Evolutionary divergence between sympatric species of southern African Hakes, *Merluccius capensis* and *M. paradoxus*.
I. Electrophoretic analysis of proteins

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We estimated the amount of genetic divergence between two morphologically similar species of southern African hake, *Merluccius capensis* and *M. paradoxus*, with the electrophoretic analysis of proteins encoded by 31 loci. Nei's genetic distance between these taxa was 0·583 (±0·160) and is typical of evolutionary divergence between well differentiated congeneric species. We found no evidence of hybrid individuals. The mean heterozygosity over 13 samples of *M. capensis* was 0·055 and over 10 samples of *M. paradoxus* was 0·067. The present sympatric distributions of these fish are most likely the result of secondary contact after speciation in allopatry or the result of repeated dispersals of ancestral populations of other Atlantic Ocean hakes to southern Africa, rather than the result of sympatric speciation. There were significant excesses of rare alleles in both species as compared with that expected for neutral alleles in species at drift-mutation equilibrium. Average heterozygosities, however, were not appreciably reduced in comparison with other marine fish. Using genetic distance and the assumptions of the molecular clock, we estimate that the lineages leading to these species diverged from one another between 7 and 13 million years ago.

INTRODUCTION

Isolation in allopatry followed by genetic drift or adaptive divergence appears to be the most common mode of speciation in sexually reproducing animals (Futuyma and Mayer, 1980). Two kinds of population events can lead to allopatric isolation in marine fish. One is the subdivision of an ancestral population into two or more large populations by geologic events or by changes in oceanic currents or temperatures, and the other is the establishment of small founder populations outside of a species' usual geographic range. The sympatric occurrence of closely related species is therefore assumed to reflect secondary contact after the formation of reproductive barriers has prevented reassimilation into a single gene pool.

In this paper we describe genetic divergence between two sympatric species of hake, *Merluccius capensis* and *M. paradoxus*, which inhabit the coas-

tal waters of southern Africa. *Merluccius capensis* is distributed between 12°S on the west coast and 31°E on the south coast. *Merluccius paradoxus*, in contrast, has a more restricted range between 18°S and 27°E (Inada, 1981). The ranges of these hakes coincide with the cool temperate waters of the Benguela Upwelling System and are bounded by the warm southward flowing Southern Equatorial Current on the west coast and the southwestward flowing Mocambique Current on the south coast. There is a broad area of bathymetric overlap; *Merluccius capensis* inhabits the continental shelf to about 400 m, and *M. paradoxus* inhabits the continental shelf and slope from about 140 m to 850 m.

The taxonomies of species in the genus *Merluccius* are in considerable disarray because many species are morphologically similar to one another. The two nominal species of southern African hake were considered to be a single species, *M. capensis*, until Franca (1954) demonstrated the existence of a second sympatric taxon, *M. paradoxus*, from a study of vertebral numbers. Subsequent morphological studies (van Eck, 1969; Bentz, 1976) and

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an electrophoretic study of non-specific proteins (Jones and Mackie, 1970) have confirmed Franca's (1954) hypothesis of two distinct taxa. The actual amount of genetic divergence between these species and their phylogenetic relationships to other species of hake, however, are still matters of much speculation (Szidat, 1961; Franca, 1971; Meyer-Rochow, 1972; Kabata and Ho, 1981).

The goal of this study is to document genetically-based electrophoretic variation in these taxa and to estimate the amount of genetic divergence between them. The following paper in this issue reports genetic divergence between these taxa as measured by the restriction-endonuclease analysis of mitochondrial DNA (Becker et al., 1988). Subsequent papers in this series will examine the phylogenetic relationships of these taxa with other Atlantic and Pacific Ocean hakes.

MATERIALS AND METHODS

We examined genetic variation in a total of 1135 individuals of Merluccius capensis from 13 locations (extending from 18°42'S, 11°48'E to 34°20'S, 25°40'E), and a total of 882 individuals of M. paradoxus from 10 locations (23°02'S, 13°05'E to 34°40'S, 25°40'E) (fig. 1). Samples of eye, liver, skeletal and cardiac muscles were held at −25°C for up to 3 months until laboratory analysis. Individuals were identified to species by gill arch tubercle morphology (van Eck, 1969). Starch-gel electrophoretic methods followed May et al. (1979), and staining protocols followed Shaw and Prasad (1970) and Harris and Hopkinson (1976). To facilitate comparison between species, alleles for each locus were designated by their electrophoretic mobilities relative to the most common allele in M. capensis for that locus. Alleles with products migrating cathodally were prefixed with a minus sign.

The gene-products of the following 31 homologous loci were examined in each species. We used a discontinuous lithium-borate tris-citric acid buffer (pH 8·1; Ridgway et al., 1970) to examine variation for creatine kinase (Ck-A and Ck-B, EC 2.7.3.2), glucosephosphate isomerase (Gpi-A and Gpi-B, EC 5.3.1.9), lactate dehydrogenase (Ldh-A, Ldh-B and Ldh-C, EC 1.1.1.27), phosphoglucomutase (Pgm-1 and Pgm-2, EC 2.7.5.1), phosphogluconate dehydrogenase (Pgd, EC 1.1.1.44), non-specific protein (Pt-1, Pt-2, Pt-3, Pt-4, Pt-5), superoxide dismutase (Sod-1, EC 1.15.1.1) and sorbitol dehydrogenase (Sdh, EC 1.1.1.14). We used a tris-citric acid buffer (pH 6·9;

Whitt, 1970) to examine glyceraldehyde phosphate dehydrogenase (Gap-1 and Gap-2, EC 1.2.1.12), glycerol-3-phosphate dehydrogenase (Gpd-A, Gpd-B and Gpd-C, EC 1.1.1.8), isocitrate dehydrogenase (Idh-A, EC 1.1.1.42), malate dehydrogenase (Mdh-A and Mdh-B, EC 1.1.1.37), malate dehydrogenase-NADP$^+$ (=malic enzyme, Me, EC 1.1.1.40), and a tris-borate-EDTA buffer (pH 8·7; Markert and Faulhaber, 1965) to examine guanine deaminase (Gda, EC 3.5.4.3), mannosephosphate isomerase (Mpi, EC 5.3.1.8), nucleoside phosphorylase (Np, EC 2.4.2.1) and peptidase (Pep-D, EC 3.4.13.9 and Pep-X, EC 3.4.?.?).

RESULTS

We identified the gene products of 31 protein-coding loci by the criteria of Allendorf and Utter (1979) and by comparison with other related teleosts. Proteins encoded by Ck-B, Gap-2, Gpd-A, Gpd-B, Ldh-C, Pep-X, Pt-1, Pt-2, Pt-3, Pt-4 and Sdh appeared as invariant bands on the gels with the same mobilities for both species. The products of Np and Pt-5 were invariant within species but were fixed for different alleles between species. Three enzymes encoded by Mpi, Pgm-1 and Pgm-2 (fig. 3) showed double-banded heterozygous phenotypes in one or both species. Ten proteins encoded by Gda, Gpd-C, Gpi-A, Gpi-B (fig. 2), Idh-A, Mdh-A, Mdh-B, Pep-D, Pgd and Sod-1 had triple-banded heterozygotes typical for dimeric enzymes in at least one species. Although a dimer, Ck-A exhibited double-banded heterozygous phenotypes typical for teleosts (Ferris and Whitt, 1978). The products of Gap-1, Ldh-A, Ldh-B and Me showed broad-banded or five-banded heterozygous phenotypes typical for tetrameric enzymes in at least one species.

The amount of genetic variation present in both species was very similar. In M. capensis, 13 of 31 loci (41·9 per cent) showed at least some polymorphism and 7 loci (22·6 per cent) had common-allele frequencies less than 0·95 in at least one sample, In M. paradoxus, 13 loci (41·9 per cent) were polymorphic, and 8 loci (25·8 per cent) were polymorphic using the 0·95 criterion of polymorphism. The proportion of expected heterozygotes at a locus may be defined by

$$h = 1 - \sum x_i^2$$

where x_i denotes the frequency of the ith allele. Average heterozygosity ($\bar{H}$) is the arithmetic mean of h over all loci including monomorphic loci. In
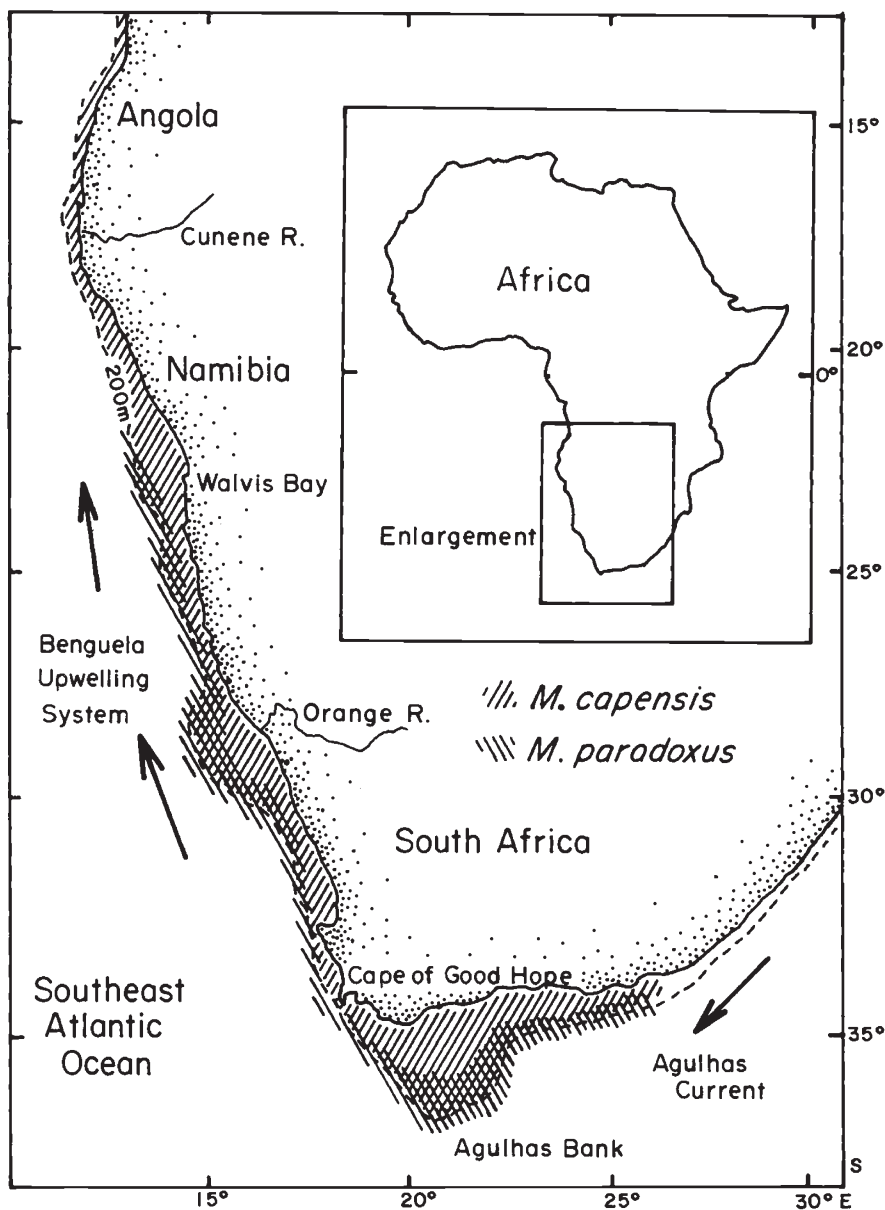
**Figure 1** Geographic distributions of southern African hakes, *Merluccius capensis* and *M. paradoxus*.

M. capensis, $\bar{H}$ per sample ranged from 0·046 to 0·055 and averaged 0·051 ($\pm$0·023) over 13 samples. $\bar{H}$ for *M. paradoxus* ranged between 0·059 and 0·070 and averaged 0·063 ($\pm$0·026) over 10 samples. The difference between these two average values of H was not significant (t-test on arcsin transforms of locus heterozygosities; Archie, 1985).

The relative amounts of subdivision at different levels of population organisation was summarised by the gene diversity statistics of Nei (1973) with the computing algorithm of Chakraborty *et al.* (1982). Total gene diversity (H_T; heterozygosity of pooled allele frequencies) was partitioned into two components

$$H_T = H_S + D_{ST}$$

where H_S is the average subpopulation heterozygosity and D_{ST} is the proportion of H_T that is due to differences between subpopulations. Therefore,

PGM
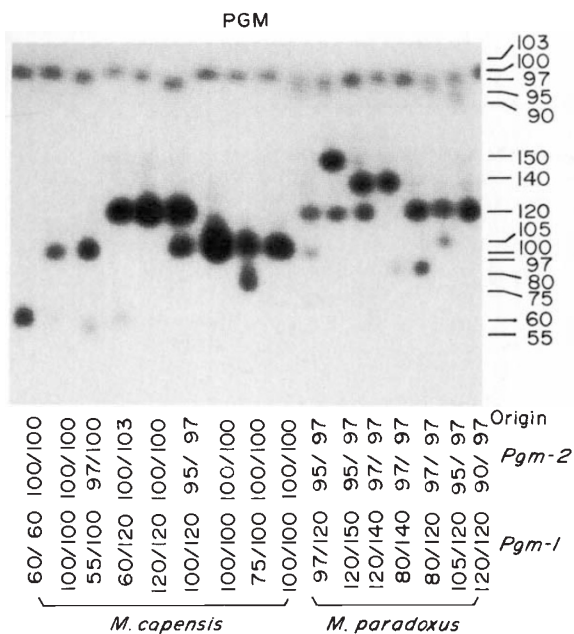
Figure 2 Electrophoretic variability for phosphoglucomutase in muscle tissues of *Merluccius capensis* (C) and *Merluccius paradoxus* (P).

GPI

Figure 3 Electrophoretic variability for glucosephosphate isomerase in muscle tissues of *Merluccius capensis* (C) and *Merluccius paradoxus* (P).

H_S/H_T is the relative proportion of the total gene diversity that is contained within subpopulations and was 0·983 and 0·987 for *M. capensis* and *M. paradoxus*, respectively. $G_{ST} = D_{ST}/H_T$ is the relative proportion of the total diversity that is due to differences between subpopulations and was 0·017 and 0·013 for *M. capensis* and *M. paradoxus*, respectively. Since these values represent very low levels of population differentiation, allele frequencies were pooled by species for further analysis (table 1).
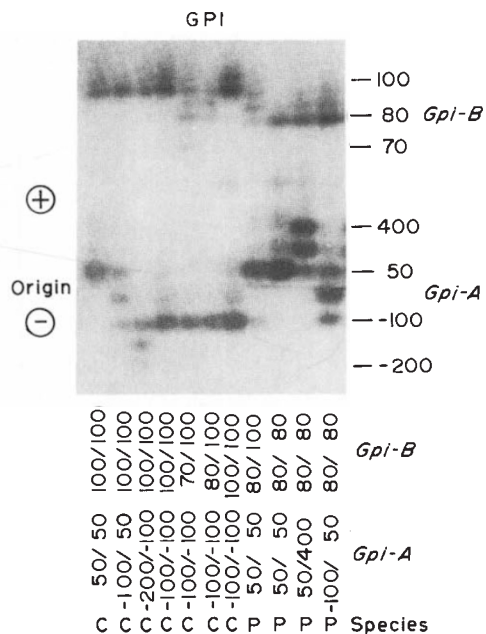
Genetic identities between taxa for a locus may be computed from allele frequency data by

$$I = J_{xy}/(J_x J_y)^{1/2}$$

where J_{xy} is the probability of identity of two alleles drawn at random, one each from populations x and y (Nei, 1972). This probability is standardised by the geometric mean of the probabilities of identity within populations x and y. An estimate of I over all loci may be computed by averaging J_{xy}, J_x and J_y over loci. Genetic distance (D) is defined as

$$D = -\log_e I.$$

Nei and Roychoudhury (1974) present a formula for computing the standard error of D. The average D (31 loci) between pairs of samples of *M. capensis*

was 0·0007 and that between pairs of samples of *M. paradoxus* was 0·0006. *I* between the two species was 0·558 (±0·160) and *D* between species was 0·583 (±0·160).

DISCUSSION

Several loci were fixed or nearly fixed for different alleles between these species and we interpret this as convincing evidence that *M. capensis* and *M. paradoxus* do not share a common gene pool. We also did not observe any individuals with hybrid genotypes. Several studies show that there is generally good agreement between traditional taxonomy and molecular distance. In some instances, however, where there is little morphological differentiation, as with the hakes in the present study, molecular distance may then be used as a guide to taxonomy. Thorpe (1982) summarised genetic distance data for 2664 pairs of taxa of invertebrates and vertebrates and found that an average *D* between congeneric species was 0·62. In a review of genetic distances specifically between fish taxa, Shaklee *et al.* (1982) found that for pairs of taxa classified as related species, *D* ranged from 0·025 to 0·65 and averaged 0·30. Thus our finding of *D* = 0·583 between these two hakes indicates that they should be treated as full species.

Table 1 Summary of alleles frequencies for southern African hakes, *Merluccius capensis* and *M. paradoxus*. Alleles are arranged according to their electrophoretic mobility beginning from the cathodal end of the gel

| Locus[1]	Species	Allele														h
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Ck-A	C	<0·001	0·198	0·004	0·797	0·001										0·327
	P	—	—	—	—	1·0										0·0
Gap-1	C	1·0	—													0·0
	P	0·002	0·998													0·004
Gda	C	0·999	0·001													0·002
	P	1·0	—													0·0
Gpd-C	C	—	1·0													0·0
	P	0·998	0·002													0·004
Gpi-A	C	—	—	0·052	0·753	0·003	0·190	0·002	—	—	—	—				0·394
	P	0·001	0·001	—	0·005	—	0·967	—	0·005	0·020	0·001	0·001				0·059
Gpi-B	C	0·001	0·015	0·001	0·027	0·001	0·956	0·001								0·081
	P	0·002	—	—	0·993	0·005	—	—								0·016
Idh-B	C	—	—	0·001	0·999											0·001
	P	0·021	0·816	—	0·163											0·308
Ldh-A	C	—	0·001	0·999	—											0·002
	P	0·001	0·002	0·985	0·012											0·030
Ldh-B	C	0·005	—	0·995												0·009
	P	0·002	0·003	0·995												0·059
Mdh-A	C	0·002	0·997	0·001												0·0
	P	—	1·0	—												0·0
Mdh-B	C	—	—	1·0												0·0
	P	0·002	0·995	0·003												0·010
Me	C	—	—	0·998	0·002	—	—									0·040
	P	0·005	0·018	0·931	—	0·043	0·003									0·131
Mpi	C	—	—	—	—	1·0	—	—	—							0·0
	P	0·011	0·173	0·010	0·656	0·001	0·143	0·003	0·003							0·520
Np	C	1·0	—													0·0
	P	—	1·0													0·0
Pep-D	C	0·005	0·358	0·010	0·057	—	0·569	0·001	—	—						0·551
	P	—	0·001	—	—	0·232	0·005	0·548	0·209	0·005						0·602
Pgd	C	—	—	—	—	1·0										0·0
	P	0·001	0·972	0·002	0·023	0·001										0·054
Pgm-1	C	0·004	0·009	0·026	—	0·003	—	0·893	0·004	0·054	—	0·007	—	—	—	0·204
	P	—	—	—	0·006	—	0·038	0·002	—	—	0·906	—	0·003	0·042	0·003	0·169
Pgm-2	C	0·002	—	0·006	—	0·984	—	0·008								0·028
	P	—	0·002	0·065	0·921	—	0·012	—								0·147
Pt-5	C	—	1·0													0·0
	P	1·0	—													0·0
Sod-1	C	—	—	—	1·0											0·0
	P	0·001	0·008	0·991	—											0·019

[1] The following loci were fixed for the same allele in both species *Ch-B*, *Gap-2*, *Gpd-A*, *Gpd-B*, *Ldh-C*, *Pep-X*, *Pt-1*, *Pt-2*, *Pt-3*, *Pt-4* and *Sdh*.

Average heterozygosity for *M. capensis* was 0.051 and that for *M. paradoxus* was 0·063 and both values were similar to those found in other fishes. Winans (1980) summarised average heterozygosity in 82 fish and found an overall average of $0·048 \pm 0·033$. In the infite alleles model of neutral mutations the expected heterozygosity in an equilibrium population is equal to $4N_e v$, where N_e is the effective population size and v is the mutation rate per locus per generation (Kimura and Crow, 1964). Fuerst *et a.* (1977) have generated theoretical distributions of locus heterozygosities for equilibrium populations having a specific average heterozygosity. A comparison of the observed distribution of locus heterozygosities in the two hakes with that expected under the infinite-alleles model for $H = 0·05$ is shown in fig. 4. This agreement suggests that these populations are in drift-mutation equilibrium.

An examination of the distributions of expected and observed allele frequencies, however, gives a different picture. When a bottleneck in population size occurs, as for instance in a small founder population, average heterozygosity declines by
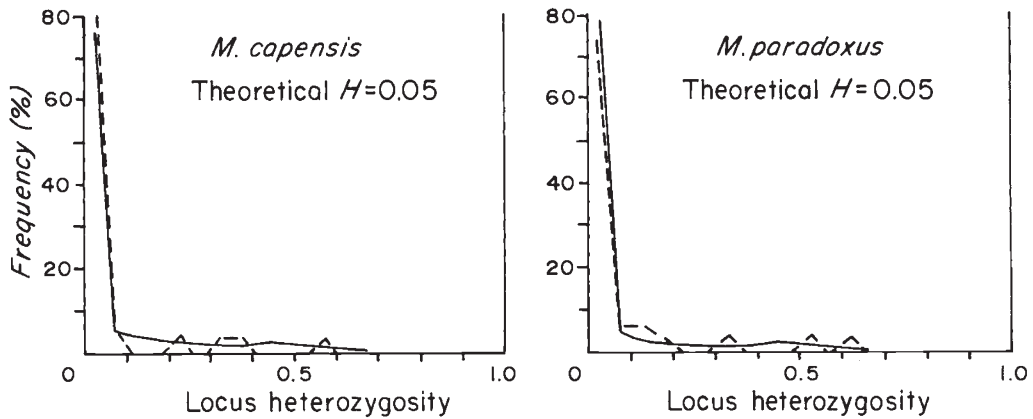
Figure 4 Comparisons of observed (broken line) locus heterozygosities with theoretical (solid line) distributions of heterozygosities expected at equilibrium in the infinite-alleles model of mutation.

$1/(2N_e)$ for a one time bottleneck (Nei *et al.*, 1975; Chakraborty and Nei, 1977). If population growth is slow after the bottleneck, heterozygosity continues to decline. With time, new alleles appear through mutation and accumulate in the population at a rate that depends upon $4N_e v$. But there is a delay in the return to original levels of heterozygosity as rare alleles drift to intermediate frequencies (Maruyama and Fuerst, 1984). Thus during the transit to equilibrium, there is an excess of low-frequency alleles compared with that expected for a given value of H in populations at drift-mutation equilibrium.

We compared the observed distributions of the number of alleles at different allele frequencies with the expected distributions under the infinite allele model of neutral mutations. The expected number of alleles with frequencies between p and q is

$$n(p, q) = \sum_{i=Np+1}^{Nq} \theta N_i / i (\theta + N - 1)_i$$

where N is the total number of genes sampled and $\theta = 4N_e v = H/(1-H)$ (p. 400, Waterson, 1984). This theoretical distribution for 0·05 frequency intervals is compared with the observed distributions of the two hakes in fig. 5. It is apparent that there is a large excess of alleles with frequencies
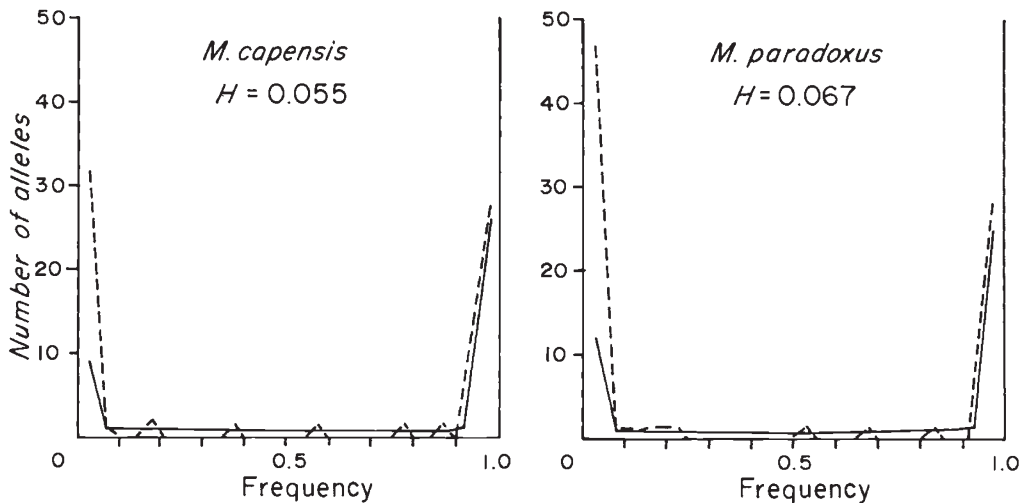
Figure 5 Comparisons of observed (broken line) numbers of alleles at 0·05 frequency intervals with the theoretical (solid line) distribution expected at equilibrium in the infinite-alleles model.

less than 0·05 for both species. The Kolmogorov-Smirnov test (Sokal and Rohlf, 1981) for fit to the theoretical distribution indicates that this deviation is highly significant for both *M. capensis* ($D_{67} = 0.337$, $P < 0.01$) and *M. paradoxus* ($D_{84} = 0.424$, $P < 0.01$).

The time needed to reach equilibrium for neutral genes is very long for species such as hakes that have large population sizes. The rate of approach to equilibrium is of the order $2v + 1/(2N_e)$ each generation. For these hakes, which presently have species effective population sizes of at least 10^8 (Payne *et al.*, 1986) and an estimated mutation rate of about 10^{-7} (Kimura and Ohta, 1971), the time to equilibrium is on the order of 10 million years or more. Thus, the effects of even an ancient bottleneck may still be apparent in allele-frequency distributions.

Genetic distance may be used to estimate the amount of time since divergence began between two taxa. Empirical calibrations of D with time suggest that a D of 1·0 is equal to about 19 million years of isolation (Carlson *et al.*, 1978; Vawter *et al.*, 1980). Critics of the electrophoretic molecular clock concept, however, have pointed out that there are large errors associated with the clock because of errors in calibration (Radinsky, 1978), because of sampling proteins with different evolutionary rates (Skibinski and Ward, 1982) and because of the stochastic nature of the clock (Fitch, 1976). Thus, we suggest that only a period of time may realistically be computed using D and its standard error to estimate when divergence most likely began. For the two southern African Hakes this period of time extends from 7·6 to 13·6 million years ago. This estimate represents the time when the lineages leading to the two species split.

There are two biogeographic scenarios that may explain the sympatric distributions of these fishes in southern Africa. The first is that past episodes of oceanic cooling (CLIMAP 1976; Shannon 1985) displaced northward and isolated ancestral populations of southern African *Merluccius*. This seems unlikely, however, because paleo-oceanographic evidence suggests that the southward-flowing warm Agulhas Current from the Indian Ocean persisted during periods of Pliocene and Pleistocene cooling (Shannon, 1985). The more likely explanation is that the two species represent different biogeographic dispersals of North Atlantic taxa along the west coast of Africa. If so, the species representing the more recent dispersal may show a greater genetic affinity to one of the North Atlantic hakes. We are, presently, collecting samples of other hakes to test this hypothesis.

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