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Biochemical Systematics of Sibling Sympatric Species of *Clathrina* (Porifera: Calcarea)

MICHELLE KLAUTAU,* ANTÓNIO M. SOLÉ-CAVA† and RADOVAN BOROJEVIC*

*Departamento de Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Caixa Postal 68021, 21941-Rio de Janeiro-RJ, Brazil;

†Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, 21941-Rio de Janeiro-RJ, Brazil and Department of Environmental and Evolutionary Biology, The University of Liverpool, U.K.

Key Word Index—*Clathrina*; Calcinea; Calcarea; Porifera; taxonomy; isozyme; electrophoresis.

Abstract—We have studied isozyme patterns in a population of calcareous sponges belonging to the genus *Clathrina*, collected at Arraial do Cabo, Rio de Janeiro, Brazil. The only slightly distinct morphotypes, that would be classified by standard systematics as conspecific, were found to belong to biologically distinct species, with no gene flow among them. We conclude that morphological criteria of spicules and general organization of the skeleton are unreliable for the classification of *Clathrina*. Moreover, comparison with specimens belonging to another morphologically well defined species indicated that genetic distances did not correlate with the observed grade of morphological differences, precluding the use of spicule-analysis to establish the relative degree of relationship among species belonging to the genus *Clathrina*, and possibly among sponges of other groups.

Introduction

The systematics of any group of organisms is limited by the number of characters usable for their description and the evolutionary stability of those characters. Usable taxonomic characters are those that display some variation within the studied group, and that are conservative enough to allow the identification of sub-groups within it. Morphological variation, the basic material of classical systematics, is usually large both within and between natural populations. This high variation has often been interpreted in the past as being the result of a large intraspecific genetic plasticity or heterogeneity, or as a consequence of subtle differentiation of morphologically related, but genetically distinct, species. This dispute has been particularly intense in studies with marine sponges, because of the paucity of unambiguous and easily measurable morphological characters and their high degree of variation (Levi, 1979). Due to the shortage of morphological characteristics for sponge systematics, analysis of other features has been used to try to obtain additional evolutionary information for the identification and classification of the group. In the class Demospongiae, the use of biochemical genetics methods has demonstrated the reproductive isolation of several sympatric populations formerly considered to be conspecific (Solé-Cava and Thorpe, 1986; Solé-Cava *et al.*, 1991, 1992; Boury-Esnault *et al.*, 1992).

Calcareous sponges of the genus *Clathrina* are a typical example of difficulties encountered in the recognition of closely related species: they are common in all shallow waters and they display a high (supposedly intraspecific) morphological diversity, both among sympatric or allopatric populations. Sponges from this genus present very few usable morphological characters, and species are often separated only by negative characteristics (Borojevic and Boury-Esnault, 1987). Since the beginning of studies on sponges that are now considered to belong to the genus

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Clathrina, different authors have attributed this diversity either to a specific differentiation (Haeckel, 1872; Dendy, 1891), or to a high morphological plasticity (Sarà, 1953; Burton, 1963). Recently, a genetic comparison of allopatric populations of *Clathrina* cf. *clathrus* and of *Clathrina* cf. *cerebrum* that would be classified as conspecific by classical morphological taxonomy, has demonstrated that subtle morphological differences did actually reflect a specific differentiation (Solé-Cava *et al.*, 1991). In the present study we have compared sympatric populations of slightly distinct morphotypes of *Clathrina* by isozyme electrophoresis, to evaluate their taxonomic status. This evaluation is then discussed in relation to the more general issue of how common is the problem of cryptic species in the Porifera.

Materials and Methods

We studied two groups of morphologically similar sponges:

(a) two morphotypes of *Clathrina* cf. *primordialis* (Haeckel, 1872);

(b) a population of *Clathrina brasiliensis* (Solé-Cava *et al.*, 1991), and some morphologically very similar sympatric sponges.

For comparisons, we also collected specimens of an apparently morphologically more homogeneous species, *C. ascandroides* (Borojevic, 1971), clearly distinct by morphological criteria from the two former groups of *Clathrina*.

The sponges were collected by scuba-diving between January and March, in Arraial do Cabo, Cabo Frio region, Rio de Janeiro, at 2–10 m depth. Samples were frozen immediately and kept at −20°C until electrophoresis, which was performed within a month after collection. Total mounts of tube walls and spicule preparations were made for the microscopical analysis of the skeleton of each individual. Discontinuities in the distribution of morphological and morphometric parameters of the spicules were used *a priori* for the delimitation of hypothetical groups (or "morphotypes") within each species, and *a posteriori*, for the search for possible subtle differences between individuals genetically clearly different.

Horizontal 12.5% starch gel electrophoresis was carried out as previously described (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; Murphy *et al.*, 1990). Twenty-seven enzyme systems were tested, but only aldehyde oxidase—AO (E.C. 1.2.3.1), acid phosphatases—ACP (E.C. 3.1.3.2), catalase—CAT (E.C. 1.11.1.6), A-esterases—A-EST (E.C. 3.1.1.1), D-esterases—D-EST (E.C. 3.1.1.1), hexokinase—HK (E.C. 2.7.1.1), malate dehydrogenase—MDH (E.C. 1.1.1.37), phosphoglucose isomerase—PGI (E.C. 5.3.1.9), and superoxide dismutase—SOD (E.C. 1.15.1.1) gave reproducible results.

Genotype frequencies were converted to gene frequencies and used to calculate heterozygosity levels and pairwise unbiased gene distances (Nei, 1978) using the program BIOSYS (Swofford and Selander, 1981).

Type specimens of *Clathrina primordialis* (Haeckel, 1872), *emend., C. aspina* sp.n. and *C. cylindractina* sp.n. were deposited at the Museum National d'Histoire Naturelle, Paris, respectively under numbers MNHN-LBIM.C.1993.1, MNHN-LBIM.C.1993.2. and MNHN-LBIM.C.1993.3. Specimens of these species were also deposited in the Porifera collection of the Department of Zoology of the Universidade Federal do Rio de Janeiro, Brazil, under numbers LABPOR.1993.1-5, LABPOR.1993.6-10 and LABPOR.1993.11-15, respectively.

Results and Discussion

Results are shown in Tables 1–6.

The most important outcome of this study is that very low levels of gene identity can be found between morphologically very similar sponge species. This conclusion agrees with the results of previous molecular systematics studies on other sponge species both in allopatry and sympatry (Solé-Cava and Thorpe, 1986; Solé-Cava *et al.*, 1991, 1992; Boury-Esnault *et al.*, 1982). It would seem, thus, that taxonomists may have been forcibly overconservative in the assignment of sponge species in the past, both due to the shortage of generally available morphological characteristics, and the dismissal of subtle differences as "intraspecific variations".

Clathrina brasiliensis and the morphologically similar morphotype were found to be genetically distinct ($I = 0.686$) (Tables 3 and 6). The low level of gene similarity and, what is more important, the existence of two diagnostic loci (Ayala, 1983) between these two sympatric populations, are positive indicators that no gene flow can be

TABLE 1. MORPHOLOGICAL CHARACTERISTICS OF THE STUDIED *CLATHRINA* SPECIES

	C. brasiliensis	*C. aspina* sp. n.
Cormus	Compact, composed of regularly anastomosed tubes	
Colour	White	
Spicules	1. Triactines equiangular, regular	
	2. Tetractins with spines on the apical actin	2. Tetractins without spines
	3. Large triactins or tripods on external tubes	
	C. primordialis	*C. cylindractina* sp. n
Cormus	Loosely anastomosed tubes	
Colour	White	
Spicules	1. Triactins equiangular, equiradiate, conical actins	2. Triactins equiangular, equiradiate, cylindrical actins
	C. ascandroides	
Cormus	Loosely anastomosed and ramified tubes	
Colour	White in life, brown in spirit	
Spicules	1. Triactins: three sizes	
	2. Tetractins: three sizes	

TABLE 2. MORPHOMETRICAL CHARACTERISTICS OF SPICULES OF THE STUDIED *CLATHRINA* SPECIES. Mean (X) and standard deviation (S) of spicule length (L) and width (W) (μm). n = Sample size

Species		Triactins		Tetractins		Tripods	
		X	S	X	S	X	S
C. brasiliensis	L	65.5	6.5	64.4	6.3	71.6	10.8
(n=130)	W	5.8	1.2	6.4	1.4	8.3	1.5
C. aspina sp. n	L	68.5	2.0	71.0	3.4	74.8	3.2
(n=20)	W	7.5	0.02	7.3	0.05	9.5	0.04
C. primordialis	L	84.0	2.4				
(n=40)	W	9.0	0.05				
C. cylindractina sp. n	L	93.5	3.7				
(n=30)	W	7.5	0.02				

occurring between them. Consequently, they must belong to different biological species. These two species, although very similar, could be distinguished by the spines on the apical actin of their tetractines, present in *C. brasiliensis* but absent in the new species (Table 1). We therefore name it *Clathrina aspina* sp.n. It must be noted that *C. brasiliensis* has been recognized as genetically highly different (I=0.287) but morphologically indistinguishable from *C. cerebrum* (Haeckel, 1872), from the Mediterranean Sea (Solé-Cava *et al.*, 1991). The subtle morphological and high genetical differences between *C. cerebrum, C. brasiliensis* and *C. aspina* sp.n. indicate that the low levels of gene identity between *Clathrina* species are not mirrored at the morphological level. Conversely, *C. ascandroides*, which is morphologically clearly distinct from the other studied species of the genus (Table 1), displayed a mean level of genetic distances comparable to that observed between the other, morphologically similar, species (Table 6).

The two morphotypes of *Clathrina* cf. *primordialis* were also found to be genetically different (Table 4). The gene identity found between them was 0.653 (Table 6), which is a typical value for congeneric species comparisons (Solé-Cava and Thorpe, 1991). No diagnostic loci were found between them, but the differences in the gene frequencies at individual loci were highly significant (binomial exact probabilities, $P<10^{-6}$), which indicates that they are not exchanging genes. Again, the absence of gene flow in these two sympatric morphotypes indicates that they must belong to different biological species. The morphological analysis has shown that the major difference

TABLE 3. GENE FREQUENCIES FOR ISOZYME LOCI: COMPARATIVE ANALYSIS FOR *C. BRASILIENSIS* AND *C. ASPINA*. (*n*) = Number of specimens analyzed

Locus	Alleles	*C. brasiliensis*	*C. aspina*
EST-1	1	0.000	0.154
	2	0.444	0.462
	3	0.444	0.269
	4	0.000	0.038
	5	0.111	0.077
	(*n*)	(9)	(13)
EST-2	1	0.700	0.737
	2	0.300	0.237
	3	0.000	0.026
	(*n*)	(10)	(19)
MDH	1	0.375	0.000
	2	0.000	0.500
	3	0.000	0.500
	4	0.625	0.000
	(*n*)	(4)	(4)
AO	1	0.400	0.000
	2	0.600	1.000
	(*n*)	(5)	(2)
D-EST-1	1	1.000	0.591
	2	0.000	0.364
	3	0.000	0.045
	(*n*)	(2)	(11)
D-EST-2	1	0.250	0.000
	2	0.667	0.731
	3	0.083	0.269
	(*n*)	(6)	(13)
HK	1	0.100	0.029
	2	0.250	0.647
	3	0.650	0.324
	(*n*)	(10)	(17)
PGI	1	0.750	0.929
	2	0.000	0.041
	3	0.250	0.000
	4	0.000	0.031
	(*n*)	(4)	(49)
CAT	1	0.929	0.000
	2	0.071	0.000
	3	0.000	0.950
	4	0.000	0.050
	(*n*)	(7)	(10)

observed between them was the shape of the spicule actins, conical in one morphotype and cylindrical in the other. This feature has already been used by Haeckel (1872) to distinguish his *C. primordialis* (conical actins) from *C. coriaceae* Montagu 1818 (cylindrical actins). This difference was considered by Topsent (1936) to be irrelevant, and *C. primordialis* was subsequently considered a junior synonym of *C. coriacea.* This synonymy was subsequently questioned, after the comparison of specimens from Brazil with those from the British Isles, *locus typicus* of *C. coriacea* (Borojevic, 1971; Borojevic and Peixinho, 1976). The present finding of these two genetically distinct species of *Clathrina* in Arraial do Cabo seems to support the importance of the shape of the actins for separating species in the genus.

We identify the specimens of *Clathrina* containing regular triactins with conical actins as *C. primordialis,* in agreement with previous identification of specimens

TABLE 4. GENE FREQUENCIES FOR ISOZYME LOCI: COMPARATIVE ANALYSIS FOR *C. PRIMORDIALIS* AND *C. CYLINDRACTINA*. (*n*) = Number of specimens analyzed

Locus	Alleles	*C. primordialis*	*C. cylindractina*
EST-1	1	0.315	0.385
	2	0.489	0.615
	3	0.054	0.000
	4	0.141	0.000
	(*n*)	(46)	(13)
EST-2	1	0.257	0.792
	2	0.700	0.208
	3	0.043	0.000
	(*n*)	(70)	(12)
MDH	1	0.700	1.000
	2	0.300	0.000
	(*n*)	(15)	(3)
AO	1	1.000	1.000
	(*n*)	(10)	(3)
D-EST-1	1	0.617	0.000
	2	0.363	0.167
	3	0.021	0.833
	(*n*)	(47)	(9)
D-EST-2	1	0.044	0.818
	2	0.956	0.182
	(*n*)	(45)	(11)
HK	1	0.073	0.200
	2	0.491	0.550
	3	0.327	0.250
	4	0.109	0.000
	(*n*)	(55)	(10)
PGI	1	0.011	0.692
	2	0.932	0.154
	3	0.045	0.154
	4	0.011	0.000
	(*n*)	(44)	(13)
CAT	1	0.338	0.000
	2	0.662	1.000
	(*n*)	(37)	(3)

TABLE 5. MEASURES OF GENETIC VARIATION FOR THE FOUR *CLATHRINA* SPECIES STUDIED. *NS* = mean sample size per locus. *NA* = mean number of alleles per locus. H_e = mean expected heterozygosity per locus (unbiased estimate). H_o = mean observed heterozygosity per locus. *P* = proportion of polymorphic loci (95% criterion). Standard errors in parentheses

Species	*NS*	*NA*	H_e	H_o	*P*
C. brasiliensis	6.2	2.2	0.419	0.278	88.9
	(1.0)	(0.2)	(0.069)	(0.096)	
C. aspina	15.1	2.7	0.385	0.378	88.9
	(4.7)	(0.4)	(0.084)	(0.113)	
C. primordialis	41.0	2.8	0.370	0.306	88.9
	(6.2)	(0.4)	(0.080)	(0.085)	
C. cylindractina	8.6	1.9	0.285	0.265	66.7
	(1.5)	(0.3)	(0.079)	(0.086)	

collected in Arraial do Cabo (Borojevic and Peixinho, 1976) and Ubatuba (Borojevic, 1971). Haeckel (1872) has attributed nearly cosmopolitan distribution to *C. primordialis*, including Rio de Janeiro, but he has not specified the *locus typicus* for the species. In view of our previous observation of a large genetical distance between South Atlantic and European species of Calcarea (Solé-Cava *et al.*, 1991), we propose the coast of Rio

TABLE 6. GENETIC IDENTITY FOR THE STUDIED *CLATHRINA* SPECIES. Below the diagonal: Nei (1978) genetic identity. Above the diagonal: Nei (1978) unbiased genetic identity

Species	(1)	(2)	(3)	(4)	(5)
(1) *C. brasiliensis*	*****	0.686	0.550	0.428	0.431
(2) *C. aspina*	0.651	*****	0.817	0.647	0.568
(3) *C. primordialis*	0.532	0.795	*****	0.653	0.436
(4) *C. cylindractina*	0.413	0.627	0.645	*****	0.434
(5) *C. ascandroides*	0.414	0.548	0.429	0.425	*****

de Janeiro as the *locus typicus* of the species. Specimens defined morphologically and biochemically in the present study are thus considered to be representative of *Clathrina primordialis* (Haeckel 1872 *emend*). The relationship between these species and similar sponges found in the Mediterranean Sea, Indian and Pacific Oceans, and attributed by Haeckel to *C. primordialis* remains to be established. On the other hand, the specimens of *Clathrina* with cylindrical actins will be named *Clathrina cylindractina* sp.n., to distinguish them from *C. coriacea* described on coasts of the British Isles.

The general conclusion of this and of our previous study on *Clathrina* from Arraial do Cabo (Solé-Cava *et al.*, 1991) is that the use of spicules morphology, and of general organization of the skeleton, is insufficient in the classification of *Clathrina*. In all the examples studied, even the very faint morphological differences were ultimately associated with genetic indication of clearly distinct species. Conversely, we could not observe any case where morphologically distinct sponges were found to belong to genetically conspecific populations. Moreover, genetic distances did not correlate with the observed grade of morphological differences between the skeleton composition of the studied species, precluding the use of spicule-analysis to establish the relative degree of relationship among species belonging to the genus. Previous studies have shown that cytological criteria may be useful to distinguish sponges at the species-level, and to obtain information of the degree of their relationship (Simpson, 1968). Extensive revisions will be necessary combining morphological, cytological and biochemical criteria to establish with certitude systematics of Calcarea. In the meantime, it would be judicious to consider even small differences in the composition of the skeleton as indicative of more extensive differences in the genetic patterns of species in the genus *Clathrina*, and possibly in other genera of sponges.

Description of the species

Clathrina primordialis (Haeckel 1872.) The cormus is composed of white, delicate, irregularly anastomosed tubes. Small specimens have a form of white ramified, occasionally anastomosed tubes, and the larger ones form massive cormi, with multiple osculi. The skeleton is composed of randomly distributed equiangular and equiradiate triactines, with conical actines (Fig. 1).

Clathrina primordialis is abundant in shallow waters, particularly in summer. It is sciaphyle, frequently found under rocks or under large *Palythoa* and *Ascidia nigra* specimens.

Clathrina cylindractina sp. n. The cormus is very similar to the *C. primordialis*, and the specimens of these two species cannot be distinguished without spicule examination. The skeleton is composed of equiangular, equiradiate triactines, with cylindrical actines (Fig. 2). The distribution of this species is also identical to that of *C. primordialis*.

Clathrina brasiliensis Solé-Cava *et al.* (1991). The cormus is sphaerical or ellipsoidal, attaining 6 cm in diameter, composed of thin, regularly anastomosed tubes. Numerous osculi are irregularly distributed over the cormus. The skeleton is composed of tetractines, and two types of triactines. Small equiangular and equi-

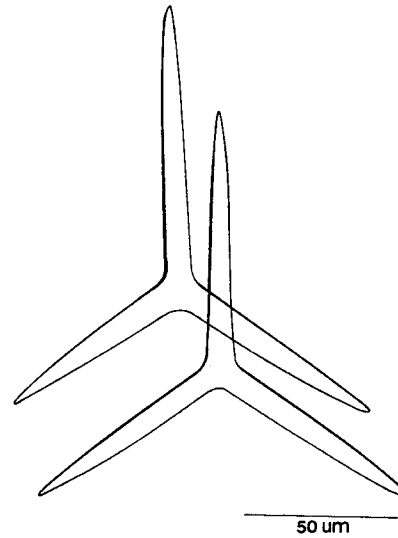

FIG. 1. *CLATHRINA PRIMORDIALIS* (Haeckel 1872). Triactines with conical actines.

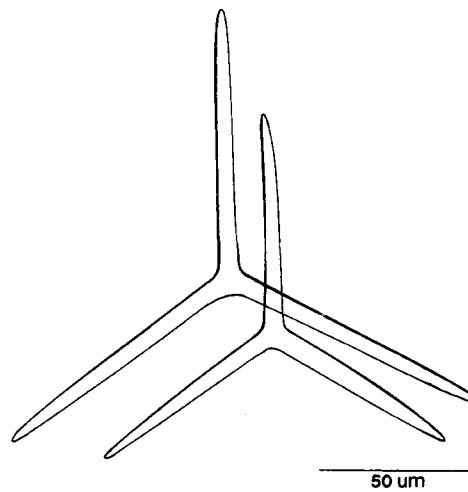

FIG. 2. *CLATHRINA CYLINDRACTINA* sp. n. Triactines with cylindrical actines.

radiate triactines are the most frequent spicule type, arranged randomly in the wall of all the tubes. Their actines are conical (Fig. 3). The tetractines bear the apical ray protruding into the lumen of the tubes. They are ornated by small spines at the distal end. The large triactines are regular or have a form of typical tripods; they are present only in the wall of outermost tubes of the cormus. This species cannot be distinguished by the morphological criteria from *C. cerebrum* (Haeckel 1872).

This species is sciaphyle, found under larger stones in shallow waters.

Clathrina aspina sp. n. The cormus and the organization of the skeleton of this species are identical to that observed in *C. brasiliensis.* The only morphological difference that we identified is the absence of spines on the apical actine of tetractines (Fig. 4).

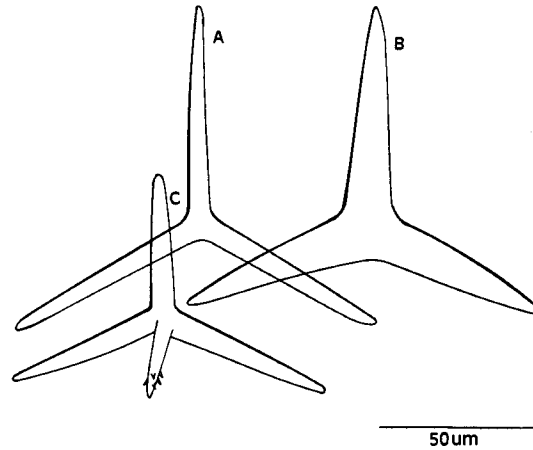

FIG. 3. *CLATHRINA BRASILIENSIS* (Solé-Cava *et al.*, 1991). (A) Small triactine. (B) Large triactine in form of a tripod. (C) Tetractine with spined apical actine.

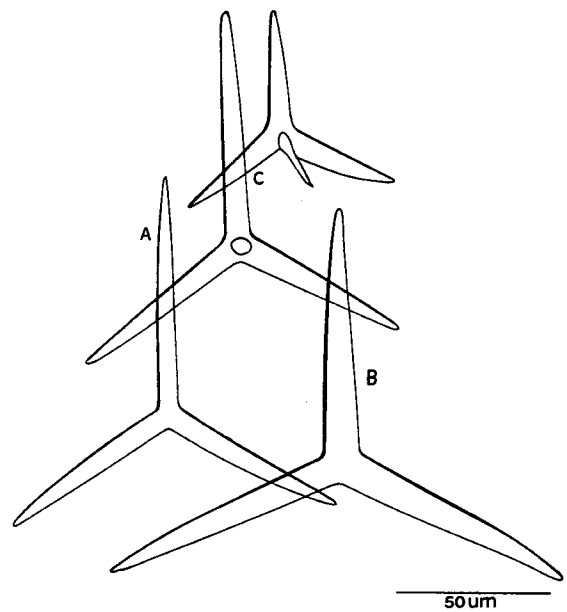

FIG. 4. *CLATHRINA ASPINA* sp. n. (A) Small triactine. (B) Large triactine. (C) Tetractines.

This species is sciaphyle, found both in shallow and warm waters, and in underwater caves of the open coast of Arraial do Cabo region (Gruta Azul), which is exposed to upwelling of deep and cold waters.

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