

Allozyme Phylogeny of *Spheniscus* Penguins

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There are four species of *Spheniscus* penguins, all with distributions limited to the Southern Hemisphere. The Humboldt (Peruvian) Penguin (*S. humboldti*) is found on the west coast of South America in the cold, upwelled waters of the Humboldt Current (Stonehouse 1975). The Magellanic Penguin (*S. magellanicus*) occurs to the south of the Humboldt Penguin on the west coast and on the south-eastern coast of South America. The Jackass Penguin (*S. demersus*) occurs on the west and south coasts of southern Africa in the cold Benguela Current, while the Galapagos Penguin (*S. mendiculus*) is restricted to the tropical Galapagos Archipelago.

The phylogenetic relations among these birds are uncertain. Murphy (1936) suggested that the Humboldt and Galapagos penguins were sister taxa and that the Magellanic and Jackass penguins were sister taxa, basing this conclusion on several morphological features and geographic distributions. However, a recent analysis of measurements of external and skeletal

structures showed that the Humboldt and the Magellanic penguins were phenetically more similar to each other and to the Jackass Penguin than to the Galapagos Penguin (Livezey 1989). The larger skeletal analysis in the latter study revealed that of the four congeners, *S. magellanicus* and *S. humboldti* were most similar (Livezey 1989). These similarities, however, may not reflect phylogenetic relations because of convergence.

In this study, we estimated a phylogenetic tree from allozyme frequencies for three of these taxa (the Humboldt, Magellanic, and Jackass penguins) and used the closely related Rockhopper (*Eudyptes chrysocome*) and Macaroni (*E. chrysolophus*) penguins from the Southern Ocean as outgroups. We also examined tissue expressions of loci to search for changes in expression during speciation (Mindell and Sites 1987) and compared the average heterozygosities among taxa to search for historical population bottlenecks.

Methods.—We collected tissues from 45 Jackass Penguins on 19 December 1986 at Stony Point (34°20'S, 18°53'E), Cape Province, South Africa about six hours after a leopard (*Pantherus pardus*) killed the birds. Samples of cardiac and breast muscle, liver, and vitreous fluid were removed for electrophoretic analysis. Four

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birds were in juvenile plumage. We also included three downy Jackass Penguins recently dead at the nest and three juveniles killed by oil pollution near Cape Town. Tissues from frozen carcasses of captive grown Magellanic and Humboldt penguins were collected at the Sea World Research Institute, San Diego, California. Macaroni and Rockhopper penguins were collected on Marion Island (46°52'S, 37°51'E). Tissue samples were held at -70°C until electrophoretic analysis. Aqueous proteins were extracted with a 0.1 M phosphate (pH = 7.4) buffer and, after centrifugation at 5,000 × g for 10 min, the clear supernatant was used for electrophoresis. Stain protocols followed Harris and Hopkinson (1976), and loci were designated numerically by the mobilities of their products beginning at the anodal end of a gel.

We detected the products of 30 loci with 22 active-enzyme stains. We used a Tris-citric acid-lithium hydroxide discontinuous buffer system (pH = 8.7; Ridgway et al. 1970) for aspartate aminotransferase (*Aat*), alcohol dehydrogenase (*Adh-1*, *Adh-2*), esterase (*Est-1*, *Est-2*, *Est-3*, *Est-4*), creatine kinase (*Ck-A*, *Ck-B*), glucocephosphate isomerase (*Gpi*), lactate dehydrogenase (*Ldh-A*, *Ldh-B*), phosphoglucomutase (*Pgm-1*, *Pgm-2*), superoxide dismutase (*Sod*), a Tris-citrate buffer (pH = 6.9; Whitt 1970) for acid phosphatase (*Acp*), adenylate kinase (*Ak*), glyceraldehydophosphate dehydrogenase (*Gap-1*, *Gap-2*, *Gap-3*), glycerol-3-phosphate dehydrogenase (*Gpd-1*, *Gpd-2*), isocitrate dehydrogenase (*Icd-A*, *Icd-B*), malate dehydrogenase (*mMdh*, *sMdh*), phosphogluconate dehydrogenase (*Pgd*), and a Trisborate-NaEDTA buffer (pH = 8.7; Markert and Faulhaber 1965) for aldolase (*Ald*), fumerase (*Fum-1*, *Fum-2*), glutamate pyruvate transaminase (*Gpt*), mannosephosphate isomerase (*Mpi*), nucleoside phosphorylase (*Np*), and peptidases with the substrates glycyl-leucine (*Pep-A*, *Pep-C*), leucyl-glycyl-glycine (*Pep-B1*, *Pep-B2*), and phenylalanyl-proline (*Pep-D1*, *Pep-D2*).

Results and Discussion.—Several enzyme systems showed developmental differences in gene expression among downy chicks, juveniles, and adults of the Jackass Penguin (Table 1). *Adh-2* and *Gap-3* were expressed only in chicks. *Adh-1*, *Icd-B*, and *Ldh-A* were not expressed in chicks. Both *Ldh-A* and *Ldh-B* were expressed in juveniles and adults, and interlocus heterotetrameric bands appeared between the homotetrameric products of each locus. All other loci were expressed in each age group. There were no differences in the tissue distributions of gene expression between the adults of *Spheniscus* and those of *Eudyptes*. No significant deviations from Hardy-Weinberg expectations were detected with the G-test and Levene's (1949) correction for small samples for any of the polymorphic loci in any of the taxa. *Ck-A*, *Est-3*, *Fum-1*, *Fum-2*, *Gpi*, *Gpd-1*, *Icd-B*, *Ldh-B*, *sMdh*, *Mpi*, *Pep-A*, *Pep-C*, *Pep-B1*, *Pgm-2*, and *Sod* were polymorphic within one or more species (Table 2). Three loci *Gap-1*, *Ldh-A*, and *Pgm-1* were fixed for different alleles between genera, and 13 loci, *Ald*, *Ak*, *Ck-B*, *Est-2*, *Est-4*,

TABLE 1. Locus expression in Jackass Penguins.^a

Locus	Muscle	Heart	Liver	Eye
<i>Acp</i>	+	++	+++	-
<i>Adh-1</i> ^b	-	-	+++	-
<i>Adh-2</i> ^c	-	-	+++	-
<i>Ak</i> ^b	+++	-	-	-
<i>Est-1</i>	-	++	+++	+
<i>Est-2</i>	-	-	++	-
<i>Est-3</i>	+	++	+++	+
<i>Est-4</i>	-	+	++	-
<i>Ck-A</i> ^b	+++	-	-	-
<i>Ck-B</i>	-	+++	-	-
<i>Fum-1</i>	++	+++	+	-
<i>Fum-2</i>	+++	++	+	-
<i>Gpi</i>	+++	+++	+++	+++
<i>Gpt</i>	+++	++	+	-
<i>Gap-1</i>	++	+++	+++	+
<i>Gap-2</i> ^b	+	-	++	-
<i>Gap-3</i> ^b	-	+	+++	-
<i>Gpd-1</i>	++	+	+++	-
<i>Gpd-2</i>	+	+	+++	-
<i>Gda</i>	++	+	+++	-
<i>Icd-A</i> ^b	+++	+++	++	-
<i>Icd-B</i>	+	++	+++	-
<i>Ldh-A</i> ^b	+++	-	++	+
<i>Ldh-B</i> ^b	-	+++	+++	-
<i>mMdh</i>	+	+++	+++	+
<i>sMdh</i>	++	+++	+	+++
<i>Mpi</i>	+++	+++	+++	++
<i>Np</i>	-	-	+	+++
<i>Pep-A</i>	++	+++	+++	+
<i>Pep-C</i>	+	+	+++	-
<i>Pep-B1</i>	+++	+++	+++	+
<i>Pep-B2</i>	+	++	+++	-
<i>Php-D1</i>	+	++	++	-
<i>Php-D2</i>	+	++	+++	-
<i>Pgd</i>	+	++	+++	+
<i>Pgm-1</i>	-	-	+++	-
<i>Pgm-2</i>	+++	+	+	-
<i>Sod</i>	++	++	+++	-

^a (-) banding absent; (+) banding just perceptible; (++) banding moderately dense; (+++) banding very dense.

^b Banding absent or much reduced in chicks.

^c Banding absent in adults.

Gap-2, *Gda*, *Icd-A*, *mMdh*, *Me-2*, *Pep-B2*, *Pep-D2*, and *Pgd* were invariant among taxa.

We estimated unbiased average heterozygosity and its variance (Nei and Roychoudhury 1974, Nei 1978) for each taxon. For the Jackass Penguin, only the birds from Stony Point were used to estimate heterozygosity, because they presumably represented a single population. Expected heterozygosities averaged over 30 loci were 0.031 and 0.046 for Rockhopper and Macaroni penguins, respectively, and 0.025, 0.054, and 0.081 for Humboldt, Magellanic and Jackass penguins, respectively. Jackknifed estimates of heterozygosities (Weir 1990) were similar to the simple averages over loci. The distributions of single-locus

TABLE 2. Allelic frequencies for penguins in the genera *Spheniscus* and *Eudyptes*.^a

Locus	Allele	Species				
		Jack-ass	Magel-lanic	Hum-boldt	Rock-hop-per	Macaroni
<i>Est-3^b</i>	90	—	—	—	1.000	—
	95	—	0.083	—	—	0.944
<i>Fum-2</i>	50	—	—	—	—	0.050
<i>Gpi</i>	600	—	—	—	0.100	—
	150	—	—	1.000	0.900	1.000
<i>Gap-1</i>	—300	0.189	—	—	—	—
	—100	1.000	1.000	1.000	—	—
<i>Gpd-1</i>	—200	—	—	—	1.000	1.000
	—5	—	—	0.333	—	—
<i>Icd-B</i>	—20	0.411	0.500	0.667	—	—
	—100	0.589	0.500	—	1.000	1.000
<i>Ldh-B</i>	150	0.378	0.083	—	—	—
<i>Ldh-A</i>	—500	—	—	—	1.000	1.000
<i>Ldh-B</i>	210	—	0.083	—	—	—
	180	—	—	—	1.000	1.000
<i>mMdh</i>	130	—	0.167	—	—	—
	—100	1.000	1.000	1.000	1.000	0.950
<i>Mpi</i>	—200	—	—	—	—	0.050
	90	—	—	—	0.150	—
<i>Pep-A</i>	105	0.533	0.167	—	—	—
	85	0.422	—	0.143	—	—
<i>Pep-C</i>	95	—	—	—	0.222	0.350
	110	—	—	—	0.778	0.650
<i>Pep-B1</i>	95	0.033	—	—	—	—
	110	—	—	—	1.000	1.000
<i>Pgm-1</i>	40	0.011	—	—	—	—
	180	—	—	—	—	0.050
<i>Pgm-2</i>	300	—	—	—	—	1.000
	150	—	—	—	0.950	1.000
<i>Sod</i>	200	—	—	—	0.050	—
	40	—	—	1.000	—	—
	105	0.044	—	—	—	—
	110	—	—	—	—	0.350
	190	—	—	—	1.000	0.650

^a *Adh-2*, *Ald*, *Ak*, *Ck-A*, *Ck-B*, *Est-2*, *Est-4*, *Gap-2*, *Gda*, *Idh-A*, *sMdh*, *Mc-2*, *Pep-D2*, and *Pgd* fixed for same allele in all taxa. Sample sizes (*n*): Jackass, 45; Magellanic, 6; Humboldt, 9; Rockhopper, 10; Macaroni, 10.

^b Frequencies for anodally migrating common (100) alleles obtained by subtraction.

heterozygosities for each of the penguin taxa (Fig. 1) showed a strong L-shaped distribution and appeared to fit the predictions of the infinite-alleles model for populations in equilibrium with mutation and drift (Fuerst et al. 1977).

To test for differences in heterozygosity (*H*) between species, we first used a *t*-test on arcsine-transformed single-locus heterozygosities, and this suggested that none of the differences in *H* was significant. However, since the power of these tests (probability of detecting a true difference) was about 30% or less (Archie 1985:fig. 6), we then used the jackknifed procedure (Weir 1990) to produce an estimate of *H*^{*} and its variance. These comparisons showed that *H*^{*} for the Jackass Penguin was significantly greater (*P* <

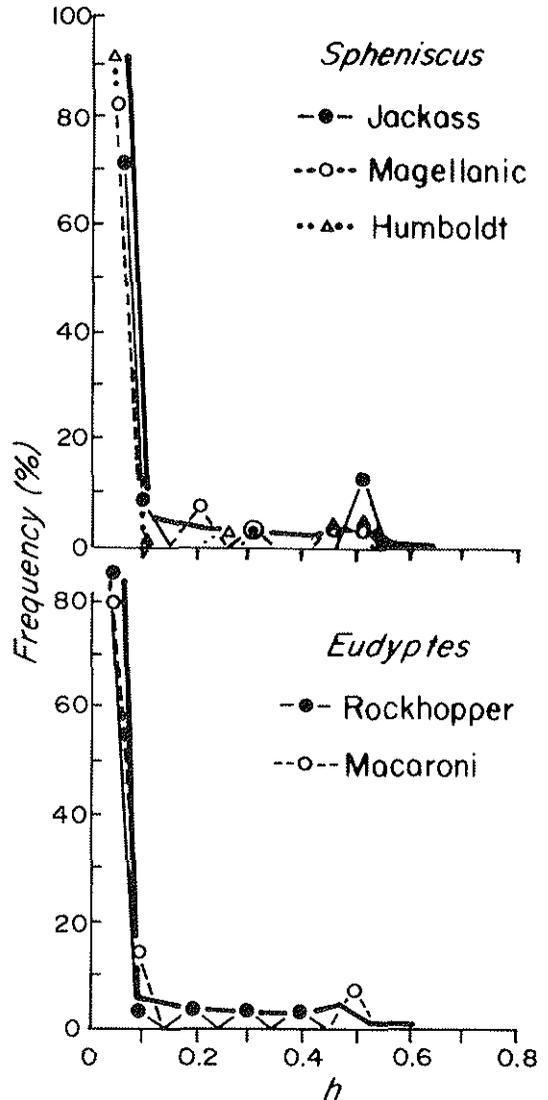


Fig. 1. Distributions of single-locus heterozygosities in *Spheniscus* and *Eudyptes* penguins.

0.05) than the values for each of the other taxa. The *H*^{*} for the Magellanic Penguin was significantly greater (*P* < 0.05) than those for the Humboldt and Rockhopper penguins, and the *H*^{*} for the Macaroni Penguin was significantly greater (*P* < 0.05) than that of the Humboldt Penguin.

The smaller heterozygosities for the Humboldt and Magellanic penguins may be because we took these samples from a captive colony, and inbreeding may have led to the loss of heterozygosity. The value of *H* of 0.081 for Jackass Penguin is larger than the average for other birds (*H* = 0.044; Evans 1987). The natural colonies of Jackass Penguins have historically been very large. For instance, a single colony on Das-

TABLE 3. Nei's modified genetic distance (Nei 1978, Hillis 1984) below diagonal and standard error above diagonal penguin-species pairs.

	Jack- ass	Magel- lanic	Hum- boldt	Rock- hopper	Maca- roni
Jackass		0.008	0.050	0.120	0.128
Magellanic	0.017		0.052	0.119	0.126
Humboldt	0.107	0.080		0.117	0.123
Rockhopper	0.389	0.370	0.357		0.050
Macaroni	0.436	0.411	0.403	0.073	

sen Island, South Africa once numbered over 1.5 million birds (Shelton et al. 1984). Both the Humboldt and Magellanic penguins also occur in large colonies (Stonehouse 1975). On the other hand, the smaller average heterozygosities for the *Eudyptes* penguins may indicate smaller populations or historical bottlenecks (Nei et al. 1975).

We inferred phylogenies from allozyme frequencies in three ways. We calculated Nei's (1978) unbiased genetic distance (Table 3) with Hillis' (1984) modified \bar{D} between pairs of taxa and used the UPGMA cluster analysis to produce a phenetic tree. Since a phenetic tree may not be the best estimate of the true phylogeny, we used two parsimony methods to produce cladistic trees: FREQPARS (Swofford and Berlocher 1987) to infer a parsimony tree directly from allelic frequencies; and PAUP (Swofford 1985) with Wagnerian parsimony and an exhaustive search to infer a tree from unordered allelic states (independent allele model). Since only 4 of the 30 loci could be ordered by allelic gains and losses with two *Eudyptes* species as outgroups, we could not produce a tree from ordered states. All of the trees had the same topology (Fig. 2). Jackass and Magellanic penguins were most closely related with a genetic distance \bar{D} of $0.017 \pm \text{SE of } 0.008$ (Table 2). The average distance between these taxa and the Humboldt Penguin was 0.094. The average \bar{D} between species of *Spheniscus* and *Eudyptes* was 0.394, and between the Rockhopper and Macaroni penguins was 0.073 ± 0.050 .

Our trees agree with the phylogeny suggested by Murphy (1936) to the extent that the Magellanic and Jackass penguins are closely related. Inferences about the Galapagos Penguin are not possible with these data; there are several positions the Galapagos Penguin may fit into the tree. The small amount of genetic divergence between the Jackass and Magellanic penguins was unexpected because these species are located on different continents. A distance of 0.017 between these taxa is more typical of that found between conspecific populations than of a distance between congeneric species (Thorpe 1982). One explanation is that there is long-distance migration and gene flow between the two species. Magellanic Penguins have traveled as far east as South Georgia in the mid-Atlantic Ocean (Tickell 1965), and penguins with two

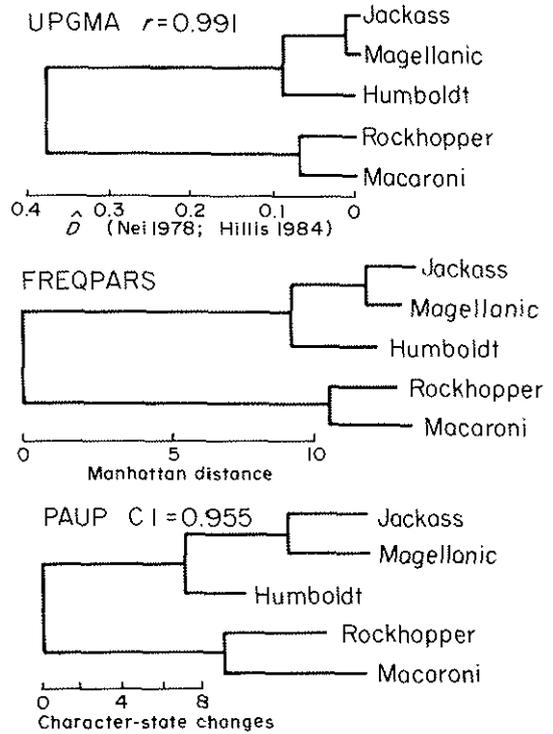


Fig. 2. Phenetic and cladistic trees for *Spheniscus* and *Eudyptes* penguins (r , cophenetic correlation; CI , consistency index).

neck bands (Magellanic Penguins?) have been reported in southern Africa (e.g., Donnelly 1965, Rowlands 1965). The second band in these latter penguins, however, is always much narrower than that of the Magellanic Penguin, and many Jackass Penguins show a partial second band that may be an atavism rather than evidence of hybridization. An alternative explanation is that these two species have only recently diverged from each other.

The relatively small genetic distances between pairs of the three *Spheniscus* penguins indicate that speciation events may have been associated with dispersal and founder events in the Pliocene or Pleistocene rather than with continental rifting between South America and Africa in the Cenozoic. Although several fossils have been described from southern South America and Africa, none is clearly ancestral to modern species (Fordyce and Jones 1990). If we assume a molecular-clock calibration of a distance (D) of 1.0 equalling five million years (Nei 1975), the two major *Spheniscus* clades diverged about 500,000 mya in the mid-Pleistocene, and the Jackass and Magellanic penguins appeared in the late Pleistocene.

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