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Genetic evidence for cryptic speciation in allopatric populations of two cosmopolitan species of the calcareous sponge genus *Clathrina*

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Abstract. Many sponge species are considered to be cosmopolitan. However, the systematics of marine sponges are very difficult because of the paucity of taxonomically useful characters, and hence the apparently cosmopolitan nature of many species may be simply a consequence of this. In this paper, geographically distant populations of two pairs of cosmopolitan calcareous sponges of the genus *Clathrina* were compared genetically. *C. clathrus* and *C. cerebrum* were collected by SCUBA diving between January and March 1989 from two localities: the Mediterranean Sea at La Vesse, near Marseille, Frances, at 9 to 12 m depth, and from the South West Atlantic at Arraial do Cabo, about 200 km east of Rio de Janeiro, at 2 to 10 m depth. Very high levels of gene divergence were found between the allegedly conspecific populations. The levels of genetic identity, *I*, observed are so low ($I = 0.128$ and 0.287) that the populations clearly cannot be considered conspecific. New species names of *C. aurea* sp. nov. and *C. brasiliensis* sp. nov. are therefore assigned to the southwest Atlantic counterparts of *C. clathrus* and *C. cerebrum*, respectively. It is concluded that, at least for the species studied, and probably for many other species in taxonomically difficult groups, the actual distributions of single species may be far more geographically restricted than is generally assumed.

Introduction

It has long been recognised that the systematics of many taxa within the Porifera are particularly hampered by the paucity of morphological characters which may be used for their classification (e.g. Levi 1973). It is noticeable that among sponges with a number of useful diagnostic features, most species are of relatively restricted distribution, whereas among those with few useful taxonomic characters many species are considered to be very widespread or cosmopolitan. There is, therefore, a suspicion that the apparently circumglobal distribution of various sponges may be merely a consequence of inadequate taxonomic study. Calcareous sponges of the genus *Clathrina* Gray (Calcarea: Calcinea) are typical examples of this kind of problem: they are very common in shallow waters, and display apparently high morphological diversity both among sympatric and allopatric populations of accepted species (Borojevic and Boury-Esnault 1987). Sponges from this genus show very few taxonomically useful morphological features and species are often separated only by the absence of particular characters considered to be diagnostic. Since early studies of the genus *Clathrina*, various authours have attributed the diversity either to high specific differentiation (Haeckel 1872) or high morphological plasticity within fewer species (Sarà 1953, Burton 1963). This has resulted in divergent schools of thought, one tending to split the populations into homogeneous species and the other lumping them in groups with highly variable morphology and wide geographical distributions.

In the present study we have examined the biochemical genetic divergence between two geographically separated but morphologically very similar populations of two species of *Clathrina*. *C. clathrus* and *C. cerebrum* are both very common in the Mediterranean, and both are also found on the coasts of Brazil. The two species are also well characterised, easily identified by diagnostic spiculation, colour and general organisation of the body (cormus) and are widely accepted as valid species (e.g. Tuzet 1973, Gaino et al. 1981). Since for both species Mediterranean and southwest Atlantic populations appear very similar, we felt that it would be interesting to establish: (1) how much evolutionary divergence has occurred between them, (2) how consistent are the taxonomic parameters currently used and, consequently, (3) whether these populations are indeed conspecific.

The electrophoretic analysis of isozymes has become a well established technique for the study of the genetic structure of natural populations in a wide range of organisms (reviewed by, e.g., Thorpe 1982, Richardson et al. 1986). The major advantages of the technique are the objectivity of the analysis, the ease with which large amounts of genetic information can be acquired quickly

and the relatively unbiased results compared to those obtained from morphological studies (Avise 1974, Solé-Cava and Thorpe 1987). The taxonomic uses of electrophoretic data are well known and the method has been extensively used to investigate cryptic speciation and interspecific relationships in many marine species (reviewed by Ward 1989). There are, however, few such studies on marine sponges (e.g. Solé-Cava and Thorpe 1986, Stoddart 1989, Solé-Cava et al. 1991), and the present work would appear to be the first biochemical genetic study of any calcareous sponge species.

Materials and methods

Clathrina clathrus and *C. cerebrum* were collected by SCUBA diving between January and March 1989 from two localities: the Mediterranean Sea at La Vesse, near Marseille, France, at 9 to 12 m depth and from the South West Atlantic at Arraial do Cabo, about 200 km east of Rio de Janeiro, at 2 to 10 m depth. After collection, the samples were transported to the laboratory in dry ice and stored at −20 °C until required for electrophoresis, which was carried out not later than one month after collection. Total mounts of tube walls and spicule preparations were made for the microscopical analysis of the skeleton.

Horizontal 12.5% starch gel electrophoresis was carried out as previously described for sponges (Solé-Cava and Thorpe 1986). The buffer system used was tris-citrate, pH 8.0 (Ward and Beardmore 1977). The staining of the gels followed standard procedures (Harris and Hopkinson 1978). Samples from each pair of allopatric populations were run side-by-side on the same gel, to allow the correct identification of alleles. Twenty-six enzyme systems were investigated. Although most of the tested enzymes had been used in at least some earlier studies of species of marine sponges (Balakirev and Manchenko 1985, Solé-Cava and Thorpe 1986, 1991a, Solé-Cava et al. 1991), only eight (acid phosphatases, ACP – EC 3.1.3.2; aldehyde oxidase, AO – EC 1.2.3.1; catalase, CAT – EC 1.11.1.6; D-esterases, D-EST – EC 3.1.1.1; α-esterases, EST – EC 3.1.1.1; hexokinase, HK – EC 2.7.1.1; phosphoglucose isomerase, PGI – EC 5.3.1.9 and superoxide dismutase, SOD – EC 1.15.1.1) could be reliably scored in all samples of *Clathrina clathrus* and *C. cerebrum* analysed. Genotype frequency data were analysed by the BIOSYS-1 programme (Swofford and Selander 1981). Because of the small sample sizes, Nei's unbiased genetic identity was used in the comparison on populations (Nei 1978), and Fisher's exact probabilities were used to test for fits to Hardy-Weinberg equilibrium (Swofford and Selander 1981).

Table 1. *Clathrina clathrus* and *C. cerebrum*. Major morphological characteristics of populations of the two species from southwest Atlantic and Mediterranean

Species and characteristic	Southwest Atlantic	Mediterranean
C. clathrus		
cormus	loosely anastomosed tubes, most with terminal oscule	loosely anastomosed peripheral large tubes collecting exhalant current, terminated with one or only few osculi
colour	greenish-yellow	light golden yellow
spicules	Triactines equiangular and equiradiate, with cylindrical actines occasionally slightly undulant, thickened at tips	
C. cerebrum		
cormus	compact, composed of regularly anastomosed tubes	
colour	white	light pink
spicules	(1) triactines equiangular, equiradiate; (2) tetractines with spined apical actine; (3) large triacts on external tubes	

Table 2. *Clathrina clathrus* and *C. cerebrum*. Mean (x) and standard deviation (s) of spicule length, L, and width, W (μm) for three spicule types in populations from Mediterranean and southwest Atlantic. Samples sizes = 300 for each population of *C. clathrus*, *150* for *C. cerebrum* from Mediterranean and 130 for *C. cerebrum* from the southwest Atlantic. Tetractine and tripod spicules are not present in *C. clathrus*

Species and locality		Triactines		Tetractines		Tripods	
		x	s	x	s	x	s
C. clathrus							
Mediterranean	L	92.15	6.80	–	–	–	–
	W	5.45	0.98	–	–	–	–
Southwest Atlantic	L	72.23	6.15	–	–	–	–
	W	5.55	1.10	–	–	–	–
C. cerebrum							
Mediterranean	L	84.50	7.15	83.33	8.80	88.89	15.15
	W	6.88	1.08	7.13	1.10	10.53	1.83
Southwest Atlantic	L	65.45	6.50	64.38	6.25	71.58	10.80
	W	5.78	1.20	6.40	1.40	8.33	1.45

Results

The morphological characters of the populations of the two speices of *Clathrina* are summarised in Table 1. In *C. clathrus*, the colour and the general morphology of the cormus were found to differ slightly between the two populations studied. However, no clear morphological differences could be observed between the two populations of *C. cerebrum*. A significant difference (Student's t-test; $P<10^{-10}$ for all pairwise comparisons, except triactine widths in *C. clathrus*) was found between the spicule sizes of each population pair (Table 2).

For samples from each collection site, 11 loci were detected for *Clathrina clathrus*, and 7 loci for *C. cerebrum* (Tables 3 and 4). The comparatively small number of enzymes that showed useful activity after electrophoresis is probably a consequence of the preservation, extraction or staining conditions not being adequate for these species. Similar problems have been reported for other sponge species (Solé-Cava and Thorpe 1986, Stoddart 1989, Sará 1991, Solé-Cava et al. 1991), and are likely to be a result of generally low levels of enzyme activity in these organisms (Solé-Cava and Thorpe 1987).

The four populations showed generally high levels of mean heterozygosity (Table 5). The observed genotype frequencies did not differ significantly ($P>0.05$) from Hardy-Weinberg expectations in any population. The putative conspecific populations studied displayed a low level of genetic similarity between them. The unbiased

Table 3. *Clathrina clathrus*. Gene frequencies for isozyme loci from Mediterranean (Marseille) and from southwest Atlantic (Rio de Janeiro) populations. (*n*): number of individuals analysed

Locus and alleles		Marseille	Rio
Acp	*1*	0.067	0.000
	2	0.133	0.088
	3	0.000	0.088
	4	0.700	0.824
	5	0.100	0.000
	(*n*)	(15)	(17)
Ao-1	*1*	0.625	0.000
	2	0.375	0.969
	3	0.000	0.031
	(*n*)	(12)	(16)
Ao-2	*1*	1.000	0.000
	2	0.000	1.000
	(*n*)	(12)	(16)
Cat	*1*	0.971	0.000
	2	0.029	1.000
	(*n*)	(17)	(16)
D-Est-1	*1*	0.115	0.000
	2	0.808	0.000
	3	0.077	0.063
	4	0.000	0.938
	(*n*)	(13)	(16)
D-Est-2	*1*	1.000	0.031
	2	0.000	0.969
	(*n*)	(13)	(16)
Est-1	*1*	0.179	0.000
	2	0.786	0.063
	3	0.036	0.938
	(*n*)	(14)	(16)
Est-2	1	0.000	0.906
	2	0.967	0.094
	3	0.033	0.000
	(*n*)	(15)	(16)
Hk	*1*	1.000	0.000
	2	0.000	1.000
	(*n*)	(15)	(15)
Pgi	*1*	1.000	0.031
	2	0.000	0.938
	3	0.000	0.031
	(*n*)	14	16
Sod	*1*	1.000	0.000
	2	0.000	0.961
	3	0.000	0.031
	(*n*)	14	16

Table 4. *Clathrina cerebrum*. Gene frequencies for isozyme loci from Mediterranean (Marseille) and from southwest Atlantic (Rio de Janeiro) populations. (*n*): number of individuals analysed

Locus and alleles		Marseille	Rio
Acp	*1*	0.000	0.227
	2	0.063	0.727
	3	0.156	0.045
	4	0.438	0.000
	5	0.281	0.000
	6	0.063	0.000
	(*n*)	(16)	(11)
D-Est-1	*1*	0.567	0.000
	2	0.433	0.000
	3	0.000	1.000
	(*n*)	(15)	(7)
D-Est-2	*1*	0.000	1.000
	2	1.000	0.000
	(*n*)	(15)	(13)
Est-1	*1*	0.545	0.056
	2	0.045	0.333
	3	0.409	0.611
	(*n*)	(11)	(9)
Est-2	*1*	0.179	0.111
	2	0.821	0.889
	(*n*)	(14)	(9)
Hk	*1*	0.679	0.000
	2	0.321	1.000
	(*n*)	(14)	(5)
Pgi	*1*	0.033	0.000
	2	0.867	0.000
	3	0.100	0.000
	4	0.000	1.000
	(*n*)	(15)	(14)

Table 5. *Clathrina clathrus* and *C. cerebrum*. Measures of genetic variation for the four populations studied. nl: number of loci analysed; ni: number of individuals studied, per locus; H_e: mean expected heterozygosity per locus (unbiased estimate); H_0: mean observed heterozygosity per locus; P: proportion of loci polymorphic (95% criterion)

Species	nl	ni	H_e	H_0	P
C. clathrus					
Mediterranean	11	15	0.165	0.166	0.364
Atlantic	11	16	0.095	0.100	0.455
C. cerebrum					
Mediterranean	7	15	0.398	0.399	0.857
Atlantic	7	10	0.170	0.176	0.423

genetic identity index of Nei (1978) between Mediterranean and Atlantic populations was 0.128 for *Clathrina clathrus* and 0.287 for *C. cerebrum*. Corresponding values of genetic distance, *D*, are 2.06 and 1.25, respectively.

Discussion

The most striking features of the results are the very low levels of genetic similarity found between the Mediterranean and the Brazilian populations of each of the two *Clathrina* species. It is clear that in neither case can these populations be considered conspecific.

Genetic divergence between populations or species may be quantified using any of various published statistical methods (see e.g. Thorpe 1979, Nei 1987). The most commonly used statistic is the genetic identity measure, *I*, and its converse genetic distance, $D (= -\log_e I)$ of Nei (1972, 1978). The scale of *I* values ranges from 1 (no difference) to 0 (no genes is common). There is a vast published literature giving levels of genetic divergence

observed between various populations and species (discussed by e.g. Thorpe 1982, 1983, Nei 1987). Usually, conspecific populations have *I* values above 0.9 and rarely as low as 0.8, whilst identity levels between congeneric species typically range from about 0.3 to 0.8, and species from different, but confamilial, genera usually show *I* values below about 0.4. Values of *D* are considered to increase with time of evolutionary divergence in a manner which may be stochastically linear. Although this relationship is open to dispute and depends upon the validity of various theoretical assumptions, there can be little doubt that isolated populations diverge genetically with time (Nei 1987, Thorpe 1989). This phenomenon forms the basis of what has come to be known as the "molecular clock hypothesis" (reviews by Wilson et al. 1977, Thorpe 1982).

The levels of genetic identity observed between the allopatric samples for each of the two *Clathrina* species are similar to those usually found between confamilial genera (Thorpe 1982, 1983), and the corresponding *D* values indicate a very long divergence time: higher than 10 m.yr for *Clathrina clathrus* and 6 m.yr for *C. cerebrum*, using a conservative calibration factor of $1D = 5$ m. yr (Nei 1987). However, since no calibration for the evolutionary clock is currently available for sponges, these values must be taken as only indicative of long separation.

The genetic identities above can be compared with those observed for all possible pairwise comparisons between a group of clearly distinct species of *Clathrina*, consisting of the two species considered here and three other well characterised species (*C. contorta* from the Mediterranean and *C. ascandroides* and *C. primordialis* from the southwest Atlantic). Between these species, genetic identity values range from 0.68 to 0.21 (authors' unpublished results). Obviously, the levels of genetic identity observed between the allopatric populations of *C. cerebrum* and *C. clathrus* studied here are around or below the lower end of the range found between other species of the genus but, although a value of 0.128 is unusually low for congeneric species, there is not, in our view, any strong argument for splitting the genus. Surprisingly low identity values have also been found between cryptic species of the demosponge genera *Suberites* (Solé-Cava and Thorpe 1986) and *Axinella* (Sole-Cava et al. 1991) and may indicate either that sponge species are morphologically conservative or that they evolve very fast at the genotypic level. Alternatively, high genetic diversity within genera may merely indicate that the hierarchical resolution in sponge taxonomy has traditionally been smaller than that employed in most other phyla.

Evolutionary rates for single-locus (isozymes) and multi-locus (morphology) systems can be very different and are, thus, hard to compare (Lewontin 1984). However, despite this difficulty, the relative rates of morphological and biochemical divergence were consistent for both *Clathrina* species studied: *C. clathrus* from the two sites were less similar both morphologically and genetically, whereas in *C. cerebrum* a higher overall morphological similarity was paralleled by a rather smaller number of genetic differences.

Notwithstanding the limitations of the isozyme analysis with a small number of loci for groups barely known genetically, and whatever the inaccurary of calibration of the "molecular clock", the results indicate that the two populations have been geographically separated for a considerable period, and that since this time they have become reproductively isolated. The genetic divergence of these populations contrasts with the alleged circumtropical distributions of other sponges of the sub-class Calcinea, such as *Leucascus simplex*, *Leucaltis clathria*, *Leucetta microraphis* and *Paramurrayona corticata* (see Borojevic and Peixinho 1976, Vacelet 1981), as well as of demosponges like *Chondrilla nucula*, *Chondrosia reniformis*, and *Spriastrella cunctatrix* (Wiedenmayer 1977).

Reid (1967) has suggested that disjunct distribution patterns in many sponge species might be related to the ancient Tethyan distribution. The Tethys disappeared in the Miocene with the closing-up of the link between the Mediterranean Sea and the Indian Ocean, and the formation of the Isthmus of Panamá. The discontinuous distribution of sponge species may be a consequence of the progressive fragmentation of the originally continuous populations in the Tethys area. If a mid-Miocene (~16 m.yr B.P.) date is accepted for the geographical separation of the Mediterranean and southwest Atlantic populations of the two sponge species studied here, a calibration of Nei's genetic distance of 1 *D* unit = about 8 to 13 m.yr can be made for sponges, but this is highly speculative and should be regarded with caution. Alternatively, Burton (1930, 1932) and Hechtel (1965) have explained the present pattern of amphi-Atlantic distribution as the result of larval migration. However, the transatlantic transport of sponge larvae must be doubtful (Reid 1967, Boury-Esnault and Lopes 1985), since the duration of the planktonic larval life does not usually exceed 5 d (Fry 1970).

The observed morphological variation between Mediterranean and southwest Atlantic populations of each of the two species has not been described previously, but is at a level which would generally be regarded as being well within the expected range for intraspecific variation. Some morphological divergence between allopatric populations is to be expected, and the only externally visible characters to vary were cormus shape and colour in *Clathrina clathrus* only. The significance of spicule differences has been the subject of much debate, but many authors (e.g. Sará 1953, Burton 1963) considered that only differences in spicule type should be regarded as diagnostic at the species level, since spicule size is considered to show wide intraspecific variation (Fry 1970, Jones 1984), and also to be influenced by the environment (Stone 1970). The present study suggests that significant differences in spicule size may, at least for some groups, conceal species level differences, but also that different species do not necessarily differ in spicule type. This latter conclusion confirms that of the earlier study of *Suberites* spp. (Solé-Cava and Thorpe 1986), where no significant differences of either spicule size or type were found between three sympatric species.

Many difficulties have been associated with the application of the biological species concept to allopatric pop-

ulations (Mayr 1987, Van Valen 1988), but, nevertheless, as discussed above, it is clear that the European and the American populations of the two sponge species cannot be conspecific. It is proposed, consequently, to split *Clathrina clathrus* into *C. clathrus* (*sensu strictu*) for Mediterranean populations and *C. aurea* sp. nov. for the southwest Atlantic form. Similarly, *C. cerebrum* is divided here into *C. cerebrum* (*sensu strictu*) (for the Mediterranean) and *C. brasiliensis* sp. nov. (for the southwest Atlantic). Type specimens for these two new species have been deposited at the Museum National d'Histoire Naturelle, Paris (MNHN-LBIM.C.1989.1 for *C. aurea* and MNHN-LBIM.C.1989.2 for *C. brasiliensis*). A detailed description of these species will be presented elsewhere (M. Klautau and R. Borojevic in preparation).

An additional notable feature of the results is the unusually high levels of genetic variation found in the four species of *Clathrina*. Although much higher than those generally found in most other groups of organisms (reviews by Nevo 1978, Nevo et al. 1984), similarly high variability has been found in several recent studies of other sponge species. The possible implications of these high levels of variation in sponges (and also in coelenterates) have been discussed in detail elsewhere (Solé-Cava and Thorpe 1989, 1991 b).

Our results have implications both for the systematics of geographically very widespread sponge species and for the interpretation of morphological differences in sponge populations. In view of the levels of genetic divergence found in the present work, the taxonomic integrity of many circumglobal sponge species may be open to doubt and hence should be regarded with caution. It is in any case improbable that any sessile shallow-subtidal invertebrate species with a short-lived larva will have such an extensive distribution. It is also clear that, as indicated by earlier genetic work on species of the demosponge genus *Suberites* (Solé-Cava and Thorpe 1986), very small differences generally regarded as being of little taxonomic significance (e.g. colour) can indicate species which may be genetically very distinct.

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