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Genetic Variation in the Cephalopod *Nautilus belauensis*

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ABSTRACT.—*Nautilus belauensis,* a phylogenetic relic, occurs along the fore-reef of Palau in Micronesia. Extracts from tentacles and adductor muscles provided material for an electrophoretic survey of enzymes and general proteins. Eighty-nine animals were screened for variation in 21 enzymes and protein systems; 14 systems gave genetically interpretable electromorphic patterns providing data on variation at 20 presumptive gene loci. Four loci were polymorphic: phosphoglucomutase-1, leucine aminopeptidase-1, glutamate-oxalate transaminase-2, and a general protein. The proportion of loci that were polymorphic (P) was 0.2; the mean heterozygosity per individual ($\bar{H}$) was 0.09. Alleles at the variable loci were segregating in close agreement with expectations for a panmictic population. No evidence for sexual or age-related allozyme variation was found. The moderate levels of genetic variability discovered are discussed in light of the traditional status of *Nautilus* as a bradytelic living fossil. [Cephalopod, electrophoresis, enzymes, evolution, genetic variation, *Nautilus belauensis,* Palau, proteins]

Nautilus is generally regarded as both a phylogenetic relic and a biogeographic relict. The Nautiloidea were diverse and well-represented among marine faunas as far back as the Ordovician Period (ca. 450 million years ago). The tightly coiled family that includes *Nautilus,* the Nautilidae, is believed to have appeared in the early Triassic (ca. 200 million years ago). The nautilids quickly radiated and were cosmopolitan during the remainder of the Mesozoic, but during the mid–late Tertiary they rapidly diminished to the present, single genus that survives at scattered sites in the Indo-Pacific region. Such a history would traditionally have led many biologists to predict that the four surviving species would be characterized by relatively low levels of genetic variation. However, "living fossils" have recently been interpreted as simply long-surviving members of ancient groups that exhibit normal, rather than reduced, rates of phyletic evolution (Stanley 1979). Such a characterization would lead us to predict that *Nautilus* possesses moderate levels of genetic polymorphism, typical of many horotelic organisms. We tested this hypothesis by conducting an electrophoretic survey of protein variation in a living species, *Nautilus belauensis* Saunders (1981). This species was first reported by Dugdale and Faulkner (1976) and was formally described by Saunders (1981a).

The surviving representatives of the tetrabranchiate cephalopods are not well known; one of the four extant species has never been seen alive (Saunders 1981b), and their deep, fore-reef habitat (typically 100–

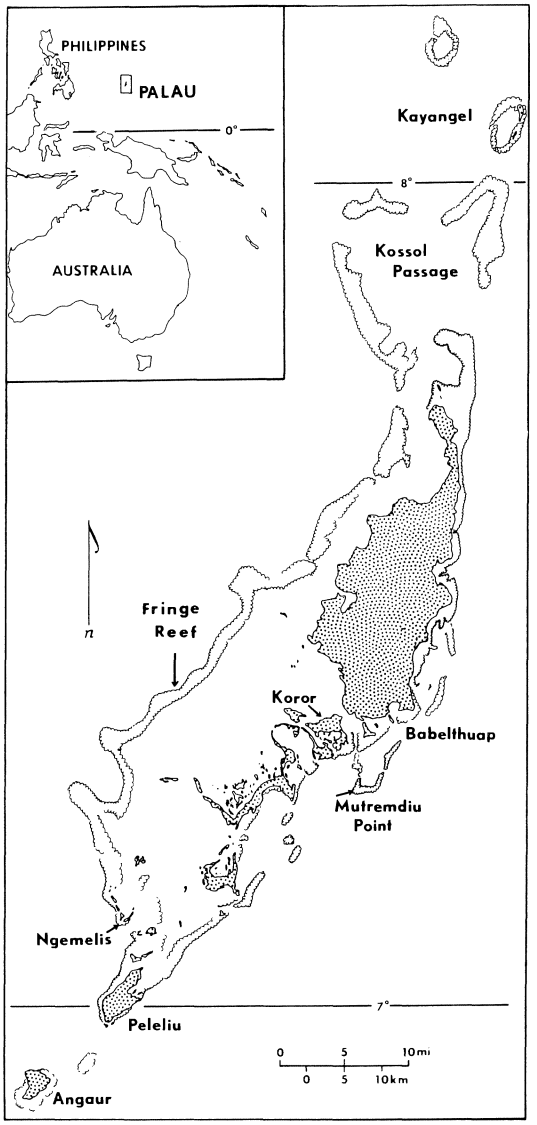

FIG. 1. Map of Palau, West Caroline Islands, Micronesia, showing location of trapping sites for *Nautilus belauensis*; Mutremdiu Point (N = 80) and Ngemelis Island (N = 9).

600 m) has made direct study difficult. Only recently have field studies begun to provide information on the ecology, behavior, and physiology of these remarkable animals. References to recent studies of *N. pompilius* in the Philippines and Fiji, of *N. macromphalus* in New Caledonia, and of *N. belauensis* in Palau are provided in Saunders (1981b). Work on the last named species (Saunders in prep., in press) provided an opportunity to undertake this preliminary study of genetic variation in *Nautilus*.

MATERIALS AND METHODS

Nautilus belauensis was trapped at two localities in Palau: Mutremdiu Point (N = 80) on the eastern side of the fringing reef and Ngemelis Island (N = 9) on the western side of the island complex (Fig. 1). Voucher specimens comprising representatives of the population sampled are reposited at the Academy of Natural Sciences of Philadelphia (ANSP 356854). Saunders and Spinosa (1979) have documented the 68 km movement of tagged animals between these sites. Data from both sites were pooled for all analyses. Animals were collected in baffle-type traps set several nights at depths of ca. 100–300 m. They are both generalistic scavengers and opportunistic predators, readily entering traps baited with a wide variety of carrion. Trapped individuals were weighed, sexed, and measured as described in Saunders and Spinosa (1978) before being tagged, "burped" to release air trapped in the body chamber, and released by a diver at a depth of 15–50 m near the trap site.

As a long-term ecological study took priority over our genetic survey, it was essential to develop a technique of tissue sampling that did not harm the animals. Preliminary studies in which we compared adductor muscle with tentacle tissue, suggested we would be able to obtain sufficient active protein from the latter. Samples were obtained by clipping the tips of 2–4 of the 34 large digital tentacles. Tentacles were obtained from all specimens and both tentacles and muscle tissues were taken from animals killed for other purposes. Evidence that tentacle clipping did not seriously harm the animals is provided by the fact that many

TABLE 1. Tissue and buffer combinations giving optimal resolution of proteins in *Nautilus belauensis.*

Enzyme/protein (E.C. #)	Electrophoretic		
	Abbrev-iation	Buffer*	Tissue
Amido black protein	ABP-1	TME	Muscle
	ABP-2	TME	Tentacle
Esterase (3.1.1.1)	EST-1	TEB	Tentacle
	EST-2	TEB	Tentacle
Glutamate-oxaloacetate transaminase (2.6.1.1)	GOT-1	TEB	Muscle
	GOT-2	TEB	Muscle
Glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12)	GAP	TME	Muscle
α-Glycerophosphate dehydrogenase (1.1.1.8)	GPD	LiOH	Muscle
Isocitrate dehydrogenase (1.1.1.42)	IDH-1	TC	Muscle
	IDH-2	TC	Tentacle
Lactate dehydrogenase (1.1.1.27)	LDH	TME	Muscle
Leucine aminopeptidase (3.4.11)	LAP-1	TC	Muscle
	LAP-2	TC	Muscle
Malate dehydrogenase (1.1.1.37)	MDH-1	TC	Tentacle
	MDH-2	TC	Tentacle
Malic enzyme (1.1.1.40)	ME	TME	Muscle
Phosphoglucomutase (2.7.5.1)	PGM-1	TC	Muscle
	PGM-2	TC	Muscle
6-Phosphogluconate dehydrogenase (1.1.1.44)	PGD	TME	Muscle
Phosphoglucose isomerase (5.3.1.9)	PGI	TEB	Tentacle
Superoxide dismutase (1.15.1.1)	SOD-1	TME	Tentacle
	SOD-2	LiOH	Muscle

* **LiOH.** Solution A: 0.03 M LiOH, 0.19 M borate, pH 8.1; Solution B: 0.008 M citrate, 0.05 M Tris, pH 8.4; 10% A plus 90% B for gel, A for electrode (4 hr, 300 V). **TC.** 0.188 M Tris, 0.065 M citrate; pH 6.8; diluted 1:10 for gels and 1:5 for electrodes (4 hr, 250 V). **TEB.** 0.5 M Tris, 0.2 M EDTA, 0.65 M borate, pH 8.0; diluted 1:10 for gels and undiluted for electrodes (4 hr, 200 V). **TME.** 0.01 M Tris, 0.01 M maleic acid, 0.01 M EDTA, 0.01 M $MgCl_2$, 0.13 M NaOH, pH 7.4; diluted 1:10 for gels and undiluted for electrodes (4 hr, 100 V).

of these animals were subsequently recaptured during the 1982 field season and showed no ill effects.

Eighty-nine animals, trapped between 3 June and 8 August 1982 at Mutremdiu Point and Ngemelis Island, were examined. For 26 of these both muscle and tentacle tissues were obtained. Tissues were held on ice in the field and then were kept frozen below −10° C until they reached San Diego in September 1982 where they were held at −70° C until they were processed for electrophoresis.

Our electrophoretic techniques are described in general terms elsewhere (Woodruff 1975; Mulvey and Vrijenhoek 1981). Tentacle and muscle tissue (0.3–0.5 g wet weight) were homogenized in 0.2 ml of grinding solution (0.01 M Tris, 0.001 M EDTA, 0.05 mM NADP, pH 7.0) using a Sonicator® cell disrupter. The homogenate was centrifuged for 20 min at 15,000 g at 4° C. The supernatant fluid was absorbed onto filter paper wicks and excess sample blotted off before the wicks were inserted into 12% horizontal starch gels. Electrophoresis was carried out until a bromophenol blue marker dye had migrated approximately 100 mm anodal to the origin. Gels were stained at 37° C following the methods of Shaw and Prasad (1970) and Harris and Hopkinson (1978). Combinations of buffers and tissues used for resolution of *Nautilus* isozymes are given in Table 1. The esterase substrate was alpha-naphthyl acetate; malate dehydrogenase and malic enzyme were

NAD-dependent and NADP-dependent, respectively.

Names of allozymes are taken from common abbreviations for the enzyme encoded by each locus. Where multiple loci occur for an enzyme (isozymes) the loci are numbered in order of decreasing anodal mobility. Italics indicate genotype designations and capital letters indicate the protein product of a locus. The bromophenol blue marker dye was used as a reference on all gels and electromorph mobilities (R_m) were calculated relative to this standard.

The proportion of polymorphic loci (P) was based on the criterion that the frequency of the most common allozyme was ≤ 0.95. Average levels of heterozygosity per individual were calculated based on Castle-Hardy-Weinberg expectations (Spiess 1977). Allozyme frequencies for the polymorphic loci were tested for their agreement with panmictic equilibrium expectations using a χ^2 statistic with expected values calculated by the small sample method of Levene (1949). The two less frequent alleles of the *Pgm-1* locus were pooled for statistical analysis. A Student's t-test was employed to compare frequencies for males and females and for immature and fully mature individuals.

Results

Sufficient tissue samples were available to survey 21 enzyme or protein systems for genetic variation. Muscle tissue consistently showed more enzyme activity than tentacle tissue per gram. Several enzymes could only be detected from muscle tissue or when at least 3 tentacles from the same individual were pooled. Unfortunately, live *Nautilus* quickly retract their tentacles into their cartilaginous sheaths when disturbed and it was very difficult to obtain more than a single piece of tentacle from some individuals. Consequently, it was not possible to examine all enzymes in all individuals.

Fourteen enzyme or general protein systems gave consistent, genetically interpretable results and provided data on variation at 20 presumptive gene loci. The following enzymes were also examined but were either undetectable (possibly because of concentration or tissue handling problems) or gave genetically uninterpretable activity patterns on the gels: alcohol dehydrogenase, aldolase, glucose dehydrogenase, hexokinase, hydroxybutyrate dehydrogenase, leucylalanine peptidase, malate dehydrogenase-1, phosphoglucomutase-2, and sorbitol dehydrogenase. Apart from the concentration effect mentioned above, muscle and tentacle samples from the same animal ($N = 26$) gave comparable results and results from the two types of tissue were consequently pooled.

The results of the electrophoretic survey of allozymic variation, the number of individuals examined, and the electromorph frequencies observed at each locus are shown in Table 2. Genetic interpretation of these results is based on conventional molecular criteria as formal inheritance experiments are presently impractical. Four loci (*Pgm-1, Lap-1, Got-2,* and *Abp-1*) were polymorphic in the population studied. The proportion of polymorphic loci in *N. belauensis* is accordingly found to be $P = 0.20$. The average level of heterozygosity per individual ($\bar{H}$) was 0.09. The χ^2 statistics indicate that frequencies at each of the four loci are consistent with Castle-Hardy-Weinberg expectations and suggest that all the animals studied were drawn from a single large randomly mating population.

Allele frequencies at the four variable loci were examined for evidence of sexual differences as mature females are smaller and have narrower shells than males (Saunders and Spinosa 1978). Student's t-test was employed to compare 18 adult males with 11 adult females and revealed no evidence for sex-associated differences at these loci despite well-marked morphological dimorphism in this species. Actually no such dif-

ferences were expected; sexual dimorphism at the loci surveyed is most unusual. Similar tests were employed to detect genetic differences between immature (white body chamber, aperture not thickened) and fully mature (thickened, blackened aperture) *Nautilus*. At each of the four polymorphic loci no significant difference between juvenile and adult animals was found ($P \geq 0.35$). We note, however, that our subsamples are small (N = 22–26) and results are preliminary.

Discussion

Since the Cretaceous there has been a general reduction in the diversity and distribution of the Nautilidae. Reasons for the reduction are unknown although the radiation of modern teleosts may have played a role (Saunders 1981b). Since genetic variation may be a key to the evolutionary fate of a species, it is of interest to investigate variation in *Nautilus*. Saunders (1981b) described morphological variation among individual populations and between isolated populations and suggested that sufficient genetic plasticity may remain in this species to permit long-term survival.

The present study demonstrates that *N. belauensis* exhibits "typical" levels of variation in electrophoretic markers. Among 93 invertebrates, Nevo (1978) reported the mean proportion of polymorphic loci (P) to be 0.397 ± 0.201. The 0.20 value for P obtained for *Nautilus* from Palau is within the range of observed values. Nevo also found the mean proportion of loci heterozygous per individual ($\bar{H}$) among invertebrates except insects was 0.1123 ± 0.0720. The value obtained for *N. belauensis*, $\bar{H} = 0.09$, is well within the range observed for other invertebrates.

The fit of the allele frequencies at each of the four polymorphic loci to Castle-Hardy-Weinberg expectations suggests that the collections made at Palau were drawn from a single randomly mating population. Saunders and Spinosa (1979) showed that while some individuals are repeatedly captured in the same location, specimens have been observed to move up to 150 km in 332 days along the Palauan fore-reef. Such movements coupled with the longevity of animals would contribute to the maintenance of a homogenous, randomly mating population of *N. belauensis* at Palau. Although their life span is not accurately established, they may take 10 years to reach maturity and they are known to live 4 years beyond that point (Saunders in press).

TABLE 2. Relative mobility (R_m) of *Nautilus belauensis* isozymes and their frequency among a population from Palau. The number of individuals examined for each locus is also provided. A negative sign indicates mobility cathodal to the origin. For polymorphic loci a χ^2 test of the fit to Castle-Hardy-Weinberg expectations is given with the associated probability (P).

Locus	No. examined	Allozyme mobility	Allozyme frequency	χ^2	P
Abp-1	25	0.21	0.60	2.46	0.13
		0.19	0.40		
Abp-2	63	0.06	1.00		
Est-1	65	0.44	1.00		
Est-2	59	0.09	1.00		
Got-1	17	0.07	1.00		
Got-2	24	−0.12	0.79	2.43	0.13
		−0.08	0.21		
Gpd	28	0.21	1.00		
Gap	43	0.14	1.00		
Idh-1	35	0.12	1.00		
Idh-2	60	0.05	1.00		
Ldh	17	0.14	1.00		
Lap-1	40	0.34	0.71	1.96	0.18
		0.27	0.29		
Lap-2	30	0.12	1.00		
Mdh-2	89	0.09	1.00		
Me	28	0.23	1.00		
Pgm-1	27	0.28	0.28	2.97	0.09
		0.26	0.17		
		0.24	0.55		
Pgd	24	0.04	1.00		
Pgi	82	0.23	1.00		
Sod-1	89	0.22	1.00		
Sod-2	25	0.04	1.00		

TABLE 3. Proportion of polymorphic loci (P) and mean proportion of loci heterozygous ($\bar{H}$) for other "living fossils." The approximate geological age of each genus in millions of years (t) is also given.

Genus	t	P	$\bar{H}$	Reference
Nautilus	30	0.20	0.09	this paper
Alligator	35	0.06	0.02	Gartside et al. (1977)
Limulus	200	0.25	0.06	Selander et al. (1970)
Lycopodium	350	0.10	0.06	Levin (1973)

Recently Masuda and Shinomiya (1983) reported electrophoretically detectable genetic variation in *N. pompilius* from the Philippines and Fiji. *N. pompilius,* the species so well known to shell collectors, is smaller than *N. belauensis* and mature specimens have shells about 170 mm in diameter versus 200 mm in *N. belauensis.* Five enzymes were examined and significant geographic differences in presumptive allele frequencies were found for two loci. The authors concluded that there may be little gene flow between populations in these two areas which are 7000 km apart. This is a very reasonable suggestion and underscores the need for more extensive geographic sampling.

Darwin (1859) and subsequent workers including Simpson (1953) regarded forms such as *Nautilus* as living fossils, and as paradoxical examples of phyletic stagnation. In 1969 *Limulus polyphemus,* the horseshoe crab, was examined to see if it had reduced levels of genetic variation that might account for its apparent slow rate of evolutionary change (Selander et al. 1970). As shown in Table 3, however, *Nautilus* and other so-called living fossils do not display significantly reduced levels of genetic variability. This unexpected result contributed to the reappraisal of macroevolutionary processes led by Eldredge and Gould (1972) and to a reinterpretation of living fossils by Stanley (1979). It now seems clear that living fossils are simply the extant members of lineages that happen to have survived with little detectable change for relatively long intervals. They are an expected, but rare, product of normal rates of phyletic evolution as predicted by the punctuational model. According to this interpretation, living fossils are expected to show typical amounts of genetic variability and not necessarily markedly less variation than other species. Thus the moderate levels of variation found in *Nautilus* present no problem to evolutionary theory.

Genetic variation, even as measured here, is a poor indicator of an organism's evolutionary past or potential. Arguments that rates of evolution and ecological niche breadth might be closely correlated with levels of enzyme polymorphism and heterozygosity have not been substantiated (Nevo 1978). It is now clear that such relationships are neither necessary nor predicted by evolutionary theory. This is strikingly illustrated by McCracken and Selander (1980) who found niche breadth to be greater in some self-fertilizing slugs with no detectable genetic variation at the 20 loci surveyed than in highly heterozygous, outcrossing species. We would therefore not expect any simple relationship between enzyme electrophoretic variation in *Nautilus* and its ecology or evolution. Earlier arguments that living fossils survived as narrow specialists in persistent microhabitats or as broad generalists unaffected by environmental change no longer seem useful and certainly cannot be supported by electrophoretic data. Nevertheless, our discovery of moderate levels of genetic variation in *N. belauensis* bodes well for the development of a genetic phylogeny of living *Nautilus.* The determination of the degree of intra- and interspecific genetic differentiation in *Nautilus* and related genera could throw considerable light on the relative age and evolution of the four surviving species of *Nautilus,* and on their rates and patterns

of genetic change. We propose to couple such genetic data with information on morphological, physiological, ecological, and behavioral variation to test Schopf's (1982) suggestion that in *Nautilus* and other living fossils the duration and extent of "arrested evolution" may have been exaggerated.

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