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Genetic and cytological divergence between colour morphs of the Mediterranean sponge *Oscarella lobularis* Schmidt (Porifera, Demospongiae, Oscarellidae)

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The demosponge *Oscarella lobularis* (Schmidt, 1862) has been reported from various localities throughout the world and is abundant in many areas. It is considered to be morphologically very variable, and morphs of different colour or texture can be found living sympatrically. Samples of a soft violet morph and of cartilaginous blue, yellow and green morphs were collected from the Mediterranean Sea, near Marseilles. The genetic divergence of the four morphs was estimated by enzyme electrophoresis, and cytological differences were studied using light and transmission electron microscopy. The cartilaginous morphs were genetically practically identical, but the soft violet morph was diagnostically different from the sympatric samples of the blue and green morphs and from the allopatric yellow morph at most of the genetic loci studied (genetic identity, $I \approx 0{\cdot}25$). The high genetic divergence was associated with consistent differences in morphology and cytology between the violet morph and the other colour morphs. This is considered to justify a specific distinction. It is concluded that the name *Oscarella lobularis* (Schmidt, 1862) should be applied to the soft violet morph only, whilst the other, cartilaginous colour morphs are referred to *Oscarella tuberculata* (Schmidt, 1868).

KEYWORDS: Genetics, cytology, sponge, *Oscarella*, Mediterranean.

Introduction

Sponges (Porifera) are almost the only major phylum where there is still no general systematic consensus even at the level of orders (Lévi, 1979). Ability to discriminate biologically meaningful taxa depends very much upon the number of available characters. In sponges, largely as a result of the considerable morphological plasticity found in most species, taxonomic work has principally relied upon skeletal features (i.e. spicules), which are considered to be less variable. This may have resulted in a decrease in the power of systematic resolution for the group, and therefore additional characters are needed (Bergquist, 1979). Earlier genetic studies on Porifera have shown that marine sponges can be highly variable (Balakirev and Manchenko, 1985; Solé-Cava

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and Thorpe, 1990) and demonstrated the unreliability of conventional criteria for the identification of sponge species (Solé-Cava and Thorpe, 1986; Solé-Cava *et al.* 1991a, b). It also appears that geographically very widespread sponge species should be regarded with caution. There is a suspicion that the allegedly circumglobal distribution of various sponges may merely be a consequence of the paucity of consistent and easily recognizable taxonomic characters (Solé-Cava *et al.*, 1991b).

Such taxonomic problems are even more apparent when dealing with sponges without a skeleton, since skeletal characters form the conventional basis for the identification of species in most sponges. One such species is the homoscleromorph sponge *Oscarella lobularis* (Schmidt 1862). This species was described from the Mediterranean Sea and since then has been claimed to occur in shallow-subtidal habitats almost throughout the world (e.g. Red Sea, Lévi, 1958; Irish Sea, Bruce *et al.*, 1963; Antarctic, Koltun, 1964; Adriatic Sea, Labate and Arena, 1965; Rützler, 1965; Far Eastern Arctic, Koltun, 1966). However, its genetic homogeneity must be open to doubt, especially since sponge larvae are generally very short-lived (Borojevic, 1970; Sara and Vacelet, 1973) and thus are unlikely to be dispersed widely. Sympatric and distinct, but supposedly conspecific, morphs of *O. lobularis* are found throughout the distribution of the species. In the present study we have examined the biochemical genetic divergence and the cytological differences between sympatric and allopatric specimens of various morphs of *O. lobularis* from the western Mediterranean Sea.

The electrophoresis of enzyme molecules is a well-established technique for the study of the genetic structure of natural populations in a wide range of animals and plants (reviewed by e.g. Thorpe, 1982; Richardson *et al.*, 1986; Nei, 1987), including many marine species (reviewed by Ward, 1989). For taxonomic studies the uses of electrophoretic data are well known (Avise, 1974; Solé-Cava and Thorpe, 1987) and the method has been extensively used to investigate cryptic speciation and interspecific relationships within many animal groups. There are many such studies on various marine invertebrates, but relatively few published on sponges (e.g. Solé-Cava and Thorpe, 1986; Sara *et al.*, 1989; Stoddart, 1989; Solé-Cava *et al.*, 1991a, b). The present work would appear to be the first use of biochemical genetic methods to study the systematics of any homoscleromorph sponge species.

Materials and methods

The sponges were collected by SCUBA diving in May 1990, from the Mediterranean Sea from two localities near Marseilles at between 6 and 12 m depth. The specimens collected could be separated according to the consistency of the body into 'soft' and 'cartilaginous' morphs. The soft specimens were violet in colour, but the cartilaginous specimens displayed some colour polymorphism. Blue and green cartilaginous specimens were collected sympatrically with the soft specimens at La Vesse (a few km west of Marseilles). Yellow cartilaginous specimens were collected from a few km east of Marseilles at the entrance of the Jarre cave (Riou). The distance between the two localities was about 10 km. Animals were taken to the laboratory in sea water in an insulated container and then immediately on arrival were frozen rapidly by immersion in liquid nitrogen. Samples were subsequently stored at $-20°C$ for 1 week before being packed in dry ice and transported to the Port Erin Marine Laboratory (Isle of Man) where the experimental work was carried out. Pieces of each specimen were preserved for cytological studies so that if any cryptic species were found by electrophoresis these could be subsequently identified.

Samples were homogenized in distilled water, and analysed by horizontal electrophoresis in 12·5% starch gels as previously described for sponges (Solé-Cava and Thorpe, 1986). The buffer used was continuous Tris-citrate system, pH 8·0 (Ward and Beardmore, 1977). The staining of the gels followed standard procedures (Harris and Hopkinson, 1978). Samples from each morph or population were run side-by-side on the same gel, to allow the correct identification of alleles. Eleven enzyme systems could be reliably scored in the samples (adenylate kinase, *Ak*—EC 2.7.4.3; alkaline phosphatase, *Alp*—EC 3.1.3.2; α-esterases, *Est*—EC 3.1.1.1; leucine aminopeptidase, *Lap*—EC 3.4.1.1; malate dehydrogenase, *Mdh*—EC 1.1.1.37; malic enzyme, *Me*—EC 1.1.1.40; mannose 6-phosphate isomerase, *Mpi*—EC 5.3.1.8; peptidases, *Pep*—EC 3.4.11.1; phosphoglucose isomerase, *Pgi*—EC 5.3.1.9; phosphoglucomutase, *Pgm*—EC 2.7.5.1 and superoxide dismutase, *Sod*—1.15.1.1).

A strong zone of polymorphic nonspecific enzyme activity, which transformed MTT to formazan, even in the absence of NAD or NADP, was observed close to the front of each gel. We have previously noticed this zone during electrophoresis of various species of ceractinomorph and tetractinomorph demosponges (e.g. *Aplysina fistularis* Pallas, *Chondrilla nucula* Schmidt, *Chondrosia reniformis* Nardo, *Pachymatisma johnstone* (Bowerbank), *Petrosia ficiformis* (Poiret); authors' unpublished results) and similar results have been found in several Australian ceractinomorph sponges (Stoddart, 1989), but the zone is apparently absent in three species of the tetractinomorph genus *Suberites* (Solé-Cava and Thorpe, 1986). This enzymatic activity may be linked to the presence of bacteria in the sponge (Stoddart, 1989). Because of doubts as to its origin, the bands of this nonspecific 'enzyme' were excluded from this study.

Genotype frequency data were analysed by the BIOSYS-1 program (Swafford and Selander, 1981). To compensate for small sample sizes, Nei's unbiased genetic identity was used in the comparison of populations (Nei, 1978), and Fisher's exact probabilities were used to test for fits to Hardy-Weinberg equilibrium (Swofford and Selander, 1981). Differences in allele frequencies between morphotypes were tested binomially.

For cytological work fixation was by standard methods (Boury-Esnault *et al.*, 1984): glutaraldehyde 2·5% in a mixture of 0·4 M cacodylate buffer and seawater (4 vol.: 5 vol.; 1120 mOsm) and post-fixation in 2% osmium tetroxide in sea water. For light and electron microscopy the specimens were embedded in Araldite. Semi-thin sections were stained with toluidine blue. Thin sections, stained with uranyl acetate and lead citrate, were observed with a transmission electron microscope (Philips EM 300) at the Unité de Recherches Neurobiologiques de l'INSERM, Marseilles.

Results

Enzyme electrophoresis

Allele frequencies for each morph of *Oscarella lobularis* are shown in Table 1. Of the 12 loci typed only two (malic enzyme, *Me*; phosphoglucomutase, *Pgm*) were monomorphic and identical over all the samples used. The leucine aminopeptidase (*Lap*) locus showed no significant differences between any of the samples, although the green and yellow morphs showed low levels of polymorphism. At a further four loci (adenylate kinase, *Ak*; alkaline phosphatase, *Alp*; esterase, *Est*; superoxide dismutase, *Sod*) no genetic variation was found within any colour morph, but the blue, green and yellow morphs were fixed for the same allele, whilst the samples of the violet morph were homozygous for a different allele. At three more loci (malate dehydrogenase, *Mdh*;

Table 1. Allele frequencies at the enzyme loci analysed in *Oscarella* c.f. *lobularis* morphotypes.

Locus	Allele	Blue	Green	Yellow	Violet
Ak	1	–	–	–	1·00
	2	1·00	1·00	1·00	–
	n	6	24	12	10
Alp	1	–	–	–	1·00
	2	1·00	1·00	1·00	–
	n	8	24	12	10
Est	1	–	–	–	1·00
	2	1·00	1·00	1·00	–
	n	6	24	12	10
Lap	1	–	0·04	0·08	–
	2	1·00	0·88	0·92	1·00
	3	–	0·08	–	–
	n	8	24	12	10
Mdh	1	–	–	–	1·00
	2	0·25	0·04	–	–
	3	0·63	0·92	1·00	–
	4	0·12	0·04	–	–
	n	8	24	12	10
Me	1	1·00	1·00	1·00	1·00
	n	6	24	12	10
Mpi	1	–	–	–	1·00
	2	0·88	1·00	1·00	–
	3	0·12	–	–	–
	n	8	24	12	10
Pep-1	1	–	–	–	0·90
	2	0·75	1·00	1·00	0·10
	3	0·25	–	–	–
	n	4	24	12	10
Pep-2	1	–	–	–	0·90
	2	–	0·08	0·08	0·10
	3	0·90	0·92	0·92	–
	4	0·10	–	–	–
	n	10	24	12	10
Pgi	1	–	–	–	1·00
	2	1·00	0·25	0·50	–
	3	–	0·75	0·50	–
	n	6	4	2	10
Pgm	1	1·00	1·00	1·00	1·00
	n	8	24	12	10
Sod	1	1·00	1·00	1·00	–
	2	–	–	–	1·000
	n	8	24	12	10
He		0·13	0·08	0·10	0·05
Ho		0·12	0·08	0·10	0·05

n = number of gene copies analysed for each locus in each population. *He* = Expected mean heterozygosity per locus (Hardy-Weinberg); *Ho* = observed mean heterozygosity per locus.

mannose phosphate isomerase, *Mpi*; phosphoglucose isomerase, *Pgi*) the violet morph was fixed for an allele common to none of the other colour morphs and for both of the two peptidase (*Pep*) loci allele frequencies of the violet morph differed significantly (binomial test; $p < 0{\cdot}05$) from those of the other morphs. No locus showed any significant ($p < 0{\cdot}05$) genetic variation between samples of any of the blue, green and yellow morphs.

Data summarizing levels of genetic variation within the population of each colour morph are also given in Table 1. No locus showed significant deviations (Fisher's exact test; $p > 0{\cdot}05$) from Hardy-Weinberg expectations for any of the four morphs sampled.

Cytology

Two distinct cytological types were observed within the morphs of *Oscarella lobularis*.

(*a*) *Soft-bodied violet specimens.* Morphology—*in vivo* it can be observed that the colour is restricted to the upper part of the lobes. Pigment is contained within the choanocytes and is in the form of granules about 1 μm in diameter. The consistency is very soft and the surface smooth with lobes up to 1 cm diameter. The inhalant ostia are scattered regularly on the surface and the lobes terminate in oscula. The choanocyte chambers, easily visible *in vivo* with a binocular microscope, have a diameter of about 95 μm.

General organization (Figs 1 and 2)—Semi-thin sections show large choanocyte chambers that open into exhalant canals lined by flagellated endopinacocytes. The mesohyl appears similar in both the ectosome and choanosome. Two cell types, one with large vacuoles and the other ovoid and without the large vacuoles, were observed, scattered around the mesohyl. Bacteria (stained violet by the toluidine blue) were also clearly distinguishable in the sections.

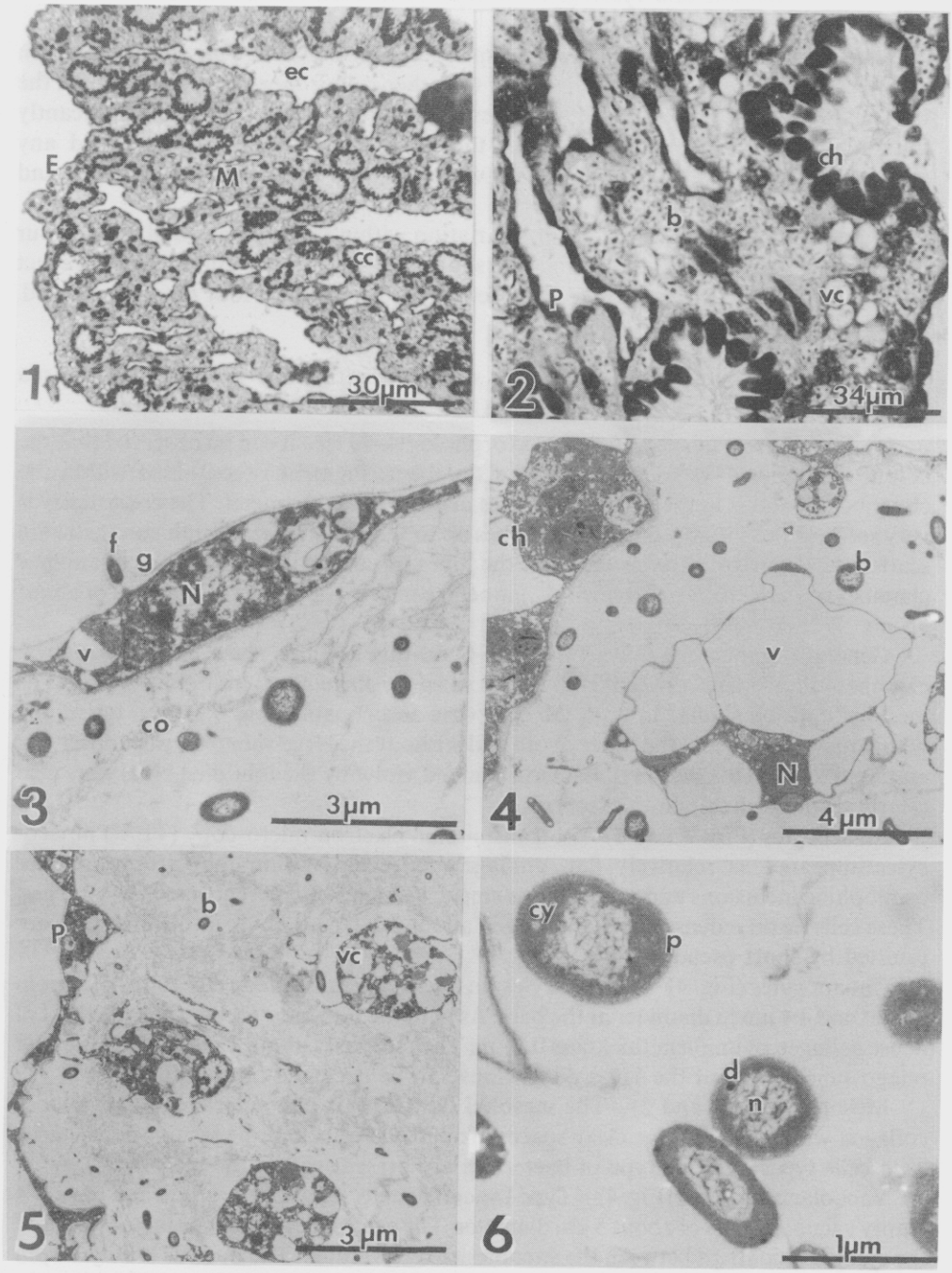
Pinacocytes (Figs 3 and 5)—In transmission electron microscopy (TEM) pinacocytes appeared as relatively flat, uniflagellated cells. Within the cytoplasm were osmiophilic inclusions and more or less empty vacuoles of different sizes up to 1·2 μm. These cells lie on a dense sheet of collagen about 0·2–0·5 μm thick into which they are inserted by short pseudopods.

Choanocytes (Fig. 4)—Choanocytes (in TEM) are truncated cells about 4·2 μm in height and 4·4 μm in diameter at the base. As with the pinacocytes, they lie on a sheet of dense collagen of uniform thickness 0·2 μm. The nucleus is about 1 μm in diameter and phagosomes of about the same dimensions can be observed in the cytoplasm.

Mesohyl (Figs 4 and 5)—The mesohyl (in TEM) is characterized by very loose collagen which leaves large clear spaces around the cells and the symbiotic bacteria. Two cells types and one type of bacterium are present within the mesohyl.

Vacuolar cells type I (Fig. 4)—Type I vacuolar cells are about 9 × 6 μm and have 1–4 empty vacuoles, each of about 5 μm diameter. The external cytoplasmic membrane and the cytoplasmic strips between the vacuoles are convoluted. The nucleus is only 1·3 μm in diameter and is located in a zone where the cytoplasm is a little more abundant. In this region mitochondria of about 0·4 μm diameter and fibrillar inclusion can be observed.

Vacuolar cells type II (Fig. 5)—The vacuolar cells type II are ovoid (4·6 × 5·9 μm) with 1 or 2 short thin pseudopods. The cytoplasm appears similar to that of the pinacocytes and contains many small, not completely empty, vacuoles (maximum 1·6 μm in diameter) and a few osmiophilic inclusions. Cells are surrounded by a zone without collagen.



FIGS 1–6. (1) Semi-thin section of a soft-violet specimen of *Oscarella*. (2) Detail of a semi-thin section of a soft-violet specimen of *Oscarella*. (3) Flagellated endopinacocytes on a thin sheet of collagen in TEM. (4) View of a vacuolar cell type I in TEM. Note the large vacuoles. (5) View of the mesohyl in TEM. It is characterized by loose collagen and clear spaces around cells and bacteria. (6) View in TEM of the symbiotic bacteria. b, bacteria; c, cytoplasm; cc, choanocyte chamber; ch, choanocytes; co, collagen; cy, cytoplasm d, dark granule; E, ectosome; ec, exhalant canal; f, flagellum; g, glycocalyx; M, mesohyl; n, nuclear zone; N, nucleus; P, flagellated pinacocytes (Fig. 2) and pinacocytes (Fig. 5); v, vacuoles; vc, vacuolar cells (Fig. 2) and vacuolar cell type II (Fig. 5).

Bacteria (Fig. 6)—Bacteria are abundant in all the regions and are rod-like about 1–1·3 μm in length and 0·5 μm in diameter. The nuclear zone is clear and contains a network of filaments of about 10 nm width. The cytoplasm is partially electron-opaque and often contains dark granules 20–70 nm in diameter. Bacteria are surrounded by a cytoplasmic membrane and an external zone, about 60 nm thick, intepreted as periplasm. Periplasm is clearer than the cytoplasm and often shows a radial arrangement.

(*b*) *Cartilaginous blue, green and yellow specimens.* Morphology—The various colours of the specimens appeared to depend upon the amount of light incident upon the areas from which they were collected. Yellow specimens were found in low light intensity areas like in the entrances to underwater caves or below overhanging rocks. Blue or green specimens were always in positions more exposed to light. Colour seems to be due to pigments which, as in the violet specimens, are included in the choanocytes. The consistency of the body could be described as semi-cartilaginous to cartilaginous. Lobes are up to 0·5 cm in diameter and the surface, as observed *in vivo* under a binocular microscope, is wrinkled or crenellated. The inhalant ostia are irregularly scattered in the valleys of the crenellations, but the choanocyte chambers, which are about 60 μm in diameter, are located on the ridges. Oscula are located at the tips of the lobes.

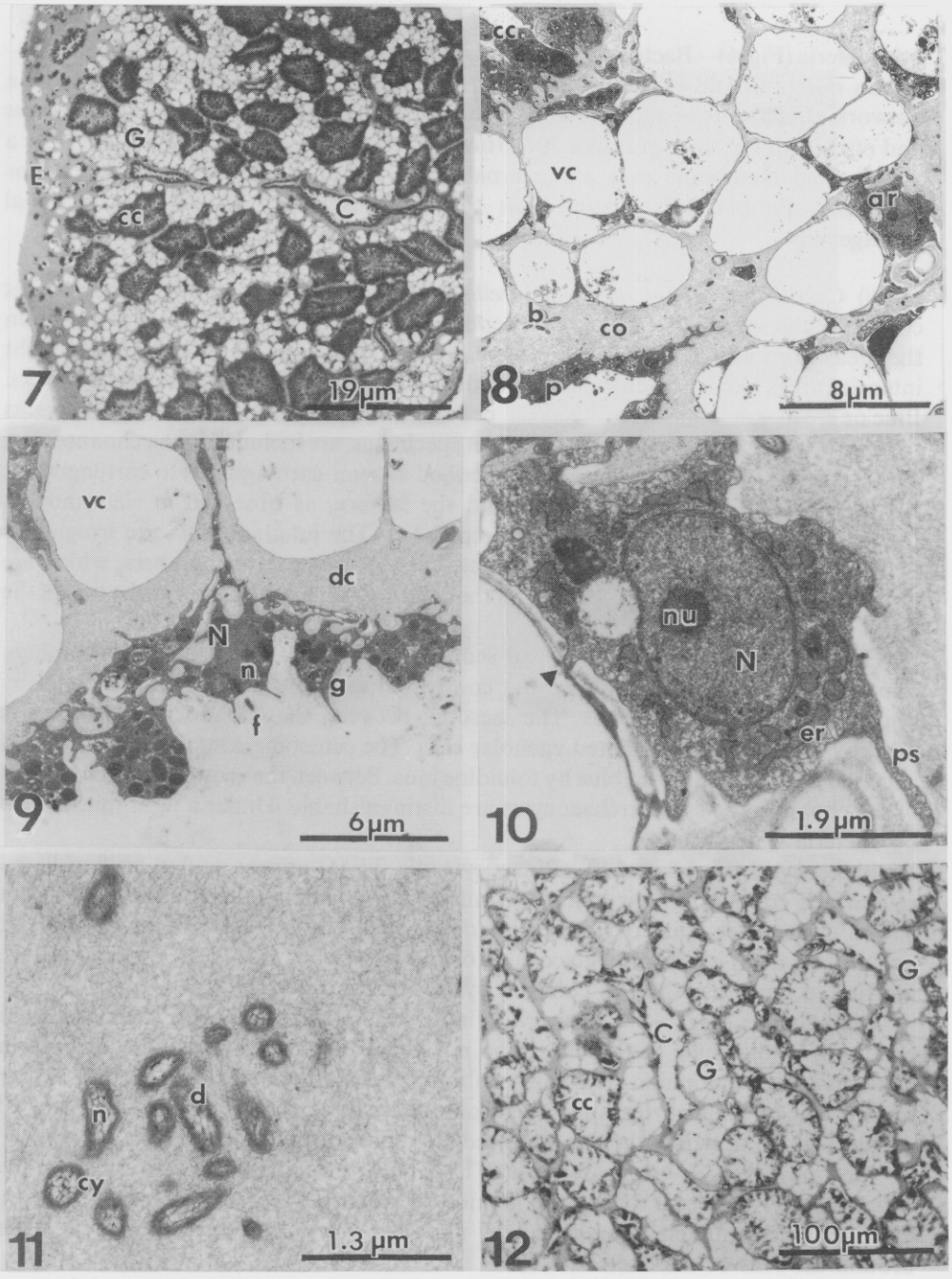
General organization (Fig. 7)—In semi-thin sections the choanocyte chambers appear contracted and open into the contracted exhalant canal which is lined by uniflagellated endopinacocytes. The mesohyl between the choanocyte chambers is filled by groups of large inflated vacuolar cells. The ectosome contains a few isolated vacuolar cells and is stained blue by toluidine blue. Between the groups of vacuolar cells of the choanosome some archaeocytes are distinguishable. Under a light microscope no bacteria were visible.

Pinacocytes (Figs 8 and 9)—Pinacocytes (in TEM) appear as flat, uniflagellated cells which contain in their cytoplasm numerous osmiophilic inclusions each about 0·6 μm in diameter. Pinacocytes lie on a dense collagen fibril mat about 0·2 μm thick and outside them is a layer of glycocalyx of about the same thickness. Pinacocytes are inserted in the dense sheet of collagen by short pseudopods.

Choanocytes (Fig. 8)—Choanocytes (in TEM) are truncated cells 5·5 μm in height and 3 μm in diameter at the base. The nucleus is 1·4–2 μm across. The cytoplasm contains a few osmiophilic inclusions of about 0·6 μm in diameter, similar to the larger ones found in pinacocytes and phagosomes. Choanocytes lie on a dense layer of collagen fibrils in which they are inserted by ramified pseudopods.

Mesophyl (Fig. 8)—Appearance (in TEM) of the mesohyl of the ectosome and the choanosome is different due to the greater abundance of the vacuolar cells (Fig. 8). Collagen is distributed homogeneously and there are no spaces without collagen around the cells or the bacteria.

Vacuolar cells (Fig. 8)—Vacuolar cells (in TEM) are about 10 × 7 μm and the most abundant cells of the mesohyl. In the choanosome they are grouped and so numerous that the mesohyl seems to be full of holes (Fig. 10). There are about 1–4 spherical vacuoles (each about 6·5 μm–8·1 μm) per cell, and these fill the cell so completely that the cytoplasm is reduced to thin strips only about 7 nm thick. The nucleus, about 2 μm in diameter, is compressed between the vacuoles. Fibrillar inclusions are also visible. The overall appearance of these cells is turgescent and the cytoplasmic membrane and the strips between the vacuoles appear very stretched. The turgidity of these cells is



FIGS 7–12. (7) Semi-thin section of a semicartilaginous blue specimen of *Oscarella*. (8) General view in TEM of mesohyl, choanocyte chamber and pinacoderm. (9) View of a flagellated endopinacocyte of an exhalent canal with characteristic stumpy microvilli on the upper surface and pseudopods penetrating the collagen network at the base. Note the glycocalyx on the upper surface. (10) View in TEM of an archaeocyte. The arrowhead shows connection between a vacuolar cell and archaeocyte. (11) View in TEM of the symbiotic bacteria embedded in the dense collagen. (12) Semi-thin section of the specimen type *Oscarella tuberculata* (Schmidt, 1868). ar, archaeocyte; b, bacteria; cc, choanocyte chamber; co, collagen; cy, cytoplasm; C, canal; d, dark granule; dc, dense collagen; ec, exhalant canal; er, ergastoplasm; E, ectosome; f, flagellum; g, glycocalyx; G, group of vacuolar cells; n, nucleolus; nu, nucleolus; N, nucleus; p, flagellated pinacocytes; ps, pseudopod; vc, vacuolar cells.

perhaps responsible for the contracted appearance of the choanocyte chambers and canals, and may result from an artifact of fixation.

Archaeocytes (Figs 8 and 10)—Archaeocytes are located between the vacuolar cells. They are about 6 μm in length and 3 μm in width with a nucleolated nucleus 2·5 μm in diameter. They have clear relationships with the vacuolar cells (Fig. 10).

Bacteria (Fig. 11)—Bacteria are visible only in TEM and are dispersed in the mesohyl. They are rod-like and relatively few in number, and each is about 0·5 μm in length and 0·25 μm in diameter. They are not surrounded by clear spaces but are completely embedded in the collagen. The nuclear zone is clear and contains a network of 10 nm filaments and is surrounded by dense cytoplasm. The walls of these bacteria appear reduced, and therefore contrast with those of the violet morphs.

Discussion

The major feature of the results is the very high level of genetic differentiation found between the soft-textured violet morph of *Oscarella lobularis* and the three other colour morphs, which were cartilaginous, and an exactly congruent pattern of cytological differentiation. Of these other morphs the green and blue were sympatric with the violet samples whilst the yellow samples were collected about 10 km to the east.

Between sympatric morphs or populations which are conspecific there should be no significant gene frequency difference at any locus since, if conspecific, individuals should be part of a single freely interbreeding population. Any difference in gene frequency between sympatric populations, other than expected sampling errors, indicates the probability that there is not random mixing of genes (panmixis) and, therefore, that the populations are likely to be different biological species. Seven of 12 loci analysed were fixed for different alleles in the soft-violet and the other forms of *Oscarella lobularis*. These constitute diagnostic loci (*sensu* Ayala, 1983) and provide a clear indication of lack of gene flow between populations and hence that these sympatric morphs are reproductively isolated. Therefore it follows that the violet morph cannot be of the same biological species, since it is not freely interbreeding with other sympatric *Oscarella lobularis*.

This conclusion, of course, depends on the assumption that his species can, at least occasionally, reproduce sexually. Fortunately *O. lobularis* is quite well studied and known to be viviparous; the sexually produced larvae are nurtured within the sponge until the final development stages, when they pass through a short ($\sim$ 2 day) free-swimming phase and then settle on rocks (Brien, 1973). Genetic data also provide strong evidence for the existence of sexual reproduction in this species, since no two individuals of either of the colour morphs presented the same genotype over all the polymorphic loci. Furthermore, the genotype frequencies observed in the samples were not inconsistent with those expected for populations in Hardy-Weinberg equilibrium. Those two facts, together with the histological evidence (Brien, 1973) demonstrate, thus, that there is at least some sexual reproduction taking place within these populations. Therefore, the existence of diagnostic genetic differences between them in sympatry is a very strong indication of their reproductive isolation.

There are many papers giving observed levels of genetic identity and divergence between various populations and species over a wide range of plants and animals (reviewed by Thorpe, 1979, 1982, 1983; Nei, 1987). In general allopatric populations of the same species have values of Nei's (1972) genetic identity, I, above about 0·9 and rarely as low as 0·8, whilst identity levels between species within a genus typically range

from about 0·3 to 0·8, and species from different, but confamilial, genera usually give I values below about 0·4. Between congeneric sponge species there are few available published estimates: the values so far obtained are in the range of 0·20–0·85 for sympatric species (Solé-Cava and Thorpe, 1986; Sara *et al.*, 1989; Solé-Cava *et al.*, 1991a), and between 0·13 and 0·29 for allopatric species (Solé-Cava *et al.*, 1991b).

Values of Nei's genetic distance, $D(= -\log_e I)$ may be stochastically related to time of evolutionary divergence, although this relationship is disputed and depends upon the validity of various theoretical assumptions. However, there can be little doubt that isolated populations diverge genetically with time (Nei, 1987; Thorpe, 1989). This putative time-related molecular genetic divergence of populations has come to be known as the 'molecular clock hypothesis' (reviews by Wilson *et al.*, 1977; Thorpe, 1982).

The genetic identity between the samples of the violet colour morph of *Oscarella lobularis* and the other putative conspecific colour morphs studied was extremely low (Table 2). The unbiased genetic identity (Nei, 1978) between the violet morph and each of the other colour morphs was around 0·25. This is within the range usually found between confamilial genera of other organisms (Thorpe, 1979, 1982, 1983; Ayala, 1983; Nei, 1987), but within the lower end of the values observed for congeneric sponge species. The corresponding value of D is around 1·4, and suggests a very long divergence time of around 7 MY even using a conservative calibration factor of $1D = 5$ MY (Nei, 1975, 1987); other workers have estimated $1D$ unit to be as high as 18–20 MY (Yang *et al.*, 1974; Sarich, 1977; Thorpe, 1982). These higher calibrations could be taken to indicate a divergence time as great as about 28 MY. However, it should be borne in mind that no calibration of the evolutionary clock is currently available for sponges, and therefore any estimates of divergence time must be taken as only indicative.

Another important point to consider is the small sample sizes used here. In the presence of diagnostic loci, even very small samples (two to five individuals) can be used to infer reproductive isolation (Nei, 1978; Gorman and Renzi, 1979; Ayala, 1983), as observed here between the cartilaginous and the other morphs. However, in order to detect more subtle differences in gene frequency, larger sample sizes would have been required (Solé-Cava *et al.*, 1985). This means that, although we can safely ascertain the isolation of the cartilaginous morph from the others (with a very low chance of making a type I error), we may be missing important differences between the other colour morphs (i.e. making a type II error—see Sokal and Rohlf (1981) for a discussion of this type of error).

Levels of genetic variability within the various colour morphs of *Oscarella lobularis* are indicated by the estimates of mean observed and expected heterozygosities per locus shown in Table 1. These values fall within the range commonly found in

Table 2. Estimates of Nei's (1972, 1978) genetic identity (above the diagonal) and genetic distance (below the diagonal) between four colour morphs of *Oscarella lobularis*.

	Blue	Green	Yellow	Violet
Blue	–	0·96	0·97	0·26
Green	0·05	–	1·00	0·25
Yellow	0·03	0·00	–	0·25
Violet	1·36	1·41	1·40	–

invertebrates (Nevo, 1978; Nevo *et al.*, 1984), but are lower than those observed in some other sponge species (Solé-Cava and Thorpe, 1989, 1991).

Two cytological patterns occur in the different morphs of *Oscarella lobularis* studied. In the soft-violet specimen the mesohyl has loose fibrils of collagen that leave clear space around cells and symbiotic bacteria. Two types of vacuolar cells are present, one of which shows inclusions which are very similar to those of the pinacocytes. Bacteria, which are visible using light microscopy, show a well-differentiated wall and all appear to be of a single type. *In vivo* the surface is smooth and the choanocyte chambers are about 95 μm in diameter. In the cartilaginous blue, green and yellow specimens the mesohyl has dense fibrils of collagen which surround the cells and the bacteria completely. Only one type of vacuolar cell is present and this forms clusters in the choanosome and is scattered in the ectosome. These cells are quite turgescent and thus contrast with the contracted appearance of the choanocyte chambers and the canals. The cytoplasm of the pinacocytes and choanocytes is similar. Numerous archaeocytes are present between the vacuolar cells of the choanosome. Symbiotic bacteria are also present, but are much smaller than those found in the violet specimens, and are not visible using light microscopy. *In vivo* the surface is wrinkled and the choanocyte chambers are about 60 μm in diameter. These two cytological types were observed not only in the specimens studied in the present work but also in other specimens collected from various other localities (authors' unpublished results). The most important macroscopic feature is not the colour but the consistency of the sponge and the relative diameter of the lobes. It appears that the differences in texture may reflect variation in the relative amounts of collagen in the sponge.

The soft specimens of *Oscarella lobularis* have a colour which varies between violet and blue. Their distribution ranges from about 15 to 35 m depth. They are apparently never observed around caves. The colour of the more cartilaginous specimens ranges from dark blue through blue to green and yellow. Red specimens are also found, although none was used in the present work. The distribution ranges from approximately 5–35 m and specimens, particularly of the yellow form, are often observed at the entrance of caves. The reproductive period is similar for both soft and cartilaginous morphs, but the larvae are white in the soft (violet) morph and pink in the cartilaginous morphs. These characters alone would not be sufficient to distinguish species, but when considered in combination with cytological differences and, particlarly, the very strong genetic evidence of reproductive isolation and genetic divergence, it is clear that there are at least two species of *Oscarella lobularis* in the Mediterranean around Marseilles. However, if, even with only two sampling sites over such a small geographic range (10 km apart), two different species occur within *Oscarella lobularis*, it is likely, given the allegedly cosmopolitan distribution of the species, that further cryptic species will be present in other parts of the world. Thus it is likely that any identification of a sponge which in the past has been assigned to *Oscarella lobularis* should be regarded with care.

A remaining problem is to decide which of the two species which we have distinguished might be that originally described as *Oscarella lobularis* by Schmidt (1862). In the original description, Schmidt (1862) mentioned that *Oscarella lobularis* had a dark violet colour and a very soft consistency. The type locality for the species is Sebenico in the northern Adriatic, but there is no mention of the depth from which it was collected. Based on this information, it appears that our soft, violet specimens correspond to Schmidt's *Oscarella lobularis*. Topsent (1895) mentioned differences of consistency between specimens collected in the Mediterranean Sea and in the English Channel, but gave no taxonomic value to this character. Schmidt (1868) described

another species under the name *Chondrosia tuberculata*, but this species was put in synonymy with *Oscarella lobularis* by Schulze (1877). In his diagnosis Schmidt specifically stated that the consistency of his *Chondrosia tuberculata* was like that of the Chondrosiidae (i.e. cartilaginous), but at the same time he said the anatomy of it was similar to that of *Oscarella lobularis*. The type specimen of *Chondrosia tuberculata* has recently been rediscovered in the Graz collection by Ruth Desqueyroux-Faúndez (Natural History Museum, Geneva). We have had the opportunity to re-examine this specimen and despite prolonged preservation (in alcohol) it was still possible to prepare histological sections. These showed that this specimen clearly corresponds to our cartilaginous *Oscarella* species (Fig. 12).

The type specimen of *Oscarella lobularis* is believed lost. However, our soft specimens correspond well with Schmidt's original description (1862) for the species. We have, therefore, assigned specimens of our soft violet species to *Oscarella lobularis* (Schmidt, 1862), and a neotype specimen has been chosen and deposited at the Museum d'Histoire Naturelle in Paris, under the number LBIM-D-1991-NBE-1. The cartilaginous specimens are referred to *Oscarella tuberculata* (Schmidt, 1868) for which the type specimen is deposited at the Natural History Museum, Geneva.

Once the substantial genetic divergence of *Oscarella tuberculata* from *O. lobularis* has been demonstrated by electrophoresis the morphological, and particularly the cytological, distinctions between the two are obvious, and it is surprising that none of the several recent authors who have examined suitable specimens has noticed the differences. *Oscarella lobularis* and *Oscarella tuberculata* are clearly illustrated (all as *Oscarella lobularis*) by Donadey (1979; plates I1 and I6, respectively), Boury-Esnault *et al.* (1984; plates 6 and 10 respectively) and Gaino *et al.* (1986; plates 23 and 1 respectively). A futher possible complication is the description of *Octavella galangaui* by Tuzet and Paris (1963). This species is allegedly distinguishable from *Oscarella lobularis* by the colour (dark blue) and the absence of flagella on the pinacocytes. As no type specimen is available this species may be open to doubt. Clearly further work is needed on *Oscarella* and related genera using genetic and cytological criteria to compare samples from a wide range of localities.

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