Canary Islands. Special thanks are given to Arnaud Estoup (Centre de Biologie et Gestion des Populations, INRA, Montpellier, France) for his advise on the genomic libraries construction for *Crambe crambe* and *Clavelina nana*; my stay in his lab was very fruitful and I learned there most of what I know on molecular techniques.

CHAPTER 5: Strong between and within population structure in the sponge *Crambe crambe* (Poecilosclerida) as revealed by microsatellite markers

5-1. Abstract

Microsatellite markers were used for the first time in a sponge species to study the population genetic structure of the encrusting sponge *Crambe crambe.* Specimens were collected in eleven locations representing most of the entire Atlanto-Mediterranean range of the species and analysed at six loci. As expected for a sessile invertebrate with lecitotrophic larvae, high levels of between population structure were found (F_{ST}=0.18) and a significant isolation-by-distance pattern was observed. More surprisingly, a strong genetic structure was found within sampled sites (F_{IS}=0.21) that may be explained by several factors including inbreeding, selfing and Wahlund effect. In spite of a sampling design planned to avoid the sampling of clones, genotypically identical individuals for the six loci were found in some locations. The significance of these potential clones is discussed and their effect on the observed pattern of population structure was assessed. Patterns of allelic distribution within population suggests the possibility of a recent colonisation of the Atlantic range from the Mediterranean Sea.

5-2. Introduction

In comparison to terrestrial environments, marine environments seem to lack obvious barriers to gene flow; hence, populations of marine species are expected to reach panmixia at a larger geographical scale than terrestrial or freshwater species. However, several studies of population structure on marine invertebrates have shown that population differentiation exists even in species with supposedly high dispersal capabilities, as found in corals (Hellberg 1994; Hellberg 1996), sea urchins (Palumbi *et al.* 1997), cuttlefish (Pérez-Losada *et al.* 2002) or squid (Shaw *et al.* 1999). This suggests that long-distance movements of larvae may be hindered by the existence of hydrological and ecological barriers, such as currents, temperature, salinity, and by behavioural responses of larvae favouring philopatry and, consequently, local differentiation.

Sponges are one of the most important benthic groups in terms of both biodiversity and biomass. Their biological characteristics, such as low dispersal potential via lecitotrophic larvae (Borojevic 1970; Sarà & Vacelet 1973) and their variable reproductive strategies including sexual and asexual reproduction (Wulff 1991; Turon *et al.* 1998; Maldonado & Bergquist 2002), as well as combinations of both (Maldonado & Uriz 1999; Vacelet 1999), make this phylum an interesting group of study for molecular and marine ecologists. How these biological features (together with species history) interact to shape the present day geographic structure of sponge populations remains unknown.

Advances in molecular technologies and the increase in the number and type of molecular markers, especially at the DNA level (Ward 1989; Palumbi 1995) make studies on structuring processes at the population level in sponges feasible. Microsatellites have proven to be one of the most informative category of DNA marker, as reduced gene flow and subtle population structure have been demonstrated when other genetic markers, e.g. mitochondrial DNA and/or protein polymorphisms, failed to detect genetic heterogeneity among geographical samples of both marine and terrestrial species (e.g. Hughes & Queller 1993; Jarne *et al.* 1994, reviewed in Estoup & Angers 1998). Although microsatellites have been developed for many marine invertebrates, there are only a few examples of population structure

studies using these markers in this group (e.g. Shaw *et al.* 1999; Brooker *et al.* 2000; Huang *et al.* 2000; Launey *et al.* 2002; Pérez-Losada *et al.* 2002; Stoner *et al.* 2002). The present study reports the first application of polymorphic microsatellite loci to study population structure in a sponge, the Atlanto-Mediterranean poecilosclerid *Crambe crambe.*

C. crambe is a very common encrusting sponge in the Western Mediterranean Sea (Uriz *et al.* 1992 and references therein) and in the Canaries (Maldonado & Uriz 1996) and Madeira (P. Wirtz pers. comm. 2002) archipelagos in the Eastern Atlantic Ocean. A few reports signal its presence in the Eastern Mediterranean, where it doesn't seem to be abundant (Schmidt 1862; Burton 1936; Saritas 1972). Ecologically, *C. crambe* is one of the best-known sponges (Turon *et al.* 1996; Becerro *et al.* 1997; Turon *et al.* 1998), and it is free of symbionts (Galera *et al.* 2000). Many sponge species are simultaneous hermaphrodites, while in many others the sexes appear to be delayed in time (Fell 1983). There are no studies on the type of hermaphroditism in *C. crambe*. Although asexual reproduction has been described for this sponge by means of fission in adult individuals (Turon *et al.* 1998), this process appears to be not as common as in other sponges (e.g. Wulff 1991)). Sexual reproduction with internal fertilization may be the main mechanism for reproduction in *C. crambe*. The sponge releases lecitotrophic larvae of the parenchymella type once they are ready to disperse. These larvae then swim in a slow corkscrew motion for about 24-72 hours in the plankton before settlement (Uriz *et al.* 1998).

In the present study, we genotyped microsatellite markers in *C. crambe* population samples collected over most of the entire range of the species to assess the level and pattern of differentiation between sampled sites. Because larval dispersal seems to be limited in invertebrates with lecitotrophic larvae and in sponges in particular (Jackson 1986; Uriz *et al.* 1998), we were also particularly keen in detecting the existence of a genetic structure at a short geographic scale, that is, within collection sites. Finally, the occurrence of asexual reproduction events (i.e. reproduction by fission of adult sponges, Turon *et al.* 1998) was tested and the effect of such reproduction events on the population structure of *C. crambe* was assessed. To our knowledge, this is the first study to use microsatellites in ascertaining population structure of a sponge.

5-3. Materials and methods

5-3-1. Sampling design

In total, 286 specimens were sampled from 11 locations covering most of the entire known distribution area of *C. crambe* (Fig 12). Fragments from about 30 individuals per location were collected by scuba diving and preserved in absolute ethanol until processed. Because asexual reproduction by fission of adult sponges has been described for *C.crambe* (Turon *et al.* 1998), individuals were taken at least five meters apart from each other to reduce the probability of sampling the same clone. Once in the laboratory, tissue samples were meticulously cleaned under the stereomicroscope of foreign organisms to avoid contaminating the extraction.

Figure 12. Map showing the locations of C.crambe sampling sites. Number of individuals sampled per location in parenthesis. MAD:Madeira (28), CAN:Canaries Islands (30), GAT: Cabo de Gata (18), BAL:Balearic Islands (30), TOS:Tossa de Mar (30), CRE: Cap de Creus (30), BNY:Banyuls sur Mer (30), MAR:Marseille (26), COR:Corsica (30), NAP:Naples (21), SIC: Sicily (13).

5-3-2. DNA extraction, amplification and genotyping

Total DNA was extracted using a protocol described in (Pascual *et al.* 1997). All samples were screened for variation at each of six polymorphic microsatellite loci (A-CR1, E-CR43, J-CR91, I-CR83, H-CR59, K-CR54) previously isolated and described for *C.crambe* by (Duran *et al.* 2002). Forward primers were 5' labelled with a fluorescent dye and PCR reactions were performed under conditions set out in (Duran *et al.* 2002). Allele sizes were estimated on an automated sequencer ABI Prism-3700 (Applied Biosystems) relative to an internal standard (EcoGen 70-500). Alleles were visualized and determined with GENESCAN^{TM} and GENOTYPER ^{TM} software.

5-3-3. Data analysis

All computations were performed using the package GENEPOP version 3.3 (Raymond & Rousset 1995b) and F-STAT version 2.9.3 (Goudet 1995). Allele frequencies were calculated for each locus and location (see Table 11). Observed and expected heterozygosity (Nei 1978) and number of alleles were calculated for each locus individually and as a multilocus estimate for each of the 11 locations. For each locus the corrected number of alleles based on the largest sample size was determined following (Ewens 1972) and using a personal program. Single and multilocus F_{is} were estimated as in (Weir & Cockerham 1984). Linkage disequilibria were tested among all pairs of loci and for all locations. Genotype distributions within samples were tested for conformity to Hardy-Weinberg expectations using Fisher's exact test.

Differentiation between pairs of samples was tested using Fisher's exact test (Raymond & Rousset 1995a). The most suitable statistic for quantifying differentiation between samples at microsatellite loci is still a matter of debate, depending upon the mutational model one chooses to apply (Estoup *et al.* 1995; e.g. Estoup & Angers 1998; Pascual *et al.* 2000; Pascual *et al.* 2001; Balloux & Lugon-Moulin 2002). Methods assuming an infinite allele model [IAM, (Kimura & Crow 1964) using F_{ST}] or a stepwise mutation model [SMM, (Kimura & Ohta 1978) using R_{ST}]

have been proposed. In view of the continuing debate, and for comparative purposes, we have quantified genetic differentiation among samples using both R_{ST} (estimated by ρ_{ST}; Michalakis & Excoffier 1996) and F_{ST} (estimated by θ; Weir & Cockerham 1984). Isolation by distance was tested through the correlation between matrices of pairwise $F_{ST}/(1-F_{ST})$ values and logarithm of the geographic distances between populations (Mantel test, 10000 permutations; Rousset 1997). Geographic distances were calculated as the shortest distance connecting the populations by sea.

Several individuals showed identical genotypes at the six loci. We were keen in testing whether such genetic identity was more likely to reflect clonal reproduction events than to be obtained by chance within a sexually reproducing population. To do this we have to estimate the probability of obtaining identical genotypes by chance alone. Although both analytical solutions (Waits *et al.* 2001) and simulation approaches (Stenberg *et al.* 2003) have been proposed to estimate such probabilities, they usually rely on the assumption of random mating, a condition rarely met in sessile invertebrates with low dispersal capabilities. An excess of homozygosity, for instance, would result in an underestimation of the true probabilities. Therefore, we used a simulation approach to estimate the probability of finding, by chance, individuals with the same multilocus genotype given the sample size, the observed allele frequencies, and the observed heterozygosity for each locus.

The program generates samples by randomly selecting alleles using the observed frequencies, but maintaining the observed heterozygosities at each locus through an iterative algorithm. In this way, we end up with samples of exactly the same size, allele frequency and heterozygosity as the observed ones for each population. This effectively mimics the effect of the factor(s) that result in the observed F_{IS} of the samples.

After generating a large number of samples (100,000), the program records the proportion of samples that have at least one group of 2, 3, and so on genotypically identical individuals. These proportions can be used to test the likelihood of the observed number of identical individuals being obtained by chance if the population is reproducing only sexually. If the proportion of generated samples

that feature groups (of a given size) of identical genotypes is below a given threshold (say, 0.05) then we can conclude that, if we find groups of that size of identical individuals in our sample, they have most likely been generated by asexual reproduction events. The only assumption in this case is that there is no linkage disequilibrium between loci. The routines were written in Turbo Pascal and the computer program used in this study is available under request from the authors.

5-4. Results

The total number of alleles per locus ranged from 5 to 26 (Table 11). There were private alleles in all populations studied but Tossa de Mar and Corsica (see Table 11). Regarding the number of alleles (Table 12) there is a trend of decrease in number towards the Western and the Eastern edges of the distribution range studied. Mean expected heterozygosities (He) over all populations were considerably high (0.605±0.05) and nine out of the 11 populations showed a significant heterozygote deficiency ($p<0.001$). The multilocus estimator values of F_{IS} ranged from 0 to 0.339 and were significantly different from zero in the nine populations that showed heterozygote deficiency (Table 12)

Table 11. Allele frequency of alleles at six microsatellite loci for eleven *C.crambe* populations. Private alleles in bold. Sample site designation in Figure 12

Locus	Allele	LOCATION									
A-CR1		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
	160	-	-	-	-	-	-	**0,017**	-	-	-	-
	162	-	-	-	-	-	-	**0,017**	-	-	-	-
	176	-	-	-	-	0,067	0,133	0,190	0,020	-	-	-
	182	0,317	0,018	-	-	-	-	-	-	-	-	-
	184	0,050	-	-	-	-	-	-	-	-	-	0,075
	188	**0,017**	-	-	-	-	-	-	-	-	-	-
	190	-	-	-	-	-	-	-	**0,120**	-	-	-
	192	-	0,018	-	0,067	0,017	0,017	-	0,020	-	-	-
	194	-	0,089	0,028	-	-	-	0,069	-	-	0,077	-
	196	-	0,071	0,194	-	0,033	-	0,034	-	-	0,038	-
	198	-	0,107	0,056	0,083	0,067	0,133	0,034	-	-	-	0,100
	200	-	0,196	0,028	0,033	-	0,033	0,034	0,020	0,250	0,192	0,025
	202	0,067	0,161	0,250	0,150	0,150	0,167	0,017	0,420	0,050	0,231	-
	204	0,100	0,071	0,111	0,017	0,067	0,083	0,017	0,160	-	0,192	0,025
	206	0,100	0,143	0,028	0,117	0,150	0,017	0,017	-	0,117	0,077	-
	208	0,133	0,089	0,111	0,150	0,067	0,033	0,034	-	0,117	0,038	0,625
	210	0,100	0,018	0,083	0,167	0,233	0,200	0,328	0,040	0,050	0,077	0,150
	212	0,017	0,018	0,056	0,167	0,100	0,133	0,121	-	0,150	-	-
	214	0,050	-	0,056	0,050	0,05	-	0,052	-	0,083	0,077	-
	216	0,017	-	-	-	-	0,050	0,017	0,020	0,017	-	-
	218	0,033	-	-	-	-	-	-	0,080	0,033	-	-
	220	-	-	-	-	-	-	-	-	0,133	-	-
	222	-	-	-	-	-	-	-	**0,040**	-	-	-
	224	-	-	-	-	-	-	-	**0,060**	-	-	-

Table 11. Continued

CR -e		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
	96	-	0,071	-	-	-	-	-	-	0,033	-	-
	98	-	-	-	-	-	-	**0,050**	-	-	-	-
	100	-	-	-	-	0,017	0,033	0,083	-	0,017	-	-
	106	0,150	0,054	-	-	-	-	-	-	-	-	-
	110	-	**0,071**	-	-	-	-	-	-	-	-	-
	114	0,767	0,768	0,389	0,217	0,533	0,183	0,517	0,019	0,500	-	0,095
	116	-	0,036	0,417	0,433	0,267	0,550	0,117	0,846	0,383	0,538	0,595
	118	-	-	0,056	0,033	-	0,050	0,017	-	-	0,077	0,119
	122	-	-	-	-	-	-	0,017	-	-	-	-
	124	-	-	-	-	-	-	-	-	-	**0,115**	-
	126	-	-	-	-	0,083	0,067	0,033	-	-	-	0,095
	128	0,017	-	0,139	0,083	-	-	-	-	-	-	-
	130	-	-	-	-	-	-	0,017	0,038	-	0,115	-
	132	-	-	-	-	-	-	-	0,019	0,033	-	-
	134	-	-	-	-	-	-	-	-	-	-	**0,095**
	136	-	-	-	-	-	-	-	**0,019**	-	-	-
	138	-	-	-	-	-	0,033	-	-	-	0,038	-
	140	-	-	-	-	0,017	-	-	0,019	0,033	0,038	-
	142	-	-	-	-	0,050	0,050	0,067	0,038	-	-	-
	144	0,017	-	-	0,150	0,033	-	0,050	-	-	-	-
	146	0,017	-	-	0,033	-	-	0,033	-	-	0,077	-
	148	-	-	-	-	-	**0,017**	-	-	-	-	-
	150	-	-	-	**0,033**	-	-	-	-	-	-	-
	152	-	-	-	**0,017**	-	-	-	-	-	-	-
	160	-	-	-	-	-	**0,017**	-	-	-	-	-
	168	**0,033**	-	-	-	-	-	-	-	-	-	-
CR - j		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
	246	-	-	-	0,033	0,250	0,150	0,200	0,327	-	0,154	0,071
	248	0,400	0,071	0,235	0,300	0,183	0,217	0,167	0,096	0,100	0,231	-
	250	-	0,089	-	0,017	-	-	-	-	-	-	0,190
	252	-	0,071	0,029	0,083	-	0,017	0,017	-	0,100	0,115	0,048
	254	0,367	0,714	0,294	0,333	0,283	0,467	0,450	0,231	0,333	0,231	0,167
	256	0,183	0,054	0,235	0,183	0,267	0,117	0,083	0,327	0,317	0,115	0,310
	258	0,017	-	-	-	-	-	-	-	-	-	-
	260	-	-	-	-	-	-	-	0,019	0,067	0,077	-
	262	-	-	-	**0,017**	-	-	-	-	-	-	-
	266	-	-	0,088	0,033	0,017	0,033	0,083	-	-	0,077	0,071
	270	0,033	-	-	-	-	-	-	-	0,083	-	0,143
	296	-	-	**0,118**	-	-	-	-	-	-	-	-
CR -h		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
	157	-	-	-	-	-	**0,017**	-	-	-	-	-
	159	0,917	0,411	0,361	0,117	0,267	0,333	0,417	0,712	0,850	0,538	0,571
	161	-	0,589	0,639	0,867	0,733	0,650	0,567	0,288	0,150	0,423	0,429
	163	-	-	-	0,017	-	-	0,017	-	-	0,038	-
	165	**0,083**	-	-	-	-	-	-	-	-	-	-
CR -k		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
	209	-	-	-	**0,017**	-	-	-	-	-	-	-
	210	0,083	-	0,611	0,317	0,167	0,183	0,167	0,038	0,083	0,385	0,368
	213	0,083	0,482	0,250	0,400	0,500	0,450	0,283	-	0,017	0,231	-
	217	-	-	-	-	0,033	0,050	0,117	-	-	-	-
	219	-	-	-	-	0,083	-	0,067	-	-	-	-
	220	-	0,214	-	0,017	-	-	-	-	-	-	0,053
	221	0,817	0,214	0,056	0,067	-	0,167	0,033	0,442	0,050	0,154	0,579
	223	0,017	-	-	-	0,067	0,050	0,233	-	0,550	-	-
	225	-	0,089	0,083	0,050	0,017	0,033	0,067	-	0,017	0,115	-
	229	-	-	-	0,133	0,133	0,067	0,033	0,519	0,283	0,115	-
CR -i												
	270	0,033	0,232	0,353	0,267	0,133	0,217	0,100	0,192	0,117	0,231	0,350
	276	-	0,054	0,206	0,217	0,267	0,317	0,300	0,288	0,167	0,231	0,225
	277	-	-	-	-	0,167	0,017	0,017	0,019	0,033	-	-
	278	0,933	0,714	0,176	0,283	0,350	0,400	0,550	0,462	0,550	0,423	0,200
	280	0,033	-	0,265	0,233	0,083	0,050	0,033	0,038	0,133	0,115	0,175
	282	-	-	-	-	-	-	-	-	-	-	**0,025**
	284	-	-	-	-	-	-	-	-	-	-	**0,025**

Table 12. Summary of genetic variation at six microsatellite loci at 11 locations for C.crambe (Fig 1). Na= number of alleles, He=expected heterozygosity (Nei 1978), Ho= observed heterozygosity, F_{IS}= inbreeding coefficient, HWE= departure from Hardy-Weinberg equilibrium, §= corrected for the sample size (Ewens 1972). Significant values (bold) after sequential Bonferroni corrections (*P<0.05, **P<0.01, ***P<0.001, ns=nonsignificant).

Locus		LOCATION											Mean Na/ Loc
		CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP	
A-CR1	Na	12	12	11	10	11	11	15	11	10	9	6	10.7
	He	0.853	0.889	0.880	0.883	0.883	0.880	0.827	0.784	0.871	0.877	0.585	
	Ho	0.633	0.892	0.777	0.900	0.533	0.433	0.310	0.680	0.400	0.690	0.100	
	F_{is}	**0.262*****	-0.001	0.120	-0.017	**0.401*****	**0.513*****	**0.636*****	0.136	**0.545*****	0.220	**0.833*****	
E-CR43	Na	6	5	4	8	7	9	11	7	6	7	5	6.8
	He	0.393	0.400	0.666	0.733	0.643	0.663	0.710	0.284	0.609	0.692	0.618	
	Ho	0.200	0.250	0.111	0.100	0.233	0.166	0.300	0.192	0.200	0.154	0.381	
	F_{is}	**0.497*****	**0.384****	**0.838*****	**0.868*****	**0.642*****	**0.752*****	**0.583*****	**0.330***	**0.676*****	**0.786*****	**0.390****	
J-CR91	Na	5	5	6	8	5	6	6	5	6	7	7	6.0
	He	0.680	0.475	0.800	0.766	0.763	0.709	0.726	0.737	0.770	0.864	0.827	
	Ho	0.366	0.535	0.529	0.700	0.733	0.666	0.566	0.731	0.600	0.769	0.904	
	F_{is}	**0.467*****	-0.125	0.348**	0.090	0.042	0.061	**0.224***	0.009	**0.224***	0.114	-0.097	
H-CR59	Na	2	2	2	3	2	3	3	2	2	3	2	2.4
	He	0.155	0.493	0.472	0.238	0.397	0.474	0.513	0.675	0.259	0.551	0.501	
	Ho	0.100	0.464	0.388	0.133	0.400	0.500	0.333	0.576	0.300	0.307	0.666	
	F_{is}	0.360	0.059	0.185	0.446*	-0.006	-0.056	0.355*	-0.390	-0.160	0.451	-0.34	
K-CR54	Na	4	4	4	7	7	7	8	3	6	5	3	5.3
	He	0.323	0.678	0.569	0.726	0.703	0.737	0.826	0.543	0.616	0.778	0.540	
	Ho	0.266	0.571	0.611	0.533	0.633	0.666	0.866	0.614	0.666	0.692	0.315	
	F_{is}	0.180	0.162	-0.075	0.269*	0.101	0.098	-0.049	-0.135	-0.081	0.115	**0.422***	
I-CR83	Na	3	3	4	4	5	5	5	5	5	4	6	4.4
	He	0.128	0.439	0.752	0.760	0.766	0.702	0.606	0.677	0.648	0.729	0.770	
	Ho	0.133	0.428	0.765	0.733	0.766	0.733	0.433	0.653	0.666	0.769	1.000	
	F_{is}	-0.036	0.028	-0.015	0.036	0	-0.046	**0.289***	0.036	-0.029	-0.057	**-0.301**	
Mean Na/ Locat		5.3	5.2	5.2	6.7	6.2	6.8	8.0	5.5	5.8	5.8	4.8	
Mean Na/ Locat§		5.3	5.2	5.9	6.7	6.2	6.8	8.0	5.7	5.8	7.4	5.2	
Mean He		0.422	0.562	0.690	0.684	0.693	0.694	0.702	0.575	0.629	0.748	0.640	
Mean Ho		0.283	0.523	0.530	0.516	0.550	0.527	0.468	0.575	0.472	0.564	0.561	
Multiloc *F*is		**0.334*****	0.073	**0.239*****	**0.251*****	**0.210*****	**0.243*****	**0.339*****	0.000	**0.255*****	**0.127***	**0.253*****	
HWE		***	ns	***	***	***	***	***	**ns**	***	***	***	

For the locus J-CR91 one individual out of 286 (0.3%) failed to amplify, for I-CR83 and K-CR54 two individuals (0.7%), and for A-CR1 three individuals (1%). In all cases, re-amplifications under less stringent reaction conditions of failed amplifications did not produce any readable genotype.

Exact tests for homogeneity of allele frequencies among all samples indicated highly significant differences between all population pairs (P<0.001), both at the individual loci and over all loci combined. Single locus as well as multi-locus estimates of F_{ST} and R_{ST} computed over all samples are given in Table 13. Global

F_{ST} and R_{ST} were high and equal to 0.18. This indicates a high level of differentiation between collected samples over the distribution range of *C. Crambe.* The different loci did not contribute equally to the interpopulation differentiation, with monolocus F_{ST} values ranging from 0.07 to 0.26 (Table 13).

Table 13. Global single locus and multilocus F_{ST} and R_{ST} values for *C.crambe* populations.

(P*<0.05,***P*<0.01,****P*<0.001)**

	Locus						
	A-CR1	E-CR	J-CR	H-CR	K-CR	I-CR	Multi-locus
F_{ST}	0.08	0.19	0.07	0.26	0.23	0.10	0.18***
R_{ST}	0.07	0.07	0.09	0.15	0.28	0.03	0.18***

Pairwise comparisons of multilocus F_{ST} ranged from 0.0217 to 0.3286 (Table 14). Regression of $F_{ST}/(1-F_{ST})$ values against the logarithm of the geographic distances in kilometers showed a positive correlation between genetic and geographic distances (Fig. 13). A Mantel test on the two matrices showed that this correlation was significant (*P*<0.02).

Table 14. Matrix of pairwise multilocus F_{ST}. For all pairwise comparisons, genetic differentiation is highly significant (Fisher's exact test, *P*<0.001)

	CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	NAP
CAN										
MAD	0.2156									
GAT	0.3085	0.1576								
BAL	0.3286	0.1461	0.0243							
TOS	0.2782	0.0987	0.0541	0.0309						
CRE	0.2761	0.1164	0.0475	0.0217	0.0272					
BNY	0.2158	0.0755	0.0815	0.0754	0.0242	0.0395				
MAR	0.3037	0.2779	0.1815	0.1879	0.1837	0.1230	0.1941			
COR	0.2049	0.1752	0.1581	0.1864	0.1464	0.1448	0.1018	0.1557		
NAP	0.2912	0.2490	0.1067	0.1345	0.1663	0.1200	0.1695	0.1495	0.1763	
SIC	0.2662	0.1634	0.0290	0.0512	0.0734	0.0223	0.0745	0.0747	0.1078	0.0842

In three out of the 11 populations some individuals sharing the same genotype for all the six loci were found. These identical individuals (potential clones) were only found within populations and are listed in Table 15. These putative clones comprised groups of only 2 or 3 individuals.

Table 15. Number of individuals per population with identical genotype (potential clones)

GENOTYPE						POPULATION		
A-CR1	E-CR43	J-CR91	H-CR59	K-CR54	I-CR83	CAN	COR	NAP
182/182	114/114	254/254	159/159	221/221	278/278	3		
200/200	114/114	252/256	159/161	223/223	276/278		2	
200/200	114/114	254/270	159/159	223/229	278/278		3	
202/206	114/114	254/254	159/159	210/223	278/278		3	
208/212	116/116	248/248	159/159	223/229	277/278		2	
212/212	114/132	248/256	159/159	223/223	278/280		2	
214/220	116/116	254/260	159/159	223/229	276/278		2	
220/220	116/116	256/256	159/159	223/229	270/280		2	
208/208	114/116	250/270	159/161	210/221	270/278			2
208/208	116/116	250/270	159/161	210/221	270/278			2
208/208	116/126	254/256	159/161	221/221	270/280			3
210/210	116/116	256/266	159/159	210/221	270/276			2
208/208	118/118	246/256	159/161	210/210	276/278			2

The proportion of samples generated by the simulation program (100,000 replicates, keeping sample size, allele frequencies and heterozygosities as observed) with at least one group of 2 individuals with the same genotype is listed in Table 16. The same figure is given for groups of 3 equal genotypes. The actual number of identical specimens found in the populations is also indicated.

The proportion of generated samples featuring one or more groups of 2 identical individuals ranges from 0.003 to 0.446. It is higher than 0.05 in the populations of Madeira, Canaries, Marseille, Corsica and Naples. The proportion of

generated samples with groups of identical multilocus genotypes of size 3 ranged from 0 to 0.052 (in Canaries). Groups of more than 3 individuals with the same genotype were not found or, if so, at very low frequencies in the generated series.

Table 16. Proportion of samples generated by the simulation featuring at least one group of 2 or one group of 3 identical individuals (Gen(2) and Gen(3), respectively), and number of clones of these sizes observed in our experimental samples (Obs(2) and Obs(3), respectively).

Clone size	CAN	MAD	GAT	BAL	TOS	CRE	BNY	MAR	COR	SIC	NAP
Gen (2)	0.446	0.293	0.007	0.016	0.008	0.030	0.018	0.251	0.052	0.003	0.087
Gen (3)	0.052	0.022	0	$5*10^{-5}$	$4*10^{-5}$	$2.3*10^{-4}$	$1.3*10^{-4}$	0.015	$5.5*10^{-4}$	0	0.002
Obs (2)	0	0	0	0	0	0	0	0	5	0	4
Obs (3)	1	0	0	0	0	0	0	0	2	0	1

One group of 3 identical individuals was observed in the sample from Canaries, where the probability of finding the same multilocus genotypes is the highest. In contrast, several groups of 2 and even 3 identical individuals appeared in the samples of Corsica and Naples, where the probability of finding such groups as a result of sexual reproduction is low. For instance, there were 5 groups of 2 identical individuals in Corsica, while in the generated series only 5% of the samples had one group of 2 individuals with the same genotype, only 0.2 % of the samples had two such groups, and none had more than 2 pairs of identical individuals.

Tests for linkage disequilibria between loci revealed, after a sequential Bonferroni correction, 13 significant p-values out of 165 comparisons, these linkage disequilibria were not uniformly distributed across populations, and were concentrated in Naples and Corsica locations. As these two populations were also the ones with more genotypically identical individuals (Table 15), we thought that this could be the cause of the linkage found, so we repeated the comparisons without the identical individuals. In this second analysis only two *P*-values remained significant ($P<10^{-5}$), one in Corsica and one in Naples not involving the same loci, suggesting that the observed linkage disequilibria were largely due to the presence of genotypically identical individuals.

In order to test the effect of these potential clones on the population structure of *C. crambe*, all analyses were repeated after removing identical individuals (keeping one individual per group). In contrast to the above results concerning linkage disequilibrium, the inter- and intra-population statistics remained very similar to the original ones (global F_{ST} =0.17; global F_{IS}=0.21; isolation by distance, P<0.02). The conclusions regarding population structure, therefore, remained regardless of the inclusion or exclusion of the potential clones, indicating that clonality is not an important structuring factor at least at the studied geographical scale.

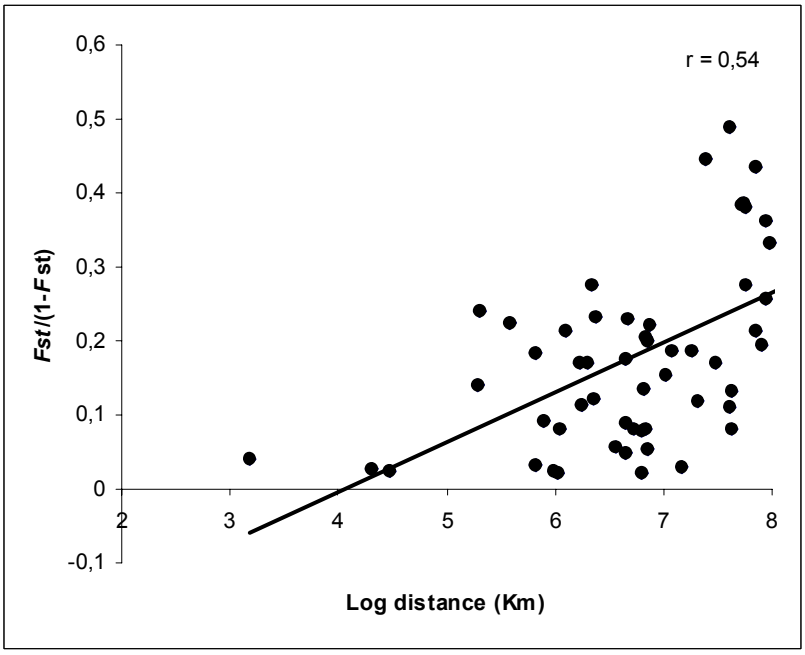

Figure 13. Relationship between genetic differentiation (computed as $F_{ST}/(1- F_{ST})$) and the logarithm of geographic distance between *C.crambe* populations. Regression line included.

5-5. Discussion

5-5-1. Within population structure

Microsatellite loci display high levels of polymorphism in *C.crambe*, providing information at both the inter and intra-population levels. This is in sharp contrast with

other genetic markers assayed on this species, such as mtDNA sequence data, which revealed a high degree of conservativeness, rendering them almost useless for population structure studies (Duran *et al. in press*).

Microsatellite markers showed heterozygote deficiency within populations, which is translated into high positive F_{IS} values. Such heterozygote deficiency could be the result of several non exclusive factors: technical factors such as the presence of non amplifying alleles (i.e. null alleles; e.g.Callen *et al.* 1993), and biological factors such as inbreeding , selfing and Wahlund effects.

Regarding the possible presence of non-amplifying alleles, it is worth mentioning that a low percentage of failed amplifications was observed. Specifically, only 8 individuals failed to amplify at a single locus, and these failures were distributed across three different loci. It is unlikely, therefore, that putative null alleles would be present at high frequency within populations, and hence influence considerably the outcomes of our analyses. Incorrect scoring of bands is also unlikely, as the use of an automated sequencer with an internal standard allowed for a high resolution for reading allele sizes.

Mating among relatives and hence inbreeding could explain heterozygote deficiency within populations. This hypothesis is even more plausible in *C. crambe* where larvae and sperm both have low dispersal capability (Uriz *et al.* 1998). Sponges are hermaphrodites, so that self-fertilization could be another explanation for the heterozygote deficiency we observed. Testing for selfing in sponges remains difficult as aquarium rearing is hardly feasible.

Although our samplings were done over only ca. $100m^2$ at each locality the existence of a Wahlund effect due to the presence of breeding subunits within each collected area could also explain the high observed F_{IS} values. A temporal Wahlund effect could also occur. Each population could represent several subunits of individuals reproducing at different times over the years leading to different breeding subunits. *C. crambe* embryos are found in some colonies from April onwards, and larvae become very abundant in August on the Catalan coast (Turon *et al.* 1996; Uriz *et al.* 1998). It seems that reproduction occurs in a continuum during spring, and therefore a reproductive lag between subpopulations may originate a Wahlund effect. More studies are needed to test this possibility.

Hence, the exact causes of the high F_{IS} values observed within sampling sites remain an open question. Addressing this issue in a precise way would require additional studies of microsatellite genotype distribution at a local geographical scale on precisely located individuals. Comparisons of population structure at different time periods would be also useful in detecting temporal structure within populations.

5-5-2. Between population structure

Highly significant levels of population differentiation were found for *C crambe populations* across its distribution range (F_{ST} and R_{ST}= 0.18). Even geographically close populations are highly differentiated (e.g. Cap de Creus and Banyuls sur Mer which are about 30 Km apart). The high number of population private alleles found provides further evidence of this high degree of structure and isolation among populations. The significant correlation between geographic and genetic distance indicates that isolation by distance is a major mechanism of differentiation in *C. crambe.* Both low dispersal potential and physical factors such as hydrological conditions are surely playing a major role in shaping the genetic structure of the species. Interestingly, Canaries and Madeira archipelagos tend to have lower number of alleles (mean of 5.25 per population) than in the Mediterranean populations (mean of 6.41 per population); moreover, for most loci, a single allele was particularly frequent (often > 0.7) in the Canaries and Madeira archipelagos. These differences in allelic patterns suggest that the Atlantic populations may have endured a more or less recent bottleneck that could reflect colonization with founder event(s) of the Canaries and Madeira archipelagos by individuals originating from the Mediterranean Sea. Note that, specific tests for bottleneck detection such as the one of Cornuet & Luikart (1996) are not adapted to our biological model, as the later test assumes Hardy-Weinberg equilibrium in the tested populations.

Because of their mutational modalities, microsatellites were found to be hardly useful at providing robust tree based evolutionary history (Estoup & Angers 1998; but see Goldstein & Pollock 1997). Hence, it would be worth using some other category of molecular markers (e.g. sequence data) that have a better memory of past evolutionary events to unravel the phylogeography of the species and to ascertain its

source of radiation. While mitochondrial DNA has a too low variability (Duran *et al. in press*), nuclear markers such as ITS sequences, coupled with nested clade analyses (Templeton 1998) have been successfully applied to phylogeographic studies in sponges (Wörheide *et al.* 2002), and may be a judicious choice to study the phylogeography of Mediterranean and Atlantic populations for *C. crambe*.

5-5-3. Asexual contribution to reproduction

As mentioned earlier, individuals were collected at least five meters apart from each other to decrease the chance of sampling the same individuals produced by fission (Turon *et al.* 1998). Some of the locations, however, showed identical individuals for the six loci genotyped (Table 15). Even if different persons were involved in sample collection, the same clear instructions were given in order to use the same protocol at all locations. Therefore, a sampling error is unlikely to explain the identical individuals found.

The probabilities of finding identical individuals by chance in sexually reproducing populations, as estimated by the simulation program, are in general low (Table 16), and only in the case of the population of the Canaries do they suggest a high chance of picking up individuals with the same genotype in samples of size 30, followed by Madeira and Marseille. The Atlantic populations behaved differently than the Mediterranean ones regarding the allelic frequencies, having for each locus one allele very frequent relative to the others, which increases the probability of identity by chance. In agreement with this, a group of three individuals with the same genotype was found in the sample from the Canaries, and they were homozygous for the most frequent alleles at each of the six loci (Table 15). The Canaries is also the only population where the probability of finding at least one group of 3 identical individuals is higher than 0.05 in the simulations performed (Table 16). All these reasons suggest that, although asexual reproduction cannot be discarded at all, the identical individuals found in Canaries may plausibly be a result of chance events in a sexually reproducing population.

The conclusion is different for the samples collected in Corsica and Naples, where the proportion of simulated samples with groups of 2 identical individuals were

5.2 and 8.7 %, respectively, and of these ca. 96% had only one pair, so the number of potential clones observed (7 and 5 respectively, and some of them of size 3) is highly unlikely to result from chance alone. In Madeira or Marseille, for instance, no identical individuals were found in spite of a much higher probability of identity in the latter populations (Table 16). It can be noted that our approach for detecting clones is a conservative one, as we record all identical genotypes found in the simulations, but clearly some genotypes are more probable than others, and in particular in Corsica and Naples some of the clones did not feature the most frequent alleles at each locus (Table 15). We therefore believe that some form of asexual reproduction may be acting in Corsica and Naples populations.

It can be noted that, if we run the simulations considering the expected heterozygosities under H-W equilibrium (instead of the observed ones), then the possibility of identity among individuals diminishes by one order of magnitude in most cases (results not shown), indicating a profound effect of the homozygosity excess of our samples in the probability of getting identical individuals by chance as a result of sexual reproduction. Hence, if random mating does not occur in a population sample, calculating probabilities of identity with methods based on this assumption would be highly misleading.

Given the slow growth of *C.crambe* and the low rates of fission observed (Turon *et al.* 1998), the presence of clonal individuals separated by at least 5 m is unlikely to be the result of fission events. Other mechanisms of asexual multiplication have to be invoked, as, for instance, accidental fragmentation with subsequent dispersal of the fragments or rafting on algal thalli. Another interesting possibility, which has not been examinated so far, is that mechanisms of asexual reproduction exist at the level of the embryos or larvae, prior to dispersal. What Corsica and Naples have in common that makes clonal reproduction more frequent than in the other locations studied remains to be explained, as well as which factors are contributing to the relative importance of asexual reproduction.

In summary, the present study reveals significant genetic structure between and within the populations of an Atlanto-Mediterranean sponge in its distribution range. Microsatellite markers also showed a marked intrapopulation differentiation in this species, revealing the existence of heterozygote deficiency. This study also

suggests the potential importance of the asexual reproduction, as clones appear to be more frequent than expected. Which mechanisms are implicated in asexual reproduction and the sizes of clones remains to be definitively explained. Capturing the intrapopulation structure and the importance of reproduction strategies in sponge populations, however, will require a specific use of these powerful molecular markers at a microgeographic level with more accurate sampling designs.

5-6. Acknowledgments

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CHAPTER 6: General conclusions

The main conclusions obtained in this study are presented in different sections; each one represents a different methodological approach on the study of *Crambe crambe* population structure. The last section summarizes the overall conclusions drawn from a comparison of the different methods.

6-1. mtDNA (COI)

6-1-1. Levels of genetic variation detected were much lower than those reported for other marine invertebrates with lecitotrophic larvae. Due to these low levels of genetic diversity, the COI gene showed high genetic similarity among some distant *C. crambe* populations.

6-1-2. The lack of variability found in the mtDNA is in agreement with other studies on diploblastic phyla, raising questions about the evolution of mtDNA in Metazoa and the presence of repair mechanisms in basal groups.

6-1-3. In spite of the genetic similarity found, low levels of gene flow were detected between close populations, indicating low dispersal of larvae.

6-1-4. mtDNA has not enough resolution to attempt studies at the intraspecific level in sponges, but it can be a good marker at the interspecific level.

6-2. Internal transcribed Spacers

6-2-1. Levels of genetic variation detected in *C. crambe* are in the range of those found in other sponge species, and lower than those detected in other marine organisms.

6-2-2. Intragenomic variability is reported in sponges for the first time.

6-2-3. Sequence type frequencies and distribution showed strong genetic structure along *C. crambe* populations.

6-2-4. Sequence type distribution suggested a strong effect of the regional hydrodynamic processes in the larval dispersal in the Mediterranean Sea.

6-2-5. Both Maximum Likelihood estimates of sequence type phylogeny and the Statistical Parsimony cladograms indicated a recent origin of the sequence types due to the relatively low amount of genetic divergence found.

6-2-6. Population history inferred by the Nested Clade Analysis involved historical events such as colonization and expansion, as well as recurrent restricted gene flow with Isolation by Distance.

6-2-7. *C. crambe* has recently invaded the Canaries and Madeira archipelagos, probably via ship transport of larvae through ballast water or fouling.

6-2-8. The Internal Transcribed Spacers are good-resolving markers for phylogeographic studies in sponges in spite of the difficulties found due to the intragenomic variability. Cloning should be routinely performed in studies using ITS to assess this variability.

6-3. Microsatellites

6-3-1. Enriched genomic libraries are a good methodology to look for polymorphic microsatellites in sponges.

6-3-2. Six polymorphic microsatellites were described and used in population genetics studies for the first time in sponges.

6-3-3. High levels of between population structure and Isolation by Distance pattern were detected.

6-3-4. Heterozygote deficiency and strong genetic structure within sampled sites was found.

6-3-5. Genotypically identical individuals for the six loci were found in some locations probably due to asexual reproduction events.

6-3-6. Differences in within population diversity and between population differentiation suggested the possibility of colonization with founder effect of the Atlantic area of distribution from the Mediterranean Sea.

6-3-7. Microsatellite markers have a good resolution for intraspecific studies and are especially powerful at the intrapopulation level. Their high variability opens the field for the study of clonality in sponges

6-4. Overall Conclusions

6-4-1. This work provides guidelines for the choice of a suitable marker for genetic studies in sponges depending on the level of resolution needed.

6-4-2. mtDNA sequences have not enough resolution to attempt studies at the intraspecific level. However, they may prove useful at the interespecific level and contribute to uncover cryptic species or to clarify the taxonomy of ill-defined species-groups. Thus, phylogenetic information can be gleaned from the analysis of mtDNA sequences.

6-4-3. ITS data performed best in intraspecific phylogeographic studies, allowing identification of patterns of diversification between populations. However, cloning should be routinely performed to overcome problems related to intragenomic variability.

6-4-4. Microsatellite markers featured the highest level of variability and were therefore suitable for intraspecific and, particularly, intrapopulation studies. As they

allow individual genotyping, they open the field for the study of clonal structure in sponge populations.

6-4-5. All markers analyzed showed a strong genetic structure in *C. crambe*, indicating small-range dispersal, philopatry and restricted gene flow with Isolation by distance. The most sensitive markers also showed intrapopulation genetic structure and heterozygote deficiency that suggests levels of inbreeding and/or population substructure at small scales, as well as the existence of clonality in some cases. The results are also consistent in pointing to the Western Mediterranean as the center of radiation of the species, which has subsequently spread into the Atlantic.

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RESUM

FILOGEOGRAFIA, FLUX GÈNIC I ESTRUCTURA POBLACIONAL DE *Crambe crambe* (PORIFERA:POECILOSCLERIDA)

Introducció

L'objectiu principal d'aquesta tesi ha estat l'estudi de l'estructura poblacional d'una esponja marina (*Crambe crambe*) a diferents nivells geogràfics i mitjançant diferents marcadors moleculars.

En molts organismes, sobretot d'ambients terrestres, s'han utilitzat marcadors moleculars com a eina per a estudiar l'estructura i connectivitat de poblacions, el potencial de dispersió o la història evolutiva de les espècies. Aquest tipus de dades són avui dia molt importants per a la gestió i conservació de les espècies i els ecosistemes. La majoria dels estudis que s'han dut a terme han utilitzat com a eina els al.lozims (proteïnes), i més recentment s'han incorporat les seqüències d'ADN mitocondrial o nuclear amb força èxit. Uns dels marcadors moleculars més novedosos han estat els microsatèl.lits, els quals estan essent desenvolupats i aplicats a més espècies cada dia amb un èxit molt elevat. Tot i l'avenç que s'ha produït en la darrera dècada pel que fa a l'ús d'eines moleculars per a l'estudi de les relacions evolutives entre espècies o entre poblacions d'una mateixa espècie, l'aplicació d'aquestes eines en organismes que habiten medis marins ha estat mínima, sobretot pel que fa als organismes invertebrats, on els estudis de genètica de poblacions són ben escassos i la major part només inclouen els al.lozims com a tècnica per a detectar l'estructura de les poblacions.

Aquesta tesi és una contribució a l'aplicació de tècniques moleculars en l'estudi de l'estructura poblacional en invertebrats marins. Per a això s'han utilitzat diferents tipus de marcadors moleculars en l'esponja *Crambe crambe* i juntament amb els coneixements ecològics i biològics que es tenen de l'espècie s'ha estudiat

l'estructura actual de les seves poblacions, i s'ha intentat esbrinar quins factors han afectat i estan afectant a aquesta estructura.

La gran majoria d'invertebrats marins són organismes bentònics, molts d'ells sèssils, on la fase de dispersió es limita a la larva. Seguir els moviments de les larves en el seu medi natural és una tasca que podríem definir com a molt difícil (per no dir gairebé impossible), així, els marcadors moleculars apareixen com a una eina perfecta per a poder seguir i caracteritzar poblacions i fins i tot individus, tant al llarg de l'espai com del temps.

El patró que s'esperaria trobar seria que els organismes amb un potencial elevat de dispersió (p.ex. els que tenen larves planctotròfiques) tindrien una baixa diferenciació poblacional, ja que s'esperaria un elevat intercanvi d'individus entre les poblacions (elevat flux gènic). Aquest exemple el podem trobar en alguns estudis com a Palumbi & Wilson (1990); Lacson (1992); Ovenden *et al.* (1992) i Russo *et al.* (1994). Per altra banda, esperaríem que les espècies amb poca capacitat dispersiva (p.ex. les que tenen larves lecitotròfiques) tinguin una estructura poblacional més marcada amb poc flux gènic i poblacions tancades. Exemples d'aquest tipus d'espècies els podem trobar a Janson & Ward (1984); Day & Bayne (1988); McMillan *et al.* (1992); Duffy (1993) i Hunt (1993). Sorprenentment també es troben uns quants casos on no es segueixen aquests patrons esperats, alguns exemples els trobem a Solé-Cava *et al.* (1994); Grant & da Silva-Tatley (1997); Uthicke & Benzie (2000) i Lazoski *et al.* (2001). Així doncs, a part del potencial de dispersió de les larves, molts altres factors (biològic, físics, ecològics...) estan actuant plegats i contribueixen a esculpir l'estructura poblacional dels invertebrats marins.

Al llarg de la darrera dècada, el nombre de treballs publicats de filogeografia i genètica de poblacions en invertebrats marins ha crescut considerablement, començant amb al.lozims, seguint amb dades de seqüencies d'ADN, i darrerament utilitzant els microsatèl.lits com una de les eines estrella. Les qüestions tractades en aquests estudis han estat variades, els temes que es tracten majoritàriament són:

1-Contrast de patrons de dispersió i reproducció

2-Estudis de l'estructura genètica i flux gènic entre les poblacions

3-Especiació i hibridació entre espècies

4-Detecció de colonitzacions i invasions

5-Estudis de filogeografia

6-Estudis d'estructura clonal en organismes amb reproducció asexual

7-Estudi d'espècies críptiques

Tot i que la majoria d'aquests estudis es poden considerar ciència bàsica, el coneixement que s'adquireix a través d'ells pot ser aplicat en temes de conservació i gestió d'espècies i ecosistemes marins, un camp que cada dia està tenint més importància, i que pot arribar a salvar moltes espècies i ecosistemes que es troben en perill degut a la "globalització" que s'està donant al planeta on vivim. Un problema ecològicament greu que s'ha desenvolupat durant aquest darrer segle és el transport antropogènic d'organismes marins, moltes vegades a escala mundial, a través de les aigües que emmagatzemen els vaixells de transport de mercaderies per tal de compensar el pes de la càrrega o a través dels bucs dels vaixells. Aquests vaixells omplen uns grans dipòsits amb aigua de mar d'una zona i no la deixen anar fins que no són al lloc on carreguen la mercaderia, on alliberen l'aigua juntament amb un munt d'organismes que poden ser de l'altra banda del món. Aquest fet pot donar lloc a invasions amb desplaçament d'espècies autòctones. Algunes de les invasions poden incloure espècies críptiques, que poden passar desapercebudes als nostres ulls i només ser detectades amb l'ús d'eines moleculars que no només poden detectar la invasió sinó també l'origen d'aquesta.

Una mostra representativa dels treballs d'ecologia molecular i filogeografia publicats fins ara en invertebrats marins es presenta a la Taula 1 del Capítol 1, on s'indica l'organisme i el tema central d'estudi.

L'espècie d'estudi: *Crambe crambe*

S'ha triat una espècie sèssil amb larva lecitotròfica degut a que el baix potencial de dispersió teòricament hauria de donar lloc a una elevada estructuració poblacional (com a mínim a gran escala geogràfica), i aquest fet ajudaria a testar la resolució dels diferents marcadors moleculars utilitzats. Un punt important era triar un organisme ben conegut des del punt de vista ecològic i biològic per tal de facilitar la interpretació de les dades moleculars. Per aquestes raons i degut a una misteriosa fascinació del grup d'Ecologia Bentònica del Departament de Biologia Animal de la

UB cap a “l’esponja vermella”, es va triar *Crambe crambe* per a aquest estudi, una de les esponges més comunes al Mediterrani.

El gènere *Crambe* (Vosmaer), que pertany a la familia de les Poecilosclèrides, inclou cinc espècies vives distribuïdes majoritàriament al Mediterrani i a la costa Est de l’Atlàntic. Una espècie fòssil de Nova Zelanda descrita sota el nom de *Vetulina oamaruensi* (Hinde & Holmes 1892) ha estat recentment proposada com a part del gènere *Crambe* (Uriz & Maldonado 1995).

L’espècie *Crambe crambe* (Schmidt 1862) és una esponja vermella incrustant (veure Figura 2, Capítol 1). Pot arribar a assolir superfícies de fins a mig metre quadrat i sempre es troba lliure de macrosimbionts. És coneguda com una esponja altament bioactiva, per aquesta raó no se li coneixen depredadors i és altament competitiva per l’espai enfront als altres invertebrats que ocupen el mateix nínxol. A més, és pràcticament lliure de microsimbionts (al contrari de la majoria d’esponges), una característica molt important si tenim en compte el risc de contaminació d’ADN de bacteris i cianobacteris que pot comportar el fet d’extreure l’ADN d’esponges que tenen el seu mesohil ple d’aquests simbionts. Pel que fa a la seva distribució, és una de les espècies més abundants del litoral Mediterrani, es troba tant a parets il·luminades com ombrívoles, des de 1 fins a 60 metres de fondària, i fins i tot pot viure a les praderies formades per la fanerògama *Posidonia oceanica.* És una de les poques espècies que pot conviure amb l’alga invasiva *Caulerpa taxifolia* a l’Oest del Mediterrani. El seu rang de distribució va des de l’Est de l’Oceà Atlàntic (Illes Canàries i Madeira) cap a l’Oest del Mediterrani, fins al Mar Adriàtic, i les costes d’Egipte i Turquia on ha estat citada una vegada (veure Figura 3, Capítol 1). L’esponja assoleix el màxim d’abundància a la zona Oest del Mediterrani on pot arribar a densitats d’uns 67 individus per metre quadrat i a una cobertura del 47% de la superfície (Turon et al. 1998)

Estudis de la dinàmica de creixement de l’esponja han mostrat que es pot arribar a reproduir asexualment per fissió d’adults i que la fusió d’adults també es pot arribar a donar, tot i això aquests processos no semblen donar-se amb suficient freqüència com per a ser rellevants a la demografia de l’esponja. En altres esponges i altres espècies d’invertebrats incrustants aquests fenòmens es donen més freqüentment (Wulff 1991).

Crambe crambe és hermafrodita i es reprodueix sexualment alliberant esperma a través dels òsculs, els espermatozous són capturats per altres esponges a través dels porus inhalants per fertilitzar els òvuls. Els embrions s'incuben i les larves són alliberades a través dels òsculs des de principis de Juny fins a finals d'Agost (al Nord Oest del Mediterrani). Les larves alliberades es belluguen fent petits cercles de manera irregular i es dispersen uns quants metres de l'esponja mare. Les larves tenen un període de vida lliure que va des de 24 a 72h (al laboratori) després del qual s'assenten amb una tendència a instal·lar-se a prop d'esponges de la mateixa espècie (Uriz *et al.* 1998).

Donat el comportament filopàtric de les larves i el seu curt temps de vida lliure, s'espera que les poblacions de *C.crambe* siguin força tancades, fins al punt de poder detectar diferenciació entre poblacions relativament properes (desenes de kilòmetres) i seguint el model d'aïllament per distància.

Marcadors moleculars utilitzats: Seqüenciació d'ADN

La seqüenciació de l'ADN, és una tècnica que s'ha expandit en ús a partir del descobriment de la Reacció en Cadena de la Polimerasa (PCR) i que permet mesurar la variabilitat genètica a partir dels canvis nucleotídics que trobem entre seqüències d'ADN de diferents espècies o individus. Per a treballar a nivell intraespecífic calen regions de l'ADN que siguin altament polimòrfiques o variables per tal de poder detectar diferències entre poblacions de la mateixa espècie. Aquestes regions es poden trobar sobretot a l'ADN mitocondrial (ADNmt) i a l'ADN ribosòmic (ADNr) els quals destaquen per ser marcadors moleculars de bona resolució per a estudis intraespecifics a la majoria de Tipus zoològics.

Seqüències d'ADN mitocondrial

El 70% dels estudis de filogeografia s'han fet utilitzant l'ADNmt (veure Figura 4, Capítol 1) degut a la seva elevada variabilitat.

Hi ha diverses hipòtesis que intenten explicar les causes de la ràpida evolució del ADNmt:

- Relaxació de la funció de les proteïnes
- Elevada taxa de mutació
- Falta d'histones

Sigui quina sigui la raó, el fet és que compleix el requisit d'elevada variabilitat necessari per a ser un bon marcador a nivell intraespecífic. A més, el seu mecanisme característic de transmissió uniparental de generació en generació (per part de la mare, amb molt poques excepcions) i generalment sense recombinació, fa que tots els canvis nucleotídics detectats siguin deguts a processos de mutació i no deguts a recombinació com podria passar al treballar amb gens nuclears. Això fa que les metodologies d'anàlisi desenvolupades per a estudis filogenètics siguin útils en el cas de treballar amb dades de gens mitocondrials.

Seqüències d'ADN ribosòmic

L'ADNr forma part d'un complex multigènic amb còpies d'unes unitats disposades en tàndem al llarg de l'ADN nuclear (veure Figura 5, Capítol 1). Cada unitat està formada pels gens ribosòmics: 18S, 5.8S i 28S. Uns "Espaiadors" separen cadascun d'aquests gens: els Espaiadors Interns Transcrits 1 i 2 (ITS 1 i 2), l'Espaiador Extern Transcrit (ETS) i l'Espaiador Intergènic (IGS). D'aquests espaiadors, els ITS1 i ITS2 són les regions de més ràpida evolució i conseqüentment han estat utilitzats per a estudis a nivell intraespecífic. El fet que estiguin envoltats de gens funcionals força conservats, fa que s'hagin aprofitat aquests gens per a amplificar fàcilment els ITS mitjançant "primers" universals.

Un problema associat a l'ús dels ITS és la seva naturalesa de còpia múltiple, ja que sovint les diverses còpies no estan homogeneitzades. Això fa que treballar amb ells sigui més costos que només seqüenciar gens de còpia única, ja que per a

fer un estudi acurat haurem de clonar i seqüenciar un nombre elevat de còpies per individu per a trobar els veritables al.lels o variants.

Els microsatèl.lits

En els darrers temps els microsatèl.lits (microsats) han esdevingut un dels marcadors moleculars més populars per a estudis intraespecífics. Consisteixen en diverses repeticions en tàndem de fins a 5 nucleòtids (p.ex. $(TG)_n$ o $(CGA)_n$) que es troben àmpliament disperses al llarg del genoma d'eucariotes i en cloroplasts dels vegetals. Sovint són molt polimòrfics degut a la variació en el nombre de repeticions de cada unitat, a més es consideren neutres ja que no se'ls coneix cap mena de funció i generalment es troben en regions no codificants, així doncs no hi hauria pressió selectiva ni ambiental que els influeixi directament.

Degut a l'elevat polimorfisme, els microsats han esdevingut una eina molt útil per a la identificació d'individus i per a tests de paternitat. També permeten fer estudis d'estructura genètica de poblacions a diferents nivells, des del biogeogràfic fins al local o intrapoblacional, arribant fins i tot a diferenciar individus. El fet que per a treballar amb microsats s'utilitze la tècnica de la PCR també ha obert la possibilitat de poder treballar amb quantitats molt menudes d'ADN que de vegades poden no estar en perfecte estat i així permeten analitzar mostres fecals o especímens de museus. Malauradament aquest gran potencial es veu limitat per factors tècnics i de temps que requereixen l'ús d'aquests marcadors, sobretot en l'estadi de desenvolupament i posada a punt del mètode per a cada espècie en estudi. Si a l'espècie d'interès no hi ha cap microsat descrit, aquests s'han de buscar i aïllar del genoma de l'espècie en qüestió, una tasca molt costosa tant des del punt de vista econòmic com de la quantitat de feina que comporta. Un altre punt negre d'aquests marcadors és el poc coneixement que es té del seu model mutacional que du a la falta de mètodes apropiats d'anàlisi de dades.

En ecologia, s'han utilitzat entre d'altres coses per a estudiar estructures poblacionals, grandàries poblacionals efectives, relacions genètiques entre poblacions, graus de parentiu entre individus, i mides de clons.

Resultats i conclusions

Poca variació genètica en seqüències d'ADNmt al llarg de l' àrea de distribució de *Crambe crambe*

És el primer treball on s'utilitza ADNmt per a l'estudi de l'estructura poblacional en esponges. S'ha analitzat un fragment de 535 parells de bases de la Subunitat I del gen mitocondrial Citocrom Oxidasa per a un total de 86 individus mostrejats a una distància mínima de 5m entre ells per a minimitzar les possibilitats de mostrejar un mateix clon. El mostreig s'ha dut a terme a 8 localitats separades entre elles des de 20 Km fins a 3000 Km al llarg del rang de distribució de l'espècie (veure Figura 6, Capítol 2).

S'ha trobat una única posició polimòrfica, que defineix dos únics haplotips. La diversitat nucleotídica és molt baixa comparada amb la d'altres invertebrats marins amb larves filopàtriques. Els dos haplotips es troben en diferents freqüències a les poblacions (Taula 3 Capítol 2) i ens permet detectar nivells baixos de flux gènic entre algunes de les poblacions properes (p.ex. Cap de Creus i Banyuls).

La hipòtesi ecològica de que la curta vida lliure de la larva duria a una gran estructura genètica de les poblacions no pot ser descartada tot i la homogeneïtat trobada amb aquest marcador molecular. De fet, en un estudi amb cnidaris on s'analitzava un gen mitocondrial es va veure que hi havia una gran similitud genètica amb les esponges (Watkins & Beckenbach 1999), aquests autors van proposar que hi deuria haver una elevada conservació de l'ADN mitocondrial en organismes diblàstics.

Altres estudis de nivell biogeogràfic en esponges amb ADN mitocondrial són necessaris abans de generalitzar, però aquests resultats semblen indicar que l'ADN mitocondrial, en el cas concret de *Crambe crambe* i possiblement en el cas de les esponges en general, és una molècula massa conservada com per a utilitzar-la com a marcador molecular en estudis a nivell intraespecífic. Per contra sembla prou variable entre espècies com per utilitzar-la per a estudis filogenètics.

Història evolutiva de l'esponja *Crambe crambe*: expansió demogràfica i invasió recent de les illes de la Macaronèsia des del Mediterrani. Inferència a partir de seqüències ITS de l'ADNr

S'ha analitzat la variació trobada en 114 individus d'onze localitats del rang de distribució de l'esponja, en un fragment de DNA ribosòmic de 647 parells de bases que inclou als ITS-1 i ITS-2. En el cas de trobar polimorfismes intraindividuals, al tractar-se d'un marcador de múltiples còpies per genoma, s'ha procedit a clonar el producte de la PCR per tal de poder detectar el màxim de seqüències diferents (s'han seqüenciat un total de 116 clons de 16 individus polimòrfics). Per primera vegada en esponges s'ha detectat variabilitat intragenòmica. En total s'han detectat 16 tipus diferents de seqüències, deguts a 8 parells de bases variables (veure Taula 6 i Figura 7, Capítol 3). La variabilitat trobada està en el rang de la detectada en altres esponges, tot i que mai abans se n'havia trobat a nivell intraindividual. En aquests individus polimòrfics, el nombre de variants trobades varia de dos a set.

L'anàlisi de les seqüències ens mostra que les poblacions de *C.crambe* estan molt diferenciades i afectades per una restricció de flux gènic degut a un fenòmen d'aïllament per distància. També suggereix que tant la curta vida larvària com l'efecte de l'hidrodinamisme de les diverses zones estan limitant la dispersió i l'intercanvi d'individus entre poblacions.

L'estudi filogeogràfic s'ha dut a terme aplicant la tècnica de l'anàlisi aniuada de clades ("nested clade analysis" o NCA). S'ha inferit una expansió continuada del rang de distribució de l'espècie (Taula 8, Capítol 3). Les anàlisis filogenètiques mostren un origen recent de les diferents seqüències trobades, aquest fet podria ser explicat per un origen recent de l'espècie o per un coll d'ampolla recent a la zona d'estudi. També s'ha detectat la invasió recent de la zona Macaronèsica (Illes Canàries i Madeira) a l'est de l'oceà Atlàntic. Aquesta zona de l'Atlàntic es caracteritza per ser una àrea amb unes comunitats afins a les del Mediterrani. El fet que *C. crambe* es trobi de forma abundant en aquests arxipèlags i que no la trobem en altres zones Atlàntiques ens du a suggerir que l'arribada de l'esponja a aquestes illes ha estat molt probablement mitjançant el transport humà, és a dir, que les larves deuen haver viatjat en vaixells. Probablement amb les aigues de llastre, haurien arribat a les illes Atlàntiques una petita quantitat de larves, donant lloc a una o més

colonitzacions amb efecte fundador, el que explicaria la reducció en nombre d'al.lels detectada respecte a les poblacions mediterrànies.

L'esponja s'hauria adaptat en aquest nou ambient on s'ha expandit fins al punt de ser una de les esponges més abundants (observació personal). En aquest cas tindríem una situació contrària a les teories biogeogràfiques que fins ara s'havien postulat pel que fa a la relació entre les comunitats bentòniques del Mediterrani i de l'Est de l'Atlàntic: sempre s'ha pensat que després de la darrera crisi de dessecació del Mar Mediterrani, hi va haver una recolonització per part de les comunitats Atlàntiques. Aquest exemple concret d'una esponja, suggereix que la colonització en el sentit contrari, degut a l'efecte del transport humà, podria ser un cas no aïllat sinó més comú del que en principi es podria haver pensat en altres invertebrats bentònics.

Descripció de microsatèl.lits polimòrfics a l'esponja *Crambe crambe* i la seva variació en dues poblacions llunyanes

Mitjançant la construcció d'una llibreria genòmica enriquida s'han caracteritzat set loci microsatèl.lit (Taula 10, Capítol 4) i s'han descrit els *primers* específics per a ells. Aquests set loci han estat testats en dues poblacions llunyanes, 30 individus de Tossa de Mar i 30 individus de Gran Canària van ser genotipats per als set loci per tal d'avaluar el grau de polimorfisme i el potencial d'aquests marcadors per a estudis de genètica de poblacions en aquesta espècie. El nombre d'al.lels per locus varia de 3 a 16 i les distribucions de les freqüències al.lèliques difereixen considerablement entre les dues poblacions (Figura 11, Capítol 4). Son els primers microsatèl.lits descrits en una esponja.

Marcadors microsatèl.lits detecten una forta estructura inter i intrapoblacional a l'esponja *Crambe crambe*

S'ha analitzat la variació trobada en 286 individus d'onze poblacions del rang de distribució de l'esponja *Crambe crambe* (Figura 12, Capítol 5) amb l'ús de sis loci microsatèl.lits específics previament descrits. Es tracta del primer estudi on s'utilitzen aquests marcadors per a l'estudi de l'estructura poblacional en esponges.

Tal i com s'esperava, per tractar-se d'un invertebrat sèssil amb larva lecitotròfica, s'han trobat nivells alts de diferenciació entre les poblacions amb un valor de F_{ST} de 0.18. La diferenciació trobada entre les poblacions Atlàntiques i Mediterrànies ens indica una colonització recent de la zona Atlàntica, probablement mitjançant aigua de llastre dels vaixells de càrrega. També s'ha detectat que la variabilitat genètica de les poblacions de l'esponja segueix un patró d'aïllament per distància (Figura 13, Capítol 5). A nivell intrapoblacional s'ha detectat un alt grau d'estructuració (F_{IS}=0.21) que podria ser causat per diferents factors com ara: nivells alts de consanguinitat deguts a la poca dispersió de les larves, un efecte Wahlund degut a una subestructuració espaial, i/o la possibilitat que es produeixi autofecundació, un fet que encara no s'ha pogut demostrar però que no es pot descartar degut a l'hermafroditisme de l'espècie.

Tot i el disseny experimental, on es va intentar evitar mostrejar clons agafant sempre esponges que estiguèssin separades més de 5 m, s'han detectat alguns individus idèntics per als sis loci en les poblacions de Canàries, Còrsega i Nàpols. S'ha elaborat un programa de simulació per estimar les probabilitats de trobar individus genotípicament iguals en una població amb reproducció només sexual tenint en compte el nombre d'al.lels per locus, la seva freqüència a les diferents poblacions i els nivells d'heterozigositat detectats. Només en el cas de la localitat de Canàries és força probable l'aparició per atzar d'individus idèntics, degut a l'elevada freqüència d'un determinat al.lel a la majoria dels loci. Aquest fet també reforça la conclusió de que hi ha hagut una colonització recent de l'Atlàntic des del Mar Mediterrani. Per a les altres localitats amb individus idèntics (Còrsega i Nàpols), la probabilitat que ho siguin degut a l'atzar és tant petita i el nombre de "clons" trobats (7 i 5, respectivament) tan alta que l'explicació més plausible és que es tracti

d'autèntics clons fruit de la reproducció asexual. Així doncs, la reproducció asexual seria molt més important del que s'hauria imaginat en un principi, i a més es donaria en diferents proporcions depenent de la localitat estudiada. Si el procés de reproducció asexual es veu limitat a processos de fissió d'individus adults o aquest increment inesperat en la freqüència de clons es degut a altres processos a nivell larvari o embrionari quedaria pendent d'esbrinar. Per tal d'aprofondir en l'importància de la reproducció asexual en relació a la reproducció sexual i en relació a l'estructura de les poblacions a petita escala s'hauria de dissenyar un mostreig intensiu a nivell intrapoblacional.

Amb aquest estudi queda palesa l'elevada resolució d'aquests marcadors moleculars, que destaquen especialment pel gran potencial que tenen per respondre qüestions a nivells microgeogràfics (p.ex. intrapoblacionals), cosa que de moment, cap altre marcador no ha permès. En particular, permetrien abordar per primera vegada de manera formal l'estudi de l'estructura clonal de les esponges.

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