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GENETIC CHARACTERIZATION OF *BACILLUS GRANDII MARETIMI* (n. subsp.) (INSECTA PHASMATODEA) IN RELATION TO ITS ECOLOGY

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ELECTROPHORESIS
GENETIC DISTANCES
MEDITERRANEAN MAQUIS
EGADI ARCHIPELAGO
STICK INSECTS

ABSTRACT – The genetic structure of two *Bacillus grandii* populations collected on Levanzo and Marettimo (Egadi Archipelago) was studied by means of the analysis of allozyme frequencies. On the basis of the rather low genetic distance value ($D = 0.075$) and of the lack of distinctive morphological characters, the sample from Levanzo appears to pertain to the Northwestern Sicilian race *B. g. benazzii*; on the contrary, the Marettimo deme is to be ascribed to the new subspecies *B. g. maretimi*, because electrophoretic data are in good agreement with morphological differentiation (body and egg). The two Northern subspecies are, in turn, well differentiated from the third Southern one, i.e. *B. g. grandii* both for trophic niche and for polymorphism parameters. *B. g. benazzii* and *B. g. maretimi* feed on lentisk bushes and show a higher level of variability than *B. g. grandii*, a bramble feeder. Factors affecting population size are considered: for *B. g. maretimi* a vegetational threshold is suggested as it seems to occur for the Levanzo population of *B. g. benazzii*, while for *B. g. grandii* and *B. g. benazzii* of the Sicilian mainland the competition with hybridogenetic strains and/or with parthenogenetic hybrids is taken into account together with their heterotic and demographic advantages. Genetic affinity relationships between the *B. grandii* races are analyzed, as well as of *B. grandii* as a species versus *B. rossius* and *B. atticus*; their very different level of genetic differentiation is evaluated and discussed.

ÉLECTROPHORÈSE
DISTANCES GÉNÉTIQUES
MAQUIS MÉDITERRANÉEN
ARCHIPEL DES ÉGADES
INSECTES-BAGUETTES

RÉSUMÉ – La structure génétique de deux populations de *Bacillus grandii* recueillies sur les îles de Marettimo et Levanzo (Archipel des Egades) a été définie pour l'analyse électrophorétique des systèmes gène-enzyme. D'après la distance génétique, les populations de Levanzo, qui ne présentent pas de traits morphologiques différentiels, appartiennent à la race nord-occidentale *B. g. benazzii*, tandis que la population de Marettimo est une sous-espèce différente, *B. g. maretimi*; ses résultats génétiques sont en accord avec les caractères morphologiques distinctifs du corps et de l'œuf. Les deux races du nord-ouest se différencient à leur tour nettement de la troisième sous-espèce, *B. g. grandii* du sud-est, soit par la niche trophique (elles consomment le lentisque alors que *B. g. grandii* le ronge seulement), soit par le plus haut polymorphisme génétique. Les populations du *B. g. benazzii* de Levanzo et de Marettimo sont peu nombreuses, peut-être à cause des facteurs qui limitent la luxuriante végétation du lentisque, tandis que pour les populations de l'île principale les interactions reproductives et la compétition avec les *Bacillus* hybrides syntopiques seraient des limites importantes pour leur consistance. Les relations d'affinité génétique indiquent clairement que entre les différentes espèces du genre *Bacillus* le parthénogénétique *B. atticus* est assez voisin du bisexué *B. grandii* ($D = 0.231$) et que les deux espèces sont très éloignées de *B. rossius* (D environ 1.5).

INTRODUCTION

Taxonomy, phylogeny and reproductive biology of several taxa of the genus *Bacillus* are currently being investigated through a multidisciplinary approach, namely the study of morphology (body and egg), karyology, gene-enzyme systems and satellite DNA (Scali and Mantovani, 1989; Man-

tovani *et al.*, 1991a, 1991b, 1992a, 1992b, 1993; Maresi *et al.*, 1991, 1992, 1993) in order to reach a natural systematics of the group. *B. grandii* is a bisexual species limited to the Sicilian area, where it has been found to be clearly differentiated into two subspecies: *B. g. grandii*, present in the Southeastern part of the island, and *B. g. benazzii* in the Northwestern one (Fig. 1) (Nascetti and Bullini, 1982a; Scali, 1989, 1991).

The two races exhibit different ecological niches, the former being found in ruderal places and field sides on bramble bushes, while the latter prefers the lentisk remnants of the Mediterranean maquis. Their body and egg morphology as well allows a neat distinction owing to differences in genitalia, cercus, eye pigmentation and chorionic fine pattern (Scali, 1991); karyologically, *B. g. grandii* and *B. g. benazzii* appear to differ only for NOR localizations (Marescalchi and Scali, 1990). Allozyme frequency analysis of the two taxa fully supported their subspecific differentiation with a Nei's genetic distance of 0.192 (Mantovani *et al.*, 1991a).

B. grandii is highly differentiated for the genetic structure (Nei's D ranging from 1.48 to 1.80), chromosome complement, egg and body morphology from *B. rossius*, the other bisexual taxon of the genus (Nascetti and Bullini, 1983; Scali and Mantovani, 1989; Mantovani *et al.*, 1991a; Scali, 1991); it is also clear that *B. atticus*, in spite of its obvious morphological distinction (Nascetti and Bullini, 1982b) and thoroughly different reproductive mode (Marescalchi *et al.*, 1993), is genetically rather close to *B. grandii* (Nascetti and Bullini, 1988; Mantovani *et al.*, 1990; Mantovani and Scali, 1991).

More recently, stick-insect collections on the Egadi Archipelago led us to the invention on Levanzo and Marettimo (Favignana gave no results) of *B. grandii* - like populations which, on morphological basis, have been identified respectively as *B. g. benazzii* and a new subspecies, *B. g. maretimi* (Scali and Mantovani, 1990 and in progress; Scali, 1991). In the present paper, we detailedly define the genetic structure of the new Egadian samples and suggest their genetic affinity relationships within the genus *Bacillus*, by means of allozyme frequency analysis.

MATERIALS AND METHODS

Analyzed animals were field collected during September-October 1988-89 on the Isle of Marettimo (16 females and 10 males) and Levanzo (6 females, 2 males), and represent a sample of larger collections raised from very similar habitats, the lentisk-bush vegetational association (lentiscetum).

Lab-rearing conditions of insects, allozyme identification, electrophoretic techniques and data evaluation methods are given in Mantovani and Scali (1991). The following enzymes were analyzed: α -glycerophosphate dehydrogenase (α GPDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6PGDH), glucose oxidase (GOX), glucose-6-phosphate dehydrogenase (G6PDH), glyceraldehyde-3-phosphate dehydrogenase (G3PDH); glutamate-oxalacetate transaminase (GOT), hexokinase (HK), adenylate kinase (ADK), phosphoglucumutase (PGM), aldolase (ALD), fumarase (FH), mannose-6-phosphate isomerase (MPI), phosphoglucose isomerase (PGI). For MDH, IDH, GOT, HK and ADK two enzyme systems were identified, so that a total number of 20 loci were analyzed in the present Egadian samples. The results obtained for the new samples have been compared with similar data previously obtained from *B. g. grandii* of the Iblean type locality (Siracusa district) and *B. g. benazzii* of the Scopello area (Mantovani *et al.*, 1991a) (Fig. 1).

In order to establish wider relationships within the genus, comparisons with two additional specific taxa have been made: firstly, with Sicilian pooled samples of *B. atticus*, including some more specimens newly collected at Cugni and Milianni (Mantovani *et al.*, 1990; Mantovani and Scali, submitted) and with bisexual and partheno-

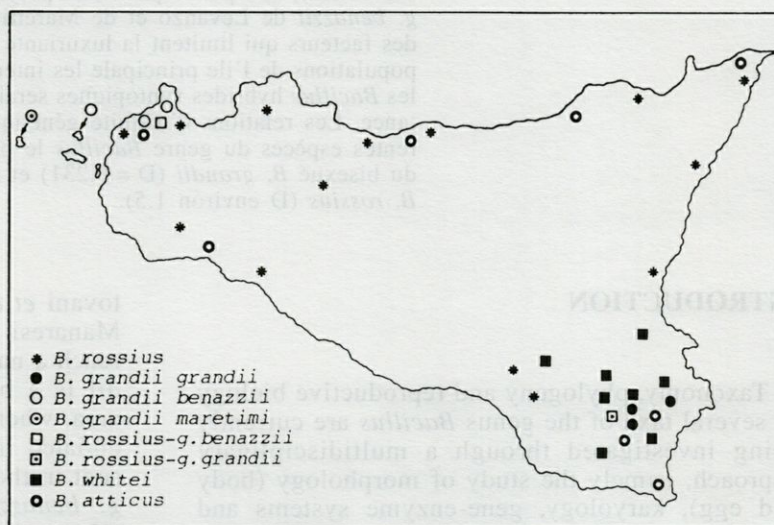


Fig. 1. - Map showing the distribution of the analyzed Sicilian taxa.

genetic demes of *B. rossius redtenbacheri* (Tinti *et al.*, 1992); secondly, with the most comprehensive pooled samples of *B. rossius* (Italian *B. r. rossius* + *B. r. redtenbacheri*) (Mantovani and Scali, 1991; Tinti *et al.*, 1992) and *B. atticus* (Italian, Croatian, Greek and Turkish populations with both diploid and triploid biotypes; Mantovani *et al.* 1990; Mantovani and Scali, 1991 and 1993).

RESULTS

Table I gives the genotype frequencies of the 18 polymorphic loci found in newly collected as well as in previously analyzed Sicilian *Bacillus*. Comparisons among *B. grandii* samples show that their genotypes are monomorphic for the same allele at 8 loci (α Gpdh, Mdh - 1, Mdh - 2, G6pdh, Got - 2, Adk - 1, Adk - 2, Ald) and also share the most common one at 6 additional loci (*Idh* - 1, *Idh* - 2, G3pdh, Got - 1, Hk - 1 and Hk - 2).

Table I. - Genotype frequencies at the 18 polymorphic loci in Sicilian *Bacillus* samples of: *B. atticus* (BATT), *B. grandii grandii* (BGGR), *B. g. benazzii* from Scopello area (BGBS) and Levanzo island (BGBL), *B. g. maretimi* (BGMA), *B. rossius redtenbacheri* bisexual samples (BREB) and parthenogenetic demes (BREP). α Gpdh and Ald are monomorphic for the same alleles in all analyzed specimens. X², where required, gives the significance of P value for Hardy-Weinberg equilibrium.

LOCUS	GENOTYPES	BATT	BGGR	BGBS	BGBL	BGMA	BREB	BREP
Mdh-1	100/100						0.91	1.00
	100/110						0.07	
	108/108	0.57	1.00	1.00	1.00	1.00		
	108/116	0.43						
	110/110							0.02
Mdh-2	χ^2							c
	96/96	1.00	1.00	1.00	1.00	1.00		
Idh-1	100/100						1.00	1.00
	96/96	0.46						
	96/100	0.54						
	100/100		1.00	0.95	0.75	0.91	1.00	1.00
Idh-2	100/105			0.05				
	103/103				0.25	0.09		
	χ^2					a		
	90/93					0.20		
	90/100					0.05		
G6pdh	93/93	1.00	1.00	1.00	1.00	0.75		
	100/100						1.00	1.00
	100/100			0.23	1.00		1.00	1.00
	100/103			0.48				
	103/103	1.00	1.00	0.29		0.56		
Gox	103/107					0.40		
	107/107					0.04		
	96/96	1.00						
	98/98			1.00	0.86	1.00		
	98/105				0.14			
G6pdh	100/100						1.00	1.00
	105/105						1.00	1.00
	96/96	0.86	1.00	1.00	1.00	1.00		
	96/98	0.09						
G3pdh	98/98	0.05						
	100/100						1.00	1.00
	100/100						1.00	1.00
	104/104	0.01		0.06				
Got-1	104/111			0.29				
	111/111	0.99	1.00	0.65	1.00	1.00		
	94/97					0.06		
	95/100						0.07	
	97/97		1.00	1.00	1.00	0.94		
Got-2	97/105	0.43						
	100/100						0.93	1.00
	105/105	0.57						
	100/100	0.99	1.00	1.00	1.00	1.00	1.00	1.00
	105/105	0.01						

Table I. - continued

LOCUS	GENOTYPES	BATT	BGGR	BGBS	BGBL	BGMA	BREB	BREP
Hk-1	100/100						1.00	1.00
	100/104					0.04		
	104/104	1.00	1.00	0.84	1.00	0.96		
	104/113			0.11				
Hk-2	113/113			0.05				
	100/100						1.00	1.00
	100/104					0.04		
	104/104	0.94	0.63	0.95	0.72	0.96		
	104/106	0.05	0.25	0.05	0.14			
Adk-1	106/106				0.14			
	108/108	0.01					0.59	0.06
	94/94						0.11	
	94/100						0.30	0.94
Adk-2	100/100	1.00	1.00	1.00	1.00	1.00		
	χ^2						a	
	96/100							0.01
	100/100						1.00	0.99
Pgm	107/107	1.00	1.00	1.00	1.00	1.00		
	96/103					0.37		
	99/103	0.74				0.21		
	100/100					0.07	1.00	0.89
	100/103					0.07		
Fh	100/105					0.07		
	103/103	0.26				0.14		
	105/105		1.00			0.07		0.11
	118/118			0.92	1.00			
	118/122			0.08				
	86/90	0.25						
Mpi	90/90	0.75						
	93/93							0.03
	95/95		1.00					
	100/100						1.00	0.97
	104/104			1.00	1.00	1.00		
Pgi	100/100						1.00	1.00
	103/103	0.89						
	103/108	0.11						
	112/112		1.00	0.88				
	112/116			0.12				
	116/116				1.00	1.00		
Gox	76/76					0.04		
	76/80					0.08		
	76/84					0.04		
	78/84			0.37				
	80/80					0.68		
	80/84					0.16		
	84/84		1.00	0.57	1.00			
	84/89	0.43		0.06				
Gox	89/89	0.57						
	97/100						0.02	
	100/100						0.98	1.00

a : P<0.001; b : 0.01<P<0.001; c : 0.05<P<0.02.

Five monomorphic loci are shared between *B. atticus* and *B. grandii* (α Gpdh, Mdh - 2, Adk - 1, Adk - 2, Ald), but only two when also *B. rossius* samples are introduced in the comparison (α Gpdh, Ald). It should be noted that at 4 loci (*Idh* - 2, *Hk* - 1, *Hk* - 2, *Pgm*) the *B. g. maretimi* sample shows alleles unknown in the other two *B. grandii* subspecies, but present in *B. rossius*.

On the whole, polymorphic loci are at the Hardy-Weinberg equilibrium, but a few exceptions can be noticed (such as those of bisexual *B. r. redtenbacheri* sample at Mdh - 1 and Adk - 1 loci and of Levanzo and Maretimo populations at *Idh* - 1 locus), always due to heterozygote deficiency.

Polymorphism parameters of the bisexuals (Table II A) show that the highest levels of variability (H_{obs} , A and P) are found in *B.g. benazzii* of Scopello and in *B.g. maretimi*. The H_{obs} value of the Levanzo sample is not significantly lower than that of the Scopello one of the same subspecies. Similarly, no significant differences among observed and expected heterozygosity are generally found in bisexual samples, the only exception being the *B.r. redtenbacheri* one, which shows a significant excess of homozygous individuals at the Mdh - 1 and Adk - 1 loci.

The genetic distances among *B. grandii* samples (Table II B) indicate a value of distance between the Scopello and Levanzo ones of 0.075 ;

Table II. - A, Allozyme polymorphism parameters in Sicilian taxa (captions as in Table I) : \bar{H}_{obs} , mean observed heterozygosity ; \bar{H}_{exp} , mean expected heterozygosity ; A, mean effective number of alleles per locus ; P, proportion of polymorphic loci. The a superscript indicates significant differences between expected and observed heterozygosity. B, Nei's genetic distances (above diagonal) and identities (below diagonal) among *B. grandii* taxa (captions as in Table I). C, Nei's genetic distances (above diagonal) and identities (below diagonal) among Sicilian *Bacillus* taxa : *B. atticus* (BATT), *B. grandii grandii* (BGGR), *B. g. benazzii* from Scopello and Levanzo (BGBE), *B. g. maretimi* (BGMA), *B. rossius redtenbacheri* bisexual samples (BREB) and parthenogenetic demes (BREP).

A

	\bar{H}_{obs}	\bar{H}_{exp}	A	P
BATT	0.153		1.19	0.55
BGGR	0.012	0.019	1.03	0.05
BGBS	0.081	0.083	1.13	0.40
BGBL	0.014	0.042	1.06	0.15
BGMA	0.079	0.099	1.21	0.40
BREB	0.013	0.032 ^a	1.05	0.20
BREP	0.000		1.02	0.15

a : $P < 0.001$

B

	BGGR	BGBS	BGBL	BGMA
BGGR	--	0.192	0.293	0.266
BGBS	0.825	--	0.075	0.136
BGBL	0.746	0.928	--	0.130
BGMA	0.766	0.873	0.878	--

C

	BATT	BGGR	BGBE	BGMA	BREB	BREP
BATT	--	0.348	0.362	0.312	1.628	1.485
BGGR	0.706	--	0.217	0.266	1.498	1.358
BGBE	0.696	0.805	--	0.109	1.338	1.232
BGMA	0.732	0.766	0.897	--	1.431	1.315
BREB	0.196	0.223	0.262	0.239	--	0.019
BREP	0.226	0.257	0.292	0.268	0.982	--

in view of the fact that the *Bacillus* interracial D values are just higher than 0.100 and that no morphological differences exist between Scopello and Levanzo specimens, we pooled them in further comparisons (see also Discussion). The higher genetic distance of the Marettimo sample clearly suggests a subspecific differentiation from both *B. g. grandii* ($D = 0.266$) and *B. g. benazzii* ($D = 0.130-0.136$). The dendrogram branching (Fig. 2), based on Table II C data, further strengthens the higher affinity of *B. g. maretimi* to *B. g. benazzii* (knot at $D = 0.109$), than to *B. g. grandii* (knot at $D = 0.241$) ; furthermore, the close relation of *B. atticus* to the *grandii* group and the very low level of differentiation between parthenogenetic and bisexual samples of *B. rossius* ($D = 0.02$) are once more evidenced.

Comparisons among specific comprehensive samples of *B. rossius*, *B. atticus* and *B. grandii*, revealed a genetic distance of 0.264 between *B. atticus* and *B. grandii*, while as much as 1.261 was obtained between the two bisexuals. An even higher differentiation was found to exist between *B. atticus* and *B. rossius* ($D = 1.498$).

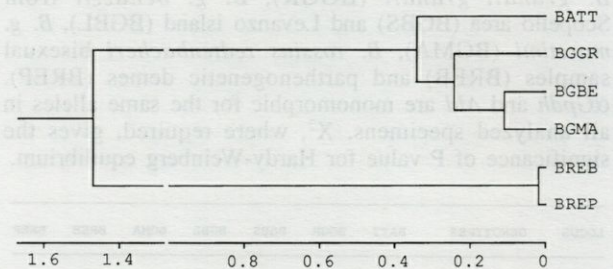


Fig. 2. - Dendrogram obtained from Nei's genetic distances, showing the genetic affinity relationships among the investigated taxa (*B. atticus* : BATT, *B. grandii grandii* : BGGR, *B. g. benazzii* : BGBE, *B. g. maretimi* : BGMA, *B. rossius redtenbacheri* bisexual samples : BREB and parthenogenetic demes : BREP).

DISCUSSION

The presented allozymic data also supported by morphological differences undoubtedly indicate a subspecific level of differentiation for the *B. grandii* population of Marettimo, thus reinforcing the idea that islands represent favourable habitats for cladogenetic processes. In turn, *B. g. maretimi* shows a higher genetic similarity to *B. g. benazzii*, than to *B. g. grandii*, although differentiated from both of them for body and egg morphology. In particular, *B. g. maretimi* lacks of the specific *B. grandii* diagnostic character represented by the femoral/tibial white patches, and its egg-chorion pattern is characterized by a fine reticulate superimposed to a surface pierced by deep pits ; this pattern sharply differs from the flat or just undu-

lated lacenet work observed in the two remaining *B. grandii* subspecies and in *B. atticus* (Scali, 1991; Scali and Mantovani, 1990 and unpublished).

Taking into account allozymic and morphological data, trophic habits and its relatively greater similarity to *B. rossius*, *B. g. maretimi* appears to be the most ancestral *B. grandii* taxon. It is again keeping in mind both morphological and allozymic observations, that the population from Levanzo can be reasonably lumped into the *B. g. benazzii* subspecies. In fact, the genetic approach alone wouldn't be fully discriminative, the genetic distance of the Levanzo sample being somewhat intermediate between the population and subspecific levels observed in *Bacillus* taxa. However, its general body and egg morphology clearly reminds of *B. g. benazzii* (Scali, 1991); therefore, to treat the Levanzo's population as a fourth race of *B. grandii*, a larger sample, eventually increasing its genetic differentiation, ought to be available.

The difference between observed and expected heterozygosity values of bisexual *B. r. redtenbacheri* may be explained by the occurrence of a certain degree of facultative parthenogenesis, which can occur even within bisexual populations of *B. rossius*, owing to unfertilized egg development (Scali, 1968; 1982; Scali and Mantovani, 1989). On the other hand, the lower heterozygosity value of the Levanzo sample compared to the Scopello one of *B. g. benazzii* is not significant and may be ascribed to the low number of individuals of the Levanzo's deme; however, the low level of polymorphism of the Southern race (*B. g. grandii*) when compared to the Northern ones (*B. g. benazzii* and *B. g. maretimi*) seems to be genuine, being repeatedly and independently reported for the former (Nascetti and Bullini, 1983; Mantovani *et al.*, 1991a). It may be possibly explained through bottlenecks due to homo- and heterospecific matings of *B. g. grandii*. In fact, besides being syntopic with the hybridogenetic *B. rossius-g. grandii* and reproductively linked to its maintenance, the Southern race is also sympatric with high density demes of the parthenogenetic *B. whitei* (= *B. rossius/g. grandii*). Since no pre-copula barriers appear to exist, *B. g. grandii* males can mate with homospecific females, hybridogens or *B. whitei* females (Mantovani and Scali, 1992; Mantovani *et al.*, 1991b, 1992a; Scali *et al.*, 1991, 1992). If homospecific matings are relatively rare, as the very low number of field-captured *B. g. grandii* females suggests, a progressive homogenization of the population gene-pool may be expected, with a consequent disappearance of rare alleles and a decreased polymorphism. For the sake of completeness, we must also mention that, although not demonstrated, also the heterotic and

demographic advantages of the hybrid clonal females (Bullini and Nascetti, 1990) could play a role in keeping *B. g. grandii* at low numbers.

B. g. benazzii as well is involved in the maintenance of the hybridogenetic *B. rossius-g. benazzii* strains (Mantovani and Scali, 1990, 1992), but it has not to cope with matings to hybrid parthenogens; as a matter of fact, its genetic variability does not seem affected. This suggests in turn that the main factor affecting genetic homogeneity of *B. g. grandii* could be represented by the great abundance of syntopic parthenogenetic *B. whitei*. It is also to be remembered that the relationships between *rossius x grandii* hybrids and *B. grandii* are further enriched by the production of androgenetic fertile individuals (both males and females) of pure paternal taxon structure (Scali *et al.*, 1991; Mantovani and Scali, 1992). The real importance of natural androgenesis is yet to be established, both quantitatively (number of androgenetic offspring/population size) and qualitatively (adaptive value), but it can be observed that androgens seem to represent a reservoir of the fathering species (Scali *et al.*, 1991; Mantovani and Scali, 1992).

It has been pointed out the low numerical consistence of *B. g. benazzii* on Levanzo Island; other, not obvious, factors than competition on behalf of heterospecific matings must affect population density there; one likely ecological parameter could be the vegetative difficulties of the food plant, which appears to suffer from the excessively dry climate. A similar line of reasoning could apply to *B. g. maretimi*. It could also be observed that, owing to the absence of other *Bacillus* taxa on the Isle of Marettimo, *B. g. maretimi* appears to be the race least menaced of extinction from reproductive competition on behalf of successful interspecific hybrids. Actually, *B. g. maretimi* seems the only taxon escaped from the very intriguing net of reticulate evolution (Grant, 1981, Grant and Grant, 1992), encompassing all other *Bacillus* taxa (Scali *et al.*, 1992).

Finally, we would like to point out that within the genus *Bacillus* extremely wide ranges of interspecific D values are found (0.264 between *B. grandii* and *B. atticus* one side and 1.498 between *B. rossius* and *B. atticus* the other). There is no doubt that a distinction at the specific level is already attained between *B. grandii* and *B. atticus* for reproductive biology and morphological characters; it could also be observed that similar D values are thought to be of interspecific rank for several other animal groups (Ayala, 1975; Bullini, 1980). On the other hand, D values around 1.500 are often within the intergeneric range; however, other parameters, such as morphological, karyological and reproductive characters, suggest keeping the three taxa within the same genus.

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