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Genetic Evaluation of the Taxonomic Status of Two Varieties of the Cosmopolitan Ascidian *Botryllus niger* (Ascidiaceae: Botryllidae)

SIMONE ARON*† and ANTONIO SOLE-CAVA†‡

*Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Bloco A, CCS, Cidade Universitaria, 21941-Rio de Janeiro-RJ, Brazil;
†Departamento de Biologia Geral, Instituto de Biologia, Universidade Federal Fluminense, C.P. 100.183, 24000-Niteroi-RJ, Brazil

Key Word Index—*Botryllus*; Ascidiacea; electrophoresis; isozymes; genetics; biochemical systematics.

Abstract—Populations of two varieties of *Botryllus niger* from three points along the southeastern coast of Brazil were compared genetically, in order to evaluate their taxonomic status. Variety *giganteum*, (two colour morphs, orange and grey) and var. *niger* were analysed for 25 enzyme systems by starch gel electrophoresis, of which nine gave reproducible results. The levels of gene variation observed were high in all populations of var. *niger* analysed ($H = 0.239$), but only moderate ($H = 0.112$) in the populations of var. *giganteum*. This may reflect observed differences in the population sizes of the two varieties. The gene identity obtained for pairwise comparisons between populations or colour morphs of the same variety was at the lower end of the range normally associated with conspecific populations ($I = 0.76–0.98$). In contrast, a high level of gene differentiation was observed between the two varieties ($I = 0.404$), with five out of 12 loci being diagnostic of each one. Since the two supposed varieties live sympatrically, this indicates that they are reproductively isolated, and therefore deserve specific status. The varietal names are thus raised here to specific level, as *Botryllus niger* and *Botryllus giganteum*.

Introduction

The intertidal colonial ascidian *Botryllus niger* (Herdman, 1866) is considered to be cosmopolitan, having been cited in the Atlantic, the Indian and the Pacific oceans, as well as the Mediterranean and the Red Sea [1–5]. This cosmopolitism could partially be the result of a broad definition for the species, which has been described under more than 20 different varietal names world wide [6, 7]. One such variety, *B. niger* var. *giganteum* (Pérès, 1949), has been described for Senegal [8–10]. Two colour morphs of that variety were also recently found in Brazil [11]. As the name indicates, this variety differs from the typical *B. niger* in zooid size and associated numbers of anatomical structures, such as the number of rows of stigmata and glandular folds. It has thus been suggested [Monniot (1990) personal communication] that var. *giganteum* might actually belong to a different biological species from *B. niger*. Both *B. niger* and its putative variety *giganteum* are very common near Rio de Janeiro, where they occur sympatrically.

The aim of this work was to evaluate the taxonomic status of *B. niger* var. *giganteum*. The approach used was the genetic interpretation of isozyme patterns, a technique widely used in systematics [12–14], and which has become well established for the analysis of systematic problems of marine benthic organisms [15–17]. Isozymes have been described for ascidians [18–20], and have been proved to behave in a Mendelian fashion [21]. However, this is the first time that isozymes have been used as an aid for the systematics of ascidians.

‡Present address: Departamento de Genetica, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Bloco A, CCS, Cidade Universitaria, 21941-Rio de Janeiro-RJ, Brazil.

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Materials and Methods

Specimens of *Botryllus niger* were collected manually in the intertidal zone in three points on the south-eastern coast of Brazil: Santos (23°57′S; 48°18′W), Rio de Janeiro (22°57′S; 43°12′W) and Vitoria (20°19′S; 40°19′W). Specimens of *B. giganteum* were collected only in Rio de Janeiro and Vitoria. The animals were recognized in the field by their external morphology and colour, and some of them were anaesthetized with menthol, fixed in 5% formalin and analysed under a binocular microscope in the laboratory, for morphological analyses and comparison with the original description of the species (Table 4). After collection the animals were put in ice and transported to the laboratory, where they were kept at −30°C for not longer than one month. The ascidians were cut into small pieces, mixed 1:1 with distilled water and ground with a glass rod in a refrigerated acrylic plate. The homogenates thus obtained were analysed by means of horizontal, 13% starch gel electrophoresis, using Tris–Citrate/Borate, pH 8.0 [22] as discontinuous buffer system. After electrophoresis the gels were sliced and developed using standard procedures [22, 23] for 25 enzyme systems, of which only nine produced interpretable results. Other buffer systems (Tris–Citric, pH 8.0 [24], Tris–EDTA–Maleate, pH 7.4 [14]) were tried, without any improvement in resolution or activity of the enzymes assayed. After staining, the gels were interpreted and fixed in 7% acetic acid for future reference.

In order to avoid problems associated with the use of small sample sizes, we employed Fisher's exact test for fits to Hardy–Weinberg equilibrium [25] and used unbiased estimates for heterozygosity and genetic identity [26]. Voucher specimens of the studied species are deposited in the Ascidiacea collection of the Zoology department from the Federal University of Rio de Janeiro, under catalogue numbers 643-C, 388-C and 457-C for *B. niger* from Santos, Urca and Vitoria, respectively; 296-C and 447-C for *B. giganteum* (orange colour morph) from Urca and Vitoria, and 293-C for *B. giganteum* (grey colour) from Urca. This was done in order to allow the correct specific assignment of the populations studied if the systematics of the genus is changed in the future.

Results

The populations of *B. niger* and *B. niger* var. *giganteum* studied showed marked differences at five out of the 12 loci analysed (Table 1). The genotypic proportions observed for all polymorphic loci were not significantly different ($P > 0.05$) from those expected populations in Hardy–Weinberg equilibrium. The levels of gene variation observed for these ascidian populations were high (Table 2), in relation to the levels normally found in animal populations [27, 28]. However, they were comparable to those observed for marine sessile invertebrates [29].

The overall levels of unbiased gene identity [26] between populations of the same variety fell within the range normally associated with conspecific populations [30–32]. Conversely, the levels of gene identity observed between different putative varieties were much lower, at the lower end of the range of identity values normally found for comparisons between congeneric species (Table 3, Fig. 1).

Discussion

The presence of a locus fixed for different alleles in sympatric populations is evidence strong enough to warrant their assignment to different biological species [33]. Four such diagnostic loci were observed between the populations of *B. niger* and its putative variety *giganteum*. One could argue that, because of the relatively small number of individuals analysed, the observed differences could be simply due to chance. It has been demonstrated, however, that for biochemical systematic studies, numbers as small as five can be used to obtain reasonably accurate (95%) estimates of genetic identity [34]. Furthermore, the maximum probability of observing, by chance alone, L loci fixed for different alleles in two samples can be calculated, as $(((1/2n1)^2)^L)^{n2}$, where $n1$ and $n2$ are the number of individuals in populations 1 and 2, respectively [35, 36]. For the samples of each species collected at Urca Beach (where they occur sympatrically) this gives, using the smallest sample sizes for each species, $P < (((1/26)^2)^4)^6$, or $P < 1.3 \times 10^{-68}$. We can safely conclude, therefore, that the populations studied clearly belong to different species. These two species can be easily distinguished morphologically (Table 4), and since the description of "var" *giganteum* is complete, the name is available, and the type specimens still exist, we decided to raise *giganteum* to specific level, as *Botryllus giganteum* [8]. The level of gene identity

TABLE 1. GENE FREQUENCIES FOR THE POPULATIONS ANALYSED

Locus		Population					
		OGU	GGU	OGV	NU	NS	NV
Cat	A	1.000	1.000	1.000	1.000	1.000	1.000
	n	10	10	4	13	8	14
Est-1	A	0.000	0.000	0.000	1.000	1.000	1.000
	B	1.000	1.000	1.000	0.000	0.000	0.000
	n	23	10	4	22	8	14
Est-2	A	0.367	0.417	0.000	0.528	0.444	0.188
	B	0.333	0.375	0.167	0.417	0.444	0.656
	C	0.300	0.208	0.833	0.056	0.111	0.156
	n	15	12	3	18	9	16
Hk	A	0.000	0.000	0.000	1.000	1.000	1.000
	B	1.000	1.000	1.000	0.000	0.000	0.000
	n	8	6	4	13	8	14
Lap	A	0.000	0.000		0.125		
	B	0.100	0.071		0.875		
	C	0.900	0.929	ND	0.000	ND	ND
	D	0.000	0.000		0.000		
	E	0.000	0.000		0.000		
	F	0.000	0.000		0.000		
	n	10	7		8		
Mdh	A	0.000	0.000	0.000	0.109	0.188	0.214
	B	0.056	0.029	0.000	0.891	0.813	0.786
	C	0.944	0.971	1.000	0.000	0.000	0.000
	n	18	17	4	23	8	14
Pep-1	A	1.000	1.000	1.000	1.000	1.000	1.000
	n	4	2	4	5	8	14
Pep-2	A	0.000	0.000	0.000	0.324	0.500	0.393
	B	0.050	0.100	0.000	0.676	0.500	0.607
	C	0.900	0.900	1.000	0.000	0.000	0.000
	D	0.050	0.000	0.000	0.000	0.000	0.000
	n	10	10	4	17	8	14
Pgi	A	0.050	0.000		0.042		
	B	0.750	0.800		0.542		
	C	0.000	0.000		0.208		
	D	0.200	0.200	ND	0.208		ND
	E	0.000	0.000		0.000		
	F	0.000	0.000		0.000		
	G	0.000	0.000		0.000		
	n	10	10		12		
Pgm	A	1.000	1.000		0.000		
	B	0.000	0.000		0.250		
	C	0.000	0.000		0.500		
	D	0.000	0.000	ND	0.250	ND	ND
	E	0.000	0.000		0.000		
	F	0.000	0.000		0.000		
	G	0.000	0.000		0.000		
	n	10	10		12		
Sod-1	A	1.000	1.000	1.000	1.000	1.000	1.000
	n	4	2	4	13	8	14
Sod-2	A	0.000	0.000	0.000	1.000	1.000	1.000
	B	1.000	1.000	1.000	0.000	0.000	0.000
	n	10	10	4	13	8	14

OGU and GGU are the orange and grey colour morphs of *B. giganteum* from Urca; OGV is the orange colour morph of *B. giganteum* from Vitoria; NU, NS and NV are the populations of *B. niger* from Urca, Santos and Vitoria, respectively. Loci for which no results could be obtained are marked ND.

TABLE 2. LEVELS OF GENE POLYMORPHISM (AND STANDARD ERRORS) FOR THE POPULATIONS ANALYSED

Population	Polymorphism (%)	Mean heterozygosity	
		Observed	Expected
OGU	41.7	0.112 (0.049)	0.133 (0.063)
GGU	41.7	0.085 (0.038)	0.117 (0.059)
OGV	11.1	0.037 (0.028)	0.037 (0.028)
NU	50.0	0.239 (0.083)	0.228 (0.079)
NS	33.3	0.204 (0.074)	0.187 (0.068)
NV	33.3	0.164 (0.059)	0.152 (0.051)

Population abbreviations as in Table 1. Because of the small sample size, the unbiased estimate [21] of the expected heterozygosities was employed.

TABLE 3. LEVELS OF GENETIC IDENTITY BETWEEN THE POPULATIONS STUDIED

Population	OGU	GGU	OGV	NU	NS	NV
OGU	*****	1.000	0.740	0.404	0.330	0.324
GGU	0.000	*****	0.729	0.406	0.330	0.324
OGV	0.301	0.316	*****	0.400	0.385	0.388
NU	0.906	0.901	0.916	*****	0.778	0.770
NS	1.109	1.109	0.954	0.251	*****	0.955
NV	1.127	1.127	0.947	0.261	0.046	*****

Above diagonal, unbiased gene identity [21]; below diagonal, genetic distance [21]. Abbreviations as in Table 1.

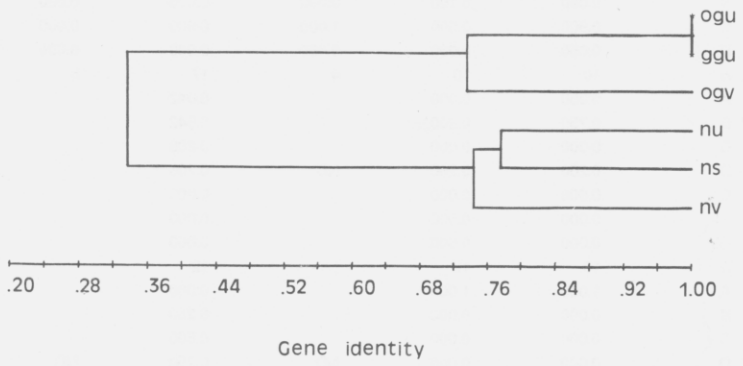

FIG. 1. UPGMA DENDROGRAM OF MEAN GENE IDENTITY BETWEEN THE STUDIED SPECIES. Abbreviations as in Table 1.

TABLE 4. MORPHOLOGICAL CHARACTERISTICS OF THE POPULATIONS ANALYSED

Species/Site	NU	NV	NS	OGU	OGV	GGU	*B. niger*	*B. giganteum*
Rows of stigmata	7–9	8–9	8–9	18–19	18	13–15	10–12	18–20
Testes	6–8	7–8	7–8	16	8–12	8–12	5–06	12–15
Gastric folds	9	9	9	11	11	11	—	11–12
Size of the zooid	9–15	9–15	11–14	34–51	26–35	28–42	15	48

The original descriptions of *B. niger* (Herman, 1886) and *B. niger* var. *giganteum* [8] are also presented, for comparison. Sizes of zooids are given in 1/10 millimetres. Abbreviations are as in Table 1.

found between these two species ($I = 0.32$) confirms further their status as distinct species (Fig. 1).

The fact that *B. giganteum* and *B. niger* are different species raises doubts about the conspecificity of the other varieties of *B. niger*. If, after subsequent study of this species, it was split further into other species, the cosmopolitism of *B. niger* might be considered to be an artifact of an over-conservative systematics. However, any number of simple studies like this one, on sympatric pairs of populations, will not be sufficient to settle the question, since in each of these studies the species most similar to the original description of *B. niger* will retain the original name, keeping its geographical distribution as inflated as before. We suggest that a comparative biochemical systematic study on the typical *B. niger* from many different geographical places be undertaken, in order to truly evaluate its presumed cosmopolitism. A similar work, on calcareous sponges, has suggested that cosmopolitism, for many marine sessile invertebrate species, may be less widespread than generally thought [37].

High levels of gene variation have been related both to selective pressures [28] and to population stochastic phenomena [38]. The ecology of the ascidians studied here is not sufficiently known to draw any conclusions as to any significant differences they might have which would explain the observed differences in heterozygosity. However, since they live sympatrically, without any obvious niche differentiation, we think that it might be fair to assume that no major differences exist in the way the environment is affecting them. On the other hand, *B. niger* seems to have a much larger population size than *B. giganteum* (personal observation) in the three sites sampled (*B. giganteum* appears to be absent in Santos). This would indicate that the results observed here conform best with those predicted by the neutralist theory. Nonetheless, the predictions of the two schools are often intermingled [39], and the number of loci analysed here is not large enough to draw any positive conclusions in favouring one school or another.

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