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Molecular Analysis of mtDNA Types in Exploited Populations of Spinner Dolphins (*Stenella longirostris*)

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ABSTRACT

Stock structure of geographic forms of the spinner dolphin (*Stenella longirostris*) in the eastern tropical Pacific (ETP) is a conservation issue. These animals are incidentally killed during yellow fin tuna purse seining by US and international fleets, and mortality quotas apportioned by stock have been established by legislative act. Four management stocks of spinner dolphins in the ETP are currently defined on the basis of morphology and geographical distribution. However, recent work proposes that two of the stocks (the northern and southern 'whitebelly' spinners) comprise a broad zone of hybridisation or clinal integration between the neighbouring endemic 'eastern' spinner and the more typical spinners residing to the west and around the world in the tropics. If this hypothesis is true, evidence of current or recent gene flow between the neighbouring 'stocks' should be apparent. To investigate this, we conducted a molecular analysis of mitochondrial DNA (mtDNA) extracted from livers of 151 spinner dolphins from the ETP and 13 spinner dolphins from the Timor Sea. We also analysed mtDNA from 10 spotted dolphins from the ETP. Liver mtDNA from each animal was digested with six restriction enzymes to deduce the individual mtDNA haplotypes. Genetic distances were then inferred within the sample pool by comparison of the individual mtDNA haplotypes in a pair-wise fashion.

The results suggest the following three points: (1) The morphologically defined neighbouring eastern forms of ETP whitebelly spinners are not genetically distinct at the level of resolution of our analysis. We found no concordance of mtDNA haplotype with either the stock type of the school or individual morphology. The mean between-form distance was not significantly different from the two mean within-form distances. Thus, introgression is likely occurring (or has recently occurred) and significant genetic interchange can be inferred. (2) Mean within-school mtDNA diversity varied from school to school, and no clustering of certain haplotypes within specific schools was detected. (3) Timor Sea spinner and the spotted dolphins have unique mitochondrial genotypes likely characteristic of their geographic and genetic separation in the case of the spinner dolphins and their species-level separation in the case of the spotted dolphins. These findings reinforce the morphologically-based hypothesis of non-uniqueness of the whitebelly form and suggest that relatively more emphasis should be placed on conservation of the endemic and presumably locally adapted eastern form.

Keywords: spinner dolphin; spotted dolphin; eastern tropical Pacific; genetics; stock identity; morphology/anatomy; management; incidental capture.

INTRODUCTION

Common sense, as well as many national and international mandates, dictates that marine mammals be managed on the basis of population or breeding units. Good management demands knowledge of the genetic as well as the spatial and temporal aspects of intraspecific population structure. It also requires awareness of how exploitation may alter this structure (Larkin, 1981; Allendorf *et al.*, 1986). Intraspecific populations have been

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variously called geographic forms, stocks, races, etc. In many situations, the populations, isolated to some unknown degree, may harbor sufficient genetic uniqueness to provide adaptable advantages within their habitat and make them morphologically distinguishable, at least in the modal sense, from each other.

Pelagic dolphins, primarily spotted dolphins (*Stenella attenuata*), spinner dolphins (*S. longirostris*), striped dolphins (*S. coeruleoalba*) and common dolphins (*Delphinus delphis*), are killed incidentally during fishing with purse seines for yellowfin tuna (*Thunnus albacares*) in the eastern tropical Pacific Ocean (ETP) (see Fig. 2). A total allowable annual kill of 20,500 animals is currently in force for the US purse-seine fleet (Marine Mammal Protection Act of 1972 [U.S.C. 1371 (a) (2)]). The total is apportioned to species and stocks[2]. The division of the stocks is based on morphology and to a lesser extent on distribution (Perrin *et al.*, 1985). In the case of the spinner dolphins, *S. longirostris*, four morphologic/geographic forms have been given stock status: Costa Rican, northern whitebelly, southern whitebelly and eastern (Perrin, 1975; Perrin *et al.*, 1985). These have formed the basis for management and assessment (Smith, 1983; Buckland and Anganuzzi, 1988). Neither tagging (Perrin *et al.*, 1979; Hedgepeth, 1985) nor genetic studies of allozymes (Sharp, 1981; Landino, 1987) have been successful in further clarifying the geographic boundaries for management purposes. Determination of exchange rates by tagging failed because of insufficient returns. Sharp's studies revealed too little electrophoretic variation to attempt a genetic definition of populations, whereas Landino's study revealed variation at seven loci but not significant departures from expected Hardy-Weinberg equilibrium values in populations subdivided by stock, possibly due to insufficient sample size. Consequently, the degree of genetic variation between the stocks and the isolation or interchange between them has been unknown for the spinner dolphins (and other dolphins) in the ETP.

In the most recent reviews of geographical variation in *Stenella* spp. in the eastern Pacific, Perrin *et al.* (1985) and Perrin (1990) hypothesised that the eastern form of *S. longirostris* constitutes a population adapted to local conditions and that the whitebelly spinner populations comprise a broad zone of hybridisation or clinal intergradation between the eastern form and the spinner dolphins of the Central and South Pacific. The latter closely resemble spinner dolphins in other tropical waters around the world (Fig. 1). They put forward two hypothetical models of evolution of the eastern form. The first model proposed differentiation in isolation during a Pleistocene cooling that may have established a trans-equatorial cool-water barrier isolating a part of the far eastern tropical Pacific. The other model suggested differentiation due to very strong selection in the oceanographically unique ETP (Au and Perryman, 1985). Characteristics of the eastern form that suggest it may comprise a locally-adapted population include pronounced morphological divergence from spinner dolphins in the rest of the world's oceans (small body and skull size, dark overall colouration obscuring the typical spinner colour pattern, and marked sexual dimorphism in shape of body and dorsal fin – Perrin, 1990) and differential life-history characteristics (breeding season – Barlow, 1984; ovulation rate, pregnancy rate, age at maturation and testis size – Perrin and Henderson, 1984). Ecological character displacement involving the spotted dolphin has been suggested as a possible mechanism for the differentiation (Perrin, 1984).

The first model proposes divergence in isolation. The whitebelly spinner forms might have arisen as a result of re-established contact and genetic interchange during the present interglacial period and thus would be a hybrid swarm. This model might involve a dynamic

[2] In recent years, an additional 60,000 to 100,000 dolphins have been killed annually by purse seiners of other nations (e.g. Hall and Boyer, 1987).

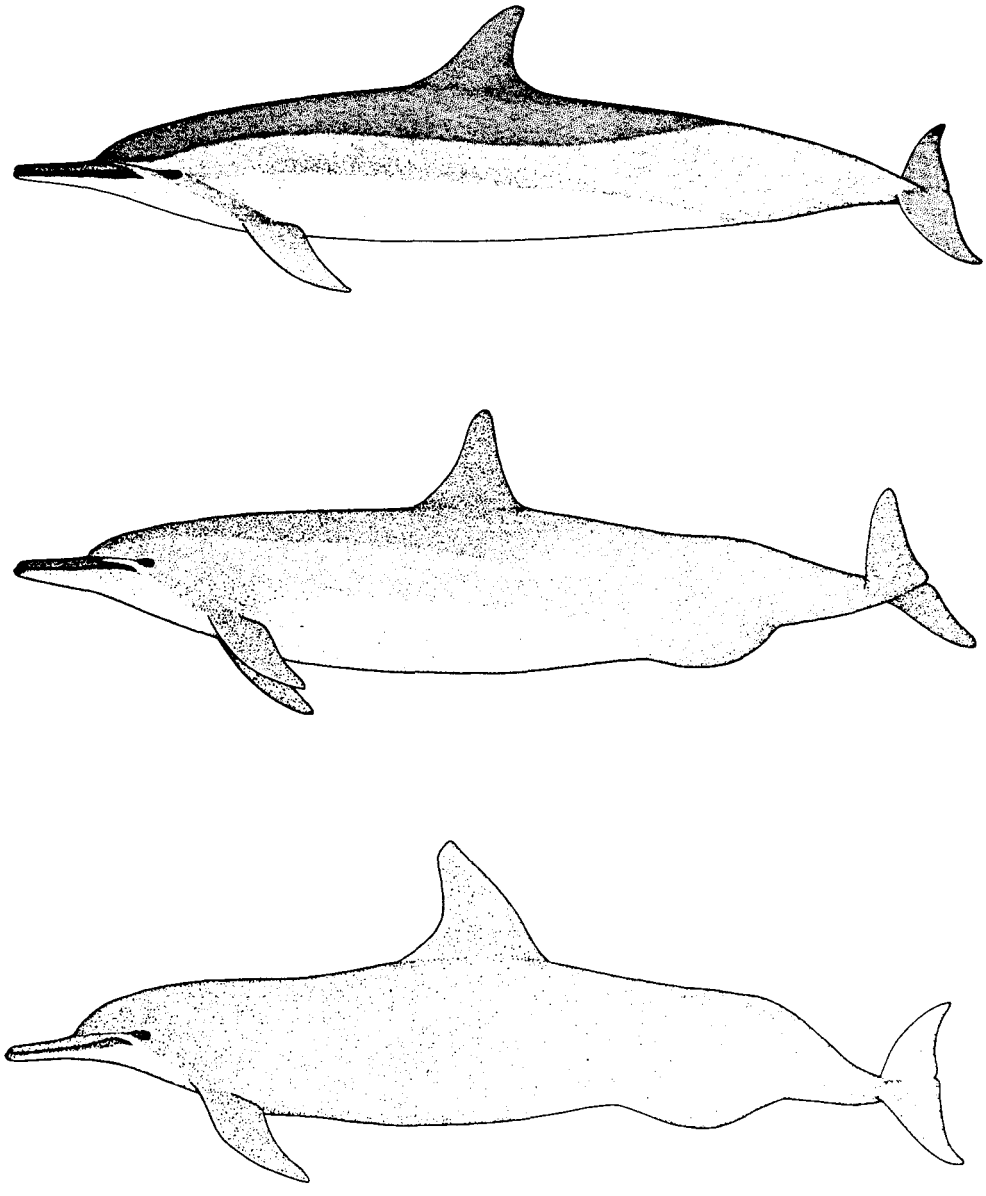

Fig. 1. Line drawing of modal appearances of the pantropical, whitebelly and eastern spinner dolphins (from Perrin, 1990).

situation, in which a much larger reservoir of 'ordinary' spinner dolphin genes located to the westward have been progressively replacing the relatively smaller eastern gene pool.

The second model proposes simple geographical differentiation without isolation. In this model, the whitebelly spinner populations might have evolved through clinal intergradation between the more primitive spinner dolphins of the Central and South

Pacific and the more specialised and derived eastern spinner dolphins. This presumably would be a relatively stationary situation in contrast to the first model. Both models propose that the whitebelly spinner form represents an intermediate form (either hybrid or intergrade) and predict substantial gene flow between the eastern and whitebelly spinner dolphin stocks.

The models proposing gene flow as an evolutionary force can be contrasted with an alternative and more conventional notion typically used in the management of marine species. This model would consider genetically isolated eastern and whitebelly stocks arising via: (1) isolation, the development of reproductive barriers, and, upon re-establishment of contact, no interchange; or (2) the slow accumulation of differences, establishing both morphological uniqueness and reproductive barriers (parapatric divergence). This hypothesis thus suggests that the whitebelly spinner form might be a relatively isolated population similar in that regard to the eastern form, rather than a hybrid form. This is the model closest to the actual current basis for management of the dolphins.

We chose to examine these alternative hypotheses of the origin of the eastern and whitebelly spinner forms (substantial gene flows vs. relative isolation) by means of mitochondrial DNA (mtDNA) analysis. The pattern of mtDNA diversity and concordance between the mtDNA genotype and the anatomic morphology, determined by the chromosomal DNA genotype, could be expected to differ between the two theoretical models. This assumption is based on observations that mtDNA can be exchanged between animal populations at a much higher rate than chromosomal DNA (Barton and Jones, 1983). Accordingly, little if any concordance between the distribution of mtDNA types and distribution of morphologic stocks might be expected in the first hypothesis postulating a substantial gene flow. In the second case, when DNA flow was halted due to reproductive isolation extending back to the Pleistocene or to parapatric divergence, some mtDNA clonal types should be unique to each population, at least those expected to have arisen since the isolation.

Quantitative genetic aspects of close taxonomic relationships can be studied through mtDNA sequence analysis. Nucleotide mutations occur about 10 times more frequently in mtDNA than in chromosomal DNA (Brown *et al.*, 1979; 1982), and therefore, analysis of mtDNA variability is more informative than the study of polymorphism in chromosomally encoded traits (Avise *et al.*, 1979b; for reviews, see Wilson *et al.*, 1985; Avise, 1986; Ferris and Berg, 1986; Avise *et al.*, 1987; Moritz *et al.*, 1987; Harrison, 1989). For the purpose of population analysis in mammals, mtDNA is assumed to be (i) inherited clonally from the female parent and (ii) identical in all somatic cells (homoplastic) (Avise *et al.*, 1987).

Our study deals with the distribution of mtDNA types among and between geographic forms and schools of the ETP spinner dolphins. We have evaluated whether these dolphins reproduce in isolation or exhibit indications of recent or ongoing reproductive interchange. For comparisons with the ETP spinner dolphins, we also obtained samples and measured mtDNA diversity of spinner dolphins from the Timor Sea off northern Australia and pantropical spotted dolphins from the ETP.

MATERIALS AND METHODS

Collection of samples

Samples for mtDNA analysis of spinner and spotted dolphins were collected by National Marine Fisheries Service and Inter-American Tuna Commission observers who regularly accompany US purse seiners fishing for yellowfin tuna and skipjack tuna (*Katsuwonus affinus*) in the ETP. In addition to their normal collection routine, observers were

requested to collect and promptly freeze (-20°C) liver samples from dolphins that were accidentally suffocated in the nets during fishing operations. Samples were returned on dry ice from Panama, where the observers disembarked.

We accumulated a collection of tissue samples from 151 spinner dolphins and 10 spotted dolphins. Collection sites were distributed across the ETP, and the samples included both the eastern and whitebelly forms of the spinner dolphin. The sample sizes range from a single animal to twenty-one animals from the same school (Fig. 2). In addition, liver tissue from 13 spinner dolphins was obtained courtesy of the late Durant Hembree[3] from animals accidentally caught and suffocated in gillnets set for sharks in the Timor Sea (12°S, 127°E; Harwood and Hembree, 1987).

We assigned one of two stock types to each ETP spinner dolphin in the sample: eastern or whitebelly[4]. This school stock identification was based on the predominating appearance of the adult members of the school from which they were captured and is determined routinely by the biologists aboard the tuna seiners for the purpose of the mortality estimates. The eastern or whitebelly designation is sometimes difficult, because the areas of distribution of these two populations overlap and on rare occasions they are even captured in the same net-set. Even in areas where only so-called 'pure' schools are found, the individuals, especially in a whitebelly school, often demonstrate a wide range of

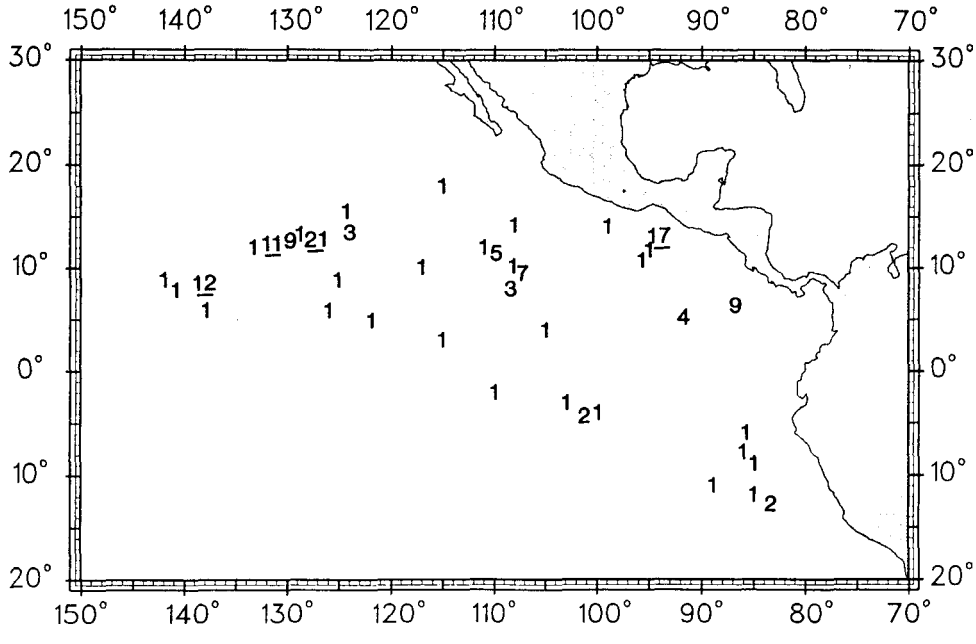

Fig. 2. Collection locations, including number captured from each school. These include eastern and whitebelly spinner dolphin as well as spotted dolphin samples (n=132). All two digit values are underlined.

[3] Western Australian Museum, Perth, Western Australia, Australia 6000.

[4] The whitebelly spinner is further subdivided into northern and southern forms based on distribution, coloration and skull morphology (Perrin *et al.*, 1985). Our sample of 45 whitebelly spinners comprised 32 individuals captured from the northern area and 13 from the southern. For our analyses, we did not stratify below the whitebelly spinner level.

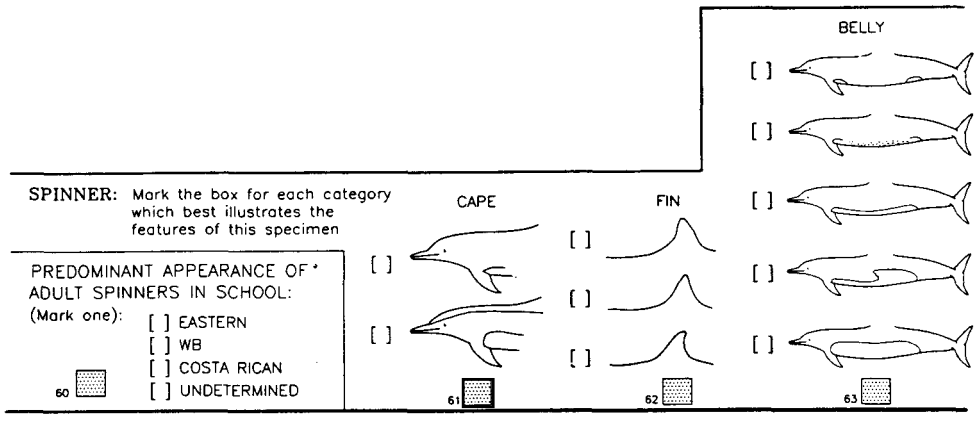

Fig. 3. A reproduction of a portion of the form used by fishery observers on purse seines to record information about individual dolphins killed during the fishing operations. It illustrates the method used to assign a numerical score based on the physical characteristics of the individual animal. It is based on the ventro-lateral colour pattern (white in axilla and genital region = 1, to complete 'whitebelly' pattern = 5), fin shape (forward canted = 1, to falcate = 3) and the easily observable absence (=1) or presence (=2) of the cape. Animals scoring 10 are considered most 'whitebelly'-like. Animals scoring 3 are most 'eastern'-like. Note also that observers are asked to judge the predominant appearance of adult spinners in the school.

characteristics, and, as would be expected in sub-specific forms, sometimes a few individuals are observed that look much like those from the other population.

We felt it important to establish whether the mtDNA haplotype is related to the morphotype (individual physical appearance) or to the school designation. Therefore, we categorised our ETP spinner dolphin samples by assigning a numerical score based on a scale designed by one of us (WFP) using the physical characteristics of the individual (its morphotype) rather than the modal characteristics of its school (Fig. 3). This morphological score ranged from 3 (most 'eastern'-like) to 10 (most 'whitebelly'-like). Each specimen thus had two morphological designations, usually the same but sometimes different.

The Timor Sea dolphins resembled a typical pan-tropical spinner dolphin (Hawaiian spinner dolphin, cf. Perrin *et al.*, 1985). However, there is some evidence that these animals (as well as other China Sea, inshore spinner dolphins) may belong to a dwarf race, attaining sexual maturity at a much smaller size than do spinner dolphins in other regions (Perrin *et al.*, 1989). The spotted dolphins were all of the northern offshore type (Perrin *et al.*, 1985).

DNA isolation and restriction analysis

The quality of DNA samples from the specimens differed considerably, probably due to post mortem handling. The longer the interval between death and freezing of the tissue sample, the greater the amount of degradation of the DNA. We had no way of knowing how much time had elapsed between death in the net, retrieval of the corpse and extraction and freezing of the liver. Although we repeatedly attempted to purify mtDNA with density gradient ultracentrifugation, we never were successful in isolating pure mtDNA uncontaminated with chromosomal DNA. (Degradation of the mtDNA

structure eliminates the buoyancy differences between the linear chromosomal DNA and the supercoiled mtDNA). Consequently, we resorted to isolation of total cell DNA, containing both the nuclear and mitochondrial. MtDNA, which comprises less than 0.1% of the total cell DNA, was then analysed by restriction mapping on Southern blots (Lansman *et al.*, 1981).

Total cell DNA was extracted using established protocols (Maniatis *et al.*, 1982), with some modification during the initial steps. Five grams of partially thawed liver tissue was minced with a razor blade and grated through taunt aluminium window screen directly into 20 to 30ml of iced 1X SSC. Cellular DNA was then prepared from SDS-solubilised cells by phenol/chloroform extraction and dialysis against a low salt buffer. The purified cellular DNA was diluted to a final concentration of 1mg/ml in a TE buffer (10mM Tris/HC1, 1mM EDTA, pH6.8) and stored at –70°C. Generally, 1 to 3mg of total cellular DNA were recovered from a 5g liver sample.

Two micrograms of the cellular DNA were used for each restriction enzyme digest. The digested DNA samples were resolved on horizontal 0.8% to 1.8% agarose gels. Two DNA fragment size standards (BRL 1kbp ladder and a recombinant mtDNA marker) were included in each gel with the dolphin DNA samples. The dolphin mtDNA fragments were visualised on Southern blots (S&S Nytron nylon membranes) using a mixture of cloned fragments of dolphin mtDNA collectively covering the entire mt genome (Southern *et al.*, 1988) as a probe. The recombinant mtDNA probe was labelled with ^{32}P by nick-translation to a specific activity of about 10^8cpm/μg. The blots were hybridised with the labelled probe ($2x10^6$cpm per blot) in the presence of 50% formamide, at 42°C, for 16 to 24hr. Stringently washed blots were then exposed to X-ray films with intensifying screens for 1 to 5 days.

Data analysis

The individual mtDNA sequence from each animal can be represented by a characteristic pattern comprised of restriction fragments generated by different enzymes. Animals showing identical composite fragment patterns are considered to belong to a single mtDNA lineage. Current models of mtDNA heredity in mammals assume that mtDNA lineage is matriarchal and clonal. The terms composite mtDNA genotype, lineage or haplotype are used here synonymously. Each different polymorphic pattern, produced by a given enzyme, was assigned an upper case letter. Considering all six enzymes, this permitted a six letter 'word' to represent each individual dolphin's composite mtDNA haplotype.

For population analysis, two approaches are generally used to analyse the relatedness of the haplotypes found within a sample: qualitative and quantitative (Avise *et al.*, 1979a,b; Lansman *et al.*, 1981; Johnson *et al.*, 1983). We employed the quantitative approach, where differences rather than similarities in the restriction patterns are compared. With this approach, mtDNA diversity is defined as sequence divergence between pairs of individuals expressed as base substitutions per nucleotide (p; Upholt, 1977). It is calculated from the proportion of the fragments shared (F) between individuals' multiband fragment patterns (Table 1). The value, F, is itself a measure of relative mtDNA difference between individuals (Avise *et al.*, 1979b; Lansman *et al.*, 1981),

$$F = (2N_{xy})/(N_x+N_y)$$

where N_x and N_y are the number of fragments in the digestion profiles of sample x and y, and N_{xy} is the number of fragments shared by the two organisms. The fewer fragments shared, the smaller the relatedness and the greater the sequence divergence,

$$p = 1 - \{[-F+(F^2+8F)^{\frac{1}{2}}]/2\}^{1/n}$$

Table 1

The letters designate each distinguishable restriction-fragment pattern produced by the given restriction endonuclease. The first five enzymes failed to show polymorphic patterns of restriction sites. The latter six did. Genome size did not in all cases add up to the approximately expected 16,300 value due to elimination of smaller than 700bp to 500bp fragments from the analysis; see text. Slight adjustments were made in the rounding of sizes to ensure that fragments that were not judged as having the same length did not have the same rounded value.

A specimen's 'haplotype' is assigned by combining the fragment pattern type of each enzyme into a six-letter composite word. For example, if a given specimen's DNA exhibited the *Bam*HI A pattern, the *Hpa*II A pattern, the *Hinc*II B pattern, the *Hinf*I A pattern, the *Ava*II A pattern and the *Hae*III A pattern, the haplotype word would be 'AABAAA'.

Enzyme	ID	Genome size	Fragment size
*Eco*RI	A	16.5	6.70,4.90,3.50,1.40
*SSt*I	A	16.5	5.80,3.40,2.60,2.50,2.20
*Bgl*II	A	15.7	6.00,3.70,3.50,1.30,1.20
*Ava*I	A	16.3	9.20,4.50,2.60
*Hind*III	A	16.6	5.70,2.80,2.60,2.00,1.80,1.70
*Bam*HI	A	16.1	9.50,5.60,1.00
	B	16.0	15.00,1.00
	C	16.1	9.50,4.15,1.40,1.00
	D	16.3	10.00,5.30,1.00
	E	16.2	5.60,5.50,4.10,1.00
	F	16.1	9.50,6.60
*Hpa*II	A	13.2	7.00,2.10,1.50,1.20,0.75,0.66
	B	13.2	6.90,2.10,1.50,1.30,0.75,0.66
	C	14.1	7.00,2.10,1.50,1.20,0.88,0.75,0.66
	D	12.5	7.00,2.10,1.50,1.20,0.66
	E	13.2	7.00,1.50,1.20,1.10,1.00,0.75,0.66
	F	13.9	7.00,2.10,1.50,1.40,1.20,0.66
	G	13.6	7.00,2.40,1.50,1.30,0.75,0.66
	H	14.4	6.90,2.30,1.50,2.30,0.75,0.66
*Hinc*II	A	16.4	6.20,4.00,3.50,0.92,0.88,0.58,0.35
	B	16.4	6.20,4.00,3.50,1.50,0.88,0.35
	C	16.2	10.00,3.50,1.50,0.88,0.35
	D	16.4	6.20,4.00,3.50,1.50,1.20
	E	16.8	4.20,4.00.3.50,2.40,0.92,0.88,0.58,0.35
	F	16.8	4.20,4.00,3.50,2.40,1.50,0.88,0.35
*Hinf*I	A	12.1	1.50,1.50,1.40,1.40,1.40,1.20,1.10,1.00,0.84,0.74
	B	12.4	1.50,1.50,1.50,1.40,1.40,1.40,1.10,1.00,0.84,0.74
	C	12.0	1.50,1.50,1.40,1.40,1.40,1.10,1.10,1.00,0.84,0.74
	D	13.1	2.40,1.80,1.70,1.50,1.50,1.40,1.20,0.84,0.74
	E	13.1	1.80,1.70,1.50,1.50,1.40,1.40,1.20,1.00,1.84,0.74
	F	12.5	1.80,1.50,1.50,1.40,1.40,1.20,1.10,1.00,0.84,0.74
	G	11.7	2.20,1.50,1.50,1.40,1.40,1.10,1.00,0.84,0.74
	H	11.0	1.50,1.50,1.40,1.40,1.40,1.20,1.00,0.84,0.74
	I	11.9	1.50,1.40,1.40,1.40,1.30,1.20,1.10,1.00,0.84,0.74
	J	12.1	2.60,1.60,1.50,1.50,1.30,1.10,0.92,0.84,0.74
	K	11.0	1.60,1.50,1.50,1.40,1.30,1.10,1.00,0.84,0.74
	L	11.9	2.40,1.60,1.50,1.50,1.30,1.10,0.92,0.84,0.74
	M	11.9	1.60,1.50,1.50,1.40,1.30,1.10,1.00,0.88,0.84,0.74
*Ava*II	A	15.8	9.00,3.50,2.80,0.54
	B	15.8	5.80,3.50,3.20,2.80,0.54
	C	16.0	4.20,3.50,3.20,2.80,1.80,0.54
	D	15.3	12.00,2.80,0.54
	E	16.0	6.70,4.20,2.80,1.80,0.54
	F	16.0	5.40,3.50,3.20,2.80,0.58,0.54
	G	16.0	4.40,4.20,2.80,2.30,1.80,0.54
	H	16.6	4.40,4.20,2.80,2.30,1.80,0.57,0.54

Table 1 continued.

Enzyme	ID	Genome size	Fragment size
*Hae*III	A	11.5	1.70,1.40,1.20,1.10,1.00,0.95,0.84,0.74,0.73
	B	10.9	1.70,1.40,1.20,1.10,1.00,0.95,0.84,0.74,0.73,0.63,0.59
	C	11.9	1.70,1.60,1.50,1.40,1.20,1.00,0.84,0.74,0.73,0.63,0.57
	D	11.5	1.70,1.40,1.20,1.10,1.00,0.95,0.84,0.74,0.73,0.69,0.59,0.57
	E	11.3	1.70,1.40,1.20,1.10,1.00,0.98,0.95,0.84,0.74,0.73,0.63
	F	10.8	1.70,1.40,1.20,1.10,1.00,0.95,0.84,0.74,0.73,0.59,0.57
	G	11.7	1.70,1.40,1.20,1.20,1.00,0.95,0.93,0.84,0.74,0.63,0.59,0.57
	H	10.6	1.70,1.40,'.20,1.10,1.00,0.95,0.74,0.73,0.63,0.59,0.57
	I	11.5	1.70,1.40,1.20,1.00,0.95,0.93,0.92,0.84,0.74,0.64,0.59,0.57
	J	11.9	1.70,1.50,1.40,1.20,1.00,0.95,0.84,0.74,0.73,0.63,0.59,0.57
	K	13.1	1.70,1.60,1.40,1.20,1.20,1.00,0.95,0.84,0.74,0.73,0.63,0.59,0.57
	L	11.2	1.70,1.40,1.20,1.00,0.95,0.84,0.80,0.74,0.73,0.63,0.59,0.57
	M	10.4	1.55,1.40,1.20,1.10,1.00,0.95,0.84,0.74,0.73,0.68,0.63,0.59,0.57
	N	9.6	2.00,1.90,1.70,1.40,1.20,1.10,1.00,0.99,0.86,0.74,0.63
	O	11.8	1.80,1.40,1.30,1.20,1.05,0.92,0.84,0.74,0.73,0.63,0.59,0.57
	P	11.3	1.80,1.50,1.30,1.20,1.05,0.92,0.84,0.74,0.73,0.63,0.59
	Q	12.8	1.80,1.40,1.30,1.20,1.05,0.96,0.92,0.84,0.74,0.73,0.66,0.63,0.57

The values of p must be calculated separately for each enzyme if length of the recognition sequence, n, varies (Lansman *et al.*, 1981). Values of n for the enzymes employed in this study are from Nei and Taijima, 1983. In our study, mean pair-wise values of p were collected and weighted by the total number of base pairs recognised by each type of enzyme (Ashley and Wills, 1987). We made estimates of sample variances of the mean pair-wise values of p by bootstrap methods, a nonparametric statistical procedure based on Monte Carlo sampling (see Appendix).

RESULTS

Restriction fragment polymorphism in mtDNA

Using an initial sample of 30 spinner dolphins, we found that 6 out of 13 tested restriction endonucleases were informative, revealing polymorphic fragment patterns consisting of 2 to 13 fragments (Table 1). Other tested enzymes either did not cut the mtDNA (*Kpn*I and *Xho*I) or failed to show polymorphic patterns of restriction sites (*Ava*I, *Bgl*II, *Eco*RI, *Hind*III, *Sst*I).

The informative six enzymes were used to digest DNA samples from 174 dolphins. Some restriction fragments, in particular those smaller than 500bp, were more difficult to visualise when yields of mtDNA were low (smaller fraction of mtDNA relative to chromosomal DNA) or background smearing was high due to the presence of degraded mtDNA. Therefore in the pattern analysis, fragments smaller than 700bp were not scored for *Hinf*I, 600bp for *Hpa*II, and 500bp for *Ava*II and *Hae*III. This was done to maximise the number of different samples analysed. In addition, individual samples were removed from the subsequent analysis if mtDNA bands in any of the six restriction digests were too faint or had too much background 'noise' to allow reasonably confident interpretation of the band pattern. After this adjustment, our reduced sample data base consisted of patterns of restriction fragments generated by the six enzymes, obtained from 143 of the animals.

Table 2

Number of individuals of all haplotypes listed for each stock sampled. The dashed lines connect stocks that share rare haplotypes (appearing only once in one of the populations). EE = eastern spinner dolphin stock designation; WB = whitebelly spinner dolphin stock designation; TIM = spinner dolphins from the Timor Sea; and SPOT = spotted dolphins.

	Stock →	EE	WB	TIM	SPOT
	Sample size →	79	45	11	8
1	AAAAAA	18	13		
2	AAAAAB	11	7		
3	AAAAAD	2			
4	AAAAAE	3			
5	AAAAAF	1			
6	AAAAAG	1	1		
7	AAAAAJ	2	1		
8	AAAAAK		1		
9	AAAAAN	1			
10	AAAADA	2			
11	AAABAA	6			
12	AAABAB	1			
13	AAACAA	1			
14	AAAFAA	1	1		
15	AAAFAB	3	1		
16	AAAGAA	1			
17	AAAHAE	1			
18	AAAIAA			2	
19	AABAAA			8	
20	AABAAB		1		
21	AACAAA		2		
22	ABAAAM	1			
23	ABABAD	1			
24	ABAKGO				1
25	ABAMGO				1
26	ACABAA		2		
27	ADABAA	1			
28	AEAAAA	1			
29	AGAAAL			1	
30	AHEJGO				2
31	AHEJGP				1
32	AHELGO				1
33	BAAAAD	1			
34	BAADBA		1		
35	BAAEBA		1		
36	BBADBA		1		
37	BBADBG	1	1		
38	BBBDBA	2			
39	BBBDBG	1			
40	BBBDBI	1			
41	BBBDFA		1		
42	BBBEBA		1		
43	BBBEBG	1	1		
44	BBBEBH		2		
45	BBBECA		1		
46	CAAAAB	3	1		
47	DBBEAA		1		
48	DBBECA	4			
49	DBBECG	4	1		
50	DBBEEG	1			
51	DBDECG	1			
52	DFABAC		1		
53	EAAAAB	1			
54	EHEJGQ				1
55	FHFJHO				1

In all, we observed 55 unique haplotypes in the reduced sample (Table 2). Frequencies of haplotypes by species and sample are given in Table 2. We found the haplotypes #24, #25, #30, #31, #32, #54 and #55 only in the spotted dolphin group, and #18, #19 and #29 only in the group of Timor Sea spinner dolphins. None of these haplotypes were found in the other 124 spinner dolphins from the ETP and none of the patterns found in them were found in the spotted or Timor Sea dolphin samples. Inspection of the haplotypes of the Timor Sea animals suggested that all of the individuals were closely related: 8 of the 11 animals had the haplotype #18; two, #19; and one, #29. A minimum of two point mutations, causing the gain/loss of two sites, inter-converts the haplotypes #18 and #19, and a minimum of four point mutations is required to inter-convert #18 and #29. The eight spotted dolphin samples showed much greater mtDNA diversity and none of the detected haplotypes predominated.

Correlations between mtDNA haplotypes and morphotypes

Within the ETP spinner samples, we found no correspondence between the mtDNA haplotype and either the school type or an individual's morphology. Intraspecific differences between spinner dolphin stocks (whitebelly or eastern) were not detected, whether stock identification was based on the individual morphotype or on the school stock. The most common haplotypes were #1 or #2. When stratified by stock (Table 2), 36% of the eastern sample vs. 45% of the whitebelly spinner sample exhibited the common haplotypes (#1 or #2) (n.s., $chi^2_{.05,1}$). When stratified by individual morphotype (Table 3), 35% of the animals with a morphotype index of 3 to 5 (very 'eastern'-like) exhibited the common haplotypes, 43% with an index of 6 to 8, and 39% with an index of 9 or 10 (very 'whitebelly'-like) exhibited the common haplotypes (n.s., $chi^2_{.05,2}$). Chi-square tests based on uncombined data either by the stock (2 x 55) or the morphotype (3 x 55), revealed no statistically significant heterogeneity. (See Roff and Bentzen, 1989, for chi-square analyses in situations where expected values within cells are very small).

Table 3

Number of individuals of all haplotypes listed for morphotype index. Totals do not add to 124, the number of ETP spinner dolphins, because some specimens did not have sufficient data to assign a morphotype score. A morphotype index of from 3 to 5 represents typical eastern spinner dolphin characteristics and from 9 to 10, typical whitebelly spinner dolphin characteristics. The dashed lines connect eastern and whitebelly morphotype groups that share rare haplotypes.

	Morphotype →	3,4,5	6,7,8	9,10
	Sample size →	49	21	36
1	AAAAAA	10	7	8
2	AAAAAB	7	2	6
3	AAAAAD			2
4	AAAAAE	2	1	
5	AAAAAF	1		
6	AAAAAG	1	1	
7	AAAAAJ	2		1
8	AAAAAK		1	
9	AAAAAN	1		
10	AAAADA	1	1	
11	AAABAA	4	1	
12	AAABAB	1		
13	AAACAA			1
14	AAAFAA	1	1	
15	AAAFAB		1	1

continued overleaf

Table 3 continued

	Morphotype →	3,4,5	6,7,8	9,10
	Sample size →	49	21	36
16	AAAGAA	1		
17	AAAHAE	1		
18	AAAIAA			
19	AABAAA			
20	AABAAB			1
21	AACAAA		1	1
22	ABAAAM			
23	ABABAD			
24	ABAKGO			
25	ABAMGO			
26	ACABAA			2
27	ADABAA		1	
28	AEAAAA	1		
29	AGAAAL			
30	AHEJGO			
31	AHEJGP			
32	AHELGO			
33	BAAAAD	1		
34	BAADBA			1
35	BAAEBA			1
36	BBADBA			1
37	BBADBG	1		1
38	BBBDBA	2		
39	BBBDBG	1		
40	BBBDBI		1	
41	BBBDFA			1
42	BBBEBA			1
43	BBBEBG	1		1
44	BBBEBH		1	1
45	BBBECA			1
46	CAAAAB	3		1
47	DBBEAA		1	
48	DBBECA	1		
49	DBBECG	3		1
50	DBBEEG			1
51	DBDECG	1		
52	DFABAC			1
53	EAAAAB	1		
54	EHEJGQ			
55	FHFJHO			

Application of a quantitative index of mtDNA diversity (p) emphasises what is apparent in the distribution of haplotypes (Table 4). The eight ETP spotted dolphins show obvious differences when compared to the ETP spinner dolphins. While the mean pair-wise mtDNA diversity within the group is similar to that of the eastern and whitebelly spinner dolphins (0.017, 0.012 and 0.015), the mean pair-wise mtDNA diversity between the spotted and the whitebelly spinner dolphins is 0.049, and between the spotted and the eastern spinner dolphins 0.044. The Timor Sea spinner dolphins when compared with the whitebelly and eastern spinner dolphins demonstrated much smaller between-group diversities, 0.011 and 0.009, respectively. The Timor Sea animals, as mentioned above, exhibit very little within-group mtDNA diversity (0.003).

The statistically inferred difference in mean pair-wise within-group mtDNA diversity of the two ETP spinner dolphin populations (0.012 vs. 0.015, Table 4), provides no extra information over that provided by a simple examination of the haplotype distribution. Insignificant mtDNA difference (0.014) is apparent between the samples stratified on school stock type. There is as much mtDNA diversity within the stocks as between them.

Table 4

MtDNA diversity among and within sample groups. Along the diagonal: within-stock sequence divergence (p, N, bootstrapped SD). Above diagonal: between sequence diversity (p, N, bootstrapped SD). EE = eastern spinner dolphin stock designation; WB = whitebelly spinner dolphin designation; TIM = spinner dolphins from the Timor Sea; and SPOT = spotted dolphins. One whitebelly animal was eliminated from the analysis due to uncertainty regarding shared bands (n=44).

	EE	WB	TIM	SPOT
EE	0.012 79 0.004	0.014 123 0.004	0.009 90 0.002	0.044 87 0.011
WB		0.015 44 0.005	0.011 55 0.003	0.049 52 0.016
TIM			0.003 11 0.002	0.038 19 0.008
SPOT				0.017 8 0.018

Diversity of mtDNA in schools

The mean within-school mtDNA diversity varied from school[5] to school. Measurements of the mean pair-wise mtDNA diversity for each school varied from zero to 0.023 (Table 5). Trends towards the clustering of certain haplotypes within specific schools were not detected (Table 6). The mtDNA diversity of each school sample was clearly independent of the size of the sample and the school size as estimated by the biologist on board the tuna vessel.

However, a weak tendency towards smaller diversities and smaller range of diversities was observed in the samples collected farthest offshore. Means of the average pair-wise mtDNA diversities from the offshore sets were 0.010 as compared to 0.015 for the inshore ones (Table 5). The range of the offshore sets was from 0.002 to 0.013, while that from the inshore sets was from 0.005 to 0.023. Some schools, like the Timor Sea sample, exhibited only a low level of mtDNA diversity and therefore appeared to have a highly specific 'tribal' character. In contrast, the character of other schools was less specific. The level of mtDNA diversity in such 'cosmopolitan' schools was equal to or greater than in the sample as a whole.

[5] Tuna purse seiners generally set their nets on what are thought to be single schools. However, mixed schools of spinner and spotted dolphins are set on as well as occasionally mixed schools of eastern and whitebelly spinner dolphins. For simplicity, we are assuming that set is synonymous with school.

Table 5

Set (school) diversity showing stock identity of school, capture location, estimated school size and within-school sequence divergence (N, p, bootstrapped SD). Symbols after the p values indicate whether the mtDNA diversity of the school is greater than, less than, or equal to that of the stock sample as a whole (0.012 for the eastern spinner dolphin samples and 0.015 for the whitebelly). Schools 1-5 were captured in the outside region where the cumulative fishing pressure is half what it is in the inside region, where schools 6-11 were captured. EE = eastern spinner dolphin stock designation; WB = whitebelly spinner dolphin stock designation; and TIM = spinner dolphins from the Timor Sea.

Set	Stock	Position		School size	N	P	SD
a.	TIM	12°S	127°E	--	4	0.0	0.0
1.	WB	9°N	138°W	500	12	0.010<	0.066
2.	EE	12°N	130°W	400	11	0.012=	0.006
3.	EE	13°N	129°W	100	9	0.008<	0.005
4.	EE	13°N	128°W	900	21	0.013>	0.005
5.	EE	13°N	124°W	20	3	0.002<	0.001
Samples combined (schools 1 - 5)					56	0.010	0.003
6.	EE	11°N	110°W	200	5	0.007<	0.004
7.	WB	8°N	109°W	20	3	0.023>	0.010
8.	EE	10°N	108°W	150	7	0.022>	0.006
9.	EE	13°N	94°W	1900	16	0.009<	0.005
10.	WB	7°N	87°W	500	5	0.019>	0.007
11.	WB	6°N	87°W	750	4	0.005<	0.004
Samples combined (Schools 6 - 11)					40	0.015	0.005

DISCUSSION

The Timor Sea spinners and the spotted dolphins appear to have unique mitochondrial genotypes as suggested by the mtDNA haplotype analysis. Samples of these two dolphin groups, albeit small, contained haplotypes that were unique to group (Table 2).

Considering current theories of population genetics, it can be expected that differences between closely related but segregated populations would be manifested in the distribution of rare haplotypes that presumably arose uniquely in the dolphin groups. We detected eight cases of a rare mtDNA haplotype shared by the eastern and whitebelly spinner dolphin populations (Table 2). In five instances, the same rare mtDNA haplotype was found both in animals having the typical eastern morphotype and the whitebelly spinner morphotype (Table 3).

The detected evidence of mtDNA exchange between the forms is surprising considering the rather large differences in their morphology. However, it has been reported that in some cases mtDNA is transmitted across taxonomic barriers, particularly during breeding in zones of hybridisation between two species inhabiting overlapping geographical areas (Ferris *et al.*, 1983; Harrison *et al.*, 1987; Harrison, 1989; Wallis and Arntzen, 1989). The populations of spinner dolphins may display distinct morphological traits by virtue of differences in their chromosomal DNA types, which were not analysed in our study. Ferris *et al.* (1983) demonstrated that at the beginning of reproductive hybridisation between two species, the exchange of mtDNA types preceded the appearance of mixed chromosomal genotypes. It is therefore possible that the spinner dolphin populations have remained separated in their reproductive chromosomal genotypes and phenotypes, while their mtDNA types have become mixed due to recent mating between the groups. If such interbreeding indeed occurs, it can be expected that the resulting gene flow may evenually

Table 6

Number of individuals of all haplotypes listed for sets (schools) sampled; the stock designation of the school, the geographic position of capture and the sample size are also listed. EE = eastern spinner dolphin and WB = whitebelly spinner dolphin.

Set:	1	2	3	4	5	6	7	8	9	10	11
Stock:	WB	EE	EE	EE	EE	EE	WB	EE	EE	WB	WB
N:	12	11	9	21	3	5	3	7	16	5	4
1 AAAAAA	2	3	3	3			1	1	5	2	1
2 AAAAAB	4	3		2	1	1			4		
3 AAAAAD				2							
4 AAAAAE			1	1	1						
5 AAAAAF			1								
6 AAAAAG	1	1									
7 AAAAAJ			1	1						1	
8 AAAAAK											1
9 AAAAAN				1							
10 AAAADA				1		1					
11 AAABAA		1	1	1	1	1			1		
12 AAABAB						1					
13 AAACAA		1									
14 AAAFAA				1							1
15 AAAFAB	1		1						2		
16 AAAGAA				1							
17 AAAHAE								1			
18 AAAIAA											
19 AABAAA											
20 AABAAB	1										
21 AACAAA											1
22 ABAAAM									1		
23 ABABAD									1		
24 ABAKGO											
25 ABAMGO											
26 ACABAA											
27 ADABAA				1							
28 AEAAAA				1							
29 AGAAAL											
30 AHEJGO											
31 AHEJGP											
32 AHELGO											
33 BAAAAD								1			
34 BAADBA											
35 BAAEBA											
36 BBADBA	1									1	
37 BBADBG			1								
38 BBBDBA								2			
39 BBBDBG											
40 BBBDBI		1									
41 BBBDFA										1	
42 BBBEBA											
43 BBBEBG											
44 BBBEBH							1				
45 BBBECA											
46 CAAAAB	1			1		1					
47 DBBEAA							1				
48 DBBECA				1				1	2		
49 DBBECG		1		1							
50 DBBEEG								1			
51 DBDECG				1							
52 DFABAC	1										
53 EAAAAB				1							
54 EHEJGQ											
55 FHFJHO											

cause disappearance of the morphological differences between the two spinner groups, unless the morphological separation is maintained by strongly differential selection pressure. Such 'swamping out' of the eastern form, if it is occurring, may be facilitated by the relatively much greater depletion of the eastern population in the tuna fishery (Smith, 1983).

Although we feel that the evidence indicates the lack of complete isolation between the two forms, there does seem to be a weak indication of geographic partitioning of the haplotypes. As pointed out by S. Palumbi (pers. comm., University of Hawaii, December 1989), our approach is suitable for detecting strong patterns but leaves weak patterns undetected. To do so requires information about the relative relationship among the haplotypes. This is obtained by making gain/loss maps (Fig. 4). We only attempted this for the three enzymes producing the patterns with the fewest fragments: *Bam*HI, *Ava*II and *Hinc*II. Nevertheless, interesting patterns emerge.

If only the ETP samples are considered, it is clear from the gain/loss maps that individuals with patterns B & D for *Bam*HI; C & E for *Ava*II; or B, C & D for *Hinc*II form distinct clades. Then the 11 individuals with haplotype #48, #49, #50 and #51 would be remarkably divergent from one another. The eight eastern individuals having haplotype #48 and #49 seem to introduce a tremendous amount of 'noise' into the calculation of the pairwise genetic diversity. Removing those from the samples produces a distribution that is significantly different from random for both the *Bam*HI B & D clades and the *Hinc*II B, C & D clades (Table 7). Thus, setting aside the outlying rare haplotypes makes clear some concordance between differences in morphology and mtDNA.

In the analysis of mtDNA diversity in schools of spinner dolphins, we had expected to find that animals sampled from one school would be more related to each other than to members of other schools, and that the observed mtDNA diversity in samples pooled at the stock level would come from inter– rather than intra-school diversity. The variation in within-school diversity that we found, if not simply due to sampling error, may be related to fishing pressure. The offshore area (west of 115°W) has experienced about half the cumulative sets that the inshore area (east of 115°W) has since the beginning of purse-seine fishing in 1969 (Punsly, 1983). This may account for the greater within-school

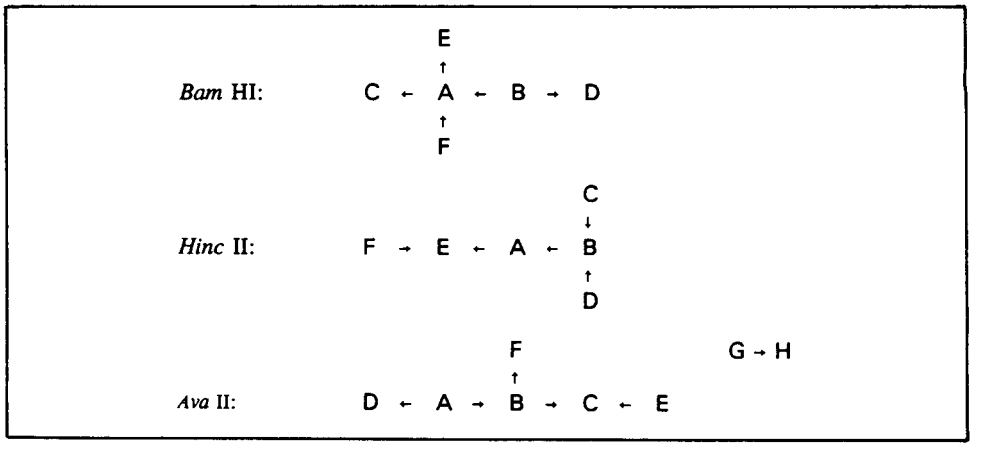

Fig. 4. Hypothesised restriction site gains and losses in *Bam*HI, *Ava*II and *Hinc*II. Patterns G and H in *Ava*II are found only in spotted dolphins in the sample and can not be related to the other patterns by the loss or gain of a single site.

Table 7

Results of goodness-of-fit tests for distribution of *Bam*HI clades B or D and *Hinc*II clades B, C or D with Haplotypes Nos 48 and 49 present and removed; see text. *Ava*II clades C or E were not present in sufficient numbers to test.

	Sample size		Frequency					
	EE	WB	Exp. EE	Exp. WB	Obs. EE	Obs. WB	Chi-sq.	Sig.
All samples								
*Bam*HI B or D	79	45	18.48	10.52	15	14	1.8018	n.s.
*Hinc*II B, C or D	79	45	15.29	8.71	13	11	0.9453	n.s.
Haplotypes Nos 48 & 49 removed from analysis								
*Bam*HI B or D	71	44	12.97	8.03	8	13	4.9698	<0.05
*Hinc*II B, C or D	71	44	10.50	6.50	6	11	5.0329	<0.05

diversity inshore. Purse-seine fishing requires herding of the dolphins with speed boats and most likely results in fragmenting of schools due to the chase or capture of partial schools.

We have suggested that Timor Sea spinner dolphins may comprise a unique group based on our measurement of their mtDNA diversity. Our sample size in this experiment was rather small, however, and may have introduced bias to interpretation of the results because, at most, only three different schools were represented (Table 6). The apparent uniqueness of the Timor Sea dolphins will therefore require further verification using a larger sample. However, it was suggested in other studies that local, isolated populations of spinner dolphins may exist in the far western Pacific Ocean. Perrin *et al.* (1989) reported a dwarf form of spinner dolphin in the Gulf of Thailand. These animals, likely mature, ranged in size from 120 to 137cm (n=10) compared with 157 to 235cm (n=2,309) in a sample of central and western Pacific Ocean spinner dolphins. The Timor Sea animals (of unknown maturity) from which our sample came ranged from 85 to 145cm (n=13).

Additional bias was introduced through selection of restriction enzymes for analysis of the mtDNA diversity. We supplemented our direct comparisons of the distributions of mtDNA haplotypes with the quantitative approach for measurement of sequence divergence (see Avise *et al.*, 1979b), but we used only those restriction enzymes which produced polymorphic patterns of restriction sites in the dolphin mtDNA. This biased enzyme selection was likely to result in overestimation of the level of mtDNA diversity within the spinner dolphin sample. Consequently, our measurements of mean pair-wise sequence divergence within the spinner dolphin sample cannot be directly compared to those from studies employing randomly selected enzymes with polymorphic as well as invariant restriction sites. However, our primary aim of detection of intraspecific differences in spinner dolphins was most effectively approached by the use of only such informative enzymes.

With the above *caveat*, our measurements of approximately 4% mtDNA divergence between the spinner and spotted dolphins is comparable to published data on mtDNA divergence at various taxonomic levels in other large or highly mobile animals. For example, six congeneric species of the horse family showed pair-wise values ranging from 3.3% to 7.8% (George and Ryder, 1986). In an extensive study of waterfowl species, the ranges of mtDNA diversity were 0.4% to 8.8% in nine *Anas* spp., 2.4% to 4.3% in four *Aythua* spp. and 10.9% between selected species belonging to different genera (Kessler

and Avise, 1984). MtDNA diversity of 9.7% to 17.5% was measured between genera and subfamilies of galliform birds (Glaus *et al.*, 1980). Based on 19 genetic loci encoding liver and muscle enzymes, Shimura and Numachi (1987) showed that the degree of isozyme divergence at a given taxonomic level (local populations, species, genera and families) in toothed whales was low in comparison to small or non-mobile taxa but very similar to that in mobile avian ones.

We conclude that the study of mtDNA variability in spinner dolphins is consistent with the hypothesis that the eastern form represents a locally adapted gene pool and the whitebelly spinner forms a hybrid swarm. In a recently completed study of colour pattern and dorsal fin shape (Perrin *et al.*, in press) it has been found that samples from the whitebelly region exhibit greater variability (higher variance) in all characters than those from the core eastern region. This, and the intermediacy of the whitebelly form between the eastern and the pantropical form in other suites of morphological characters (Perrin, 1990), support the hybrid-swarm hypothesis. The hypothesis has significant implications for management. If the eastern spinner stock indeed represents a gene pool uniquely adapted to the ETP habitat, arguably it should receive more stringent protection than the hybrid whitebelly spinner dolphin stocks. This is especially true if the eastern spinner stock has been and currently is more heavily exploited than the whitebelly spinner stock, as indicated by past assessments (Smith, 1983; Buckland and Anganuzzi, 1988), because such skewed exploitation may amplify any trend towards the swamping of the unique eastern form by immigration from the west.

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APPENDIX

Estimates of sample variances of the mean pair-wise values of p were made by bootstrap methods, a nonparametric statistical procedure based on Monte Carlo sampling (Efron and Tibshirani, 1986). In doing so, we attempted to deal with both error components of the mean pair-wise values: (1) the sampling variance associated with the selection of the sample from the population, and (2) the stochastic variance associated with the probabilistic methods for relating fragment information and nucleotide substitutions. Nei and Taijima, 1981 provide theoretical methods for calculating these variance components, however our data, derived from fragment rather than site information, does not meet the assumptions required by their methods. For the bootstrap methods we employed, the variance estimates must be viewed with caution for some of the small sample sizes.

The way we used the bootstrap concept to estimate variance was simple. The observed sample of (X_1, X_2,....X_N) allows (N/2)*(N-1) pair-wise comparisons of mtDNA diversity calculated from the patterns of restriction fragments generated by the six restriction enzymes. From these pair-wise comparisons, a mean can be calculated. For the bootstrapped estimate, an equal-sized sample is randomly drawn *with replacement* from the observed sample (X'_1, X'_2,...X'_N). And, a new six enzyme panel is randomly drawn, again with replacement, from the six enzymes, e.g. *Hpa*II, *Bam*HI, *Hin*fI, *Bam*HI, *Ava*II, *Hin*fI. Individual p values between individuals of the new bootstrapped sample are then recalculated using information from the fragment patterns generated by the six randomly chosen enzymes and from these, a mean pair-wise p value was determined. We repeated the process 1,000 times, and the distribution of resulting mean pair-wise p values was used to estimate the variance of the mtDNA diversities of original sample groups.