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RESEARCH ARTICLE

Mitochondrial cytochrome b variation in sleeper sharks (Squaliformes: Somniosidae)

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Abstract Sleeper sharks are a poorly studied group of deep-sea sharks. The subgenus, *Somniosus*, contains three morphologically similar species: *S. microcephalus* found in the Arctic and North Atlantic; *S. pacificus* in the North Pacific; and *S. antarcticus* in the Southern Ocean. These sharks have been reported mainly in temperate to polar waters and occasionally in subtropical locations. They have not been recorded in tropical waters. This study investigates the relationships among the accepted species of *Somniosus* through analysis of mitochondrial *cytochrome b* sequence variation. Seventy-five samples were examined from four sampling locations in the North Pacific, Southern Ocean and North Atlantic. Twenty-one haplotypes were found. A minimum spanning parsimony network separated these haplotypes into two divergent clades, an *S. microcephalus* and an *S. pacificus/antarcticus* clade, strongly supporting the distinction of *S. microcephalus* as a separate species from the Pacific sleeper shark species. Analysis of genetic structure within the *S. pacificus/antarcticus* clade (analysis of molecular variance, allele frequency comparisons, and a nested clade analysis) showed limited or no differences amongst three populations. Further examination of genetic variation at more variable mtDNA and nuclear markers is needed to examine the species status of *S. pacificus* and *S. antarcticus*.

Introduction

Sleeper sharks (genus *Somniosus*; subgenus *Somniosus*) are poorly studied, large (adults reaching 4 m or more in total length) "deep-sea", or perhaps more accurately, "cold

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water", sharks. The subgenus, *Somniosus*, contains three species: the Greenland shark, *S. microcephalus*, found in the Arctic and North Atlantic; the Pacific sleeper shark, *S. pacificus*, in the North Pacific; and the recently resurrected *S. antarcticus*, in the Southern Hemisphere (Yano et al. 2004). These species differ morphologically in a number of characters including the ratio of the interdorsal space to the prebranchial length, which can be used to separate *S. microcephalus* from *S. pacificus* and *S. antarcticus*, and in a number of continuous characters such as the height of the first and second dorsal fins, the number of turns in the spiral valve and the number of precaudal vertebrae (Yano et al. 2004).

Sleeper sharks have been reported mainly from temperate to polar waters; however, sharks have been noted in northern subtropical locations. *S. pacificus* has been found as far south as Baja California in the eastern Pacific (Compagno 1984) and most recently in the subtropical waters off the Island of Taiwan in the western Pacific (Wang and Yang 2004). Similarly, *S. microcephalus* has been recorded off the coast of Portugal (Compagno 1984) and off Savannah Georgia (Herdendorf and Berra 1995) in the Atlantic. They have been recorded only at depth in subtropical observations (Wang and Yang 2004; Herdendorf and Berra 1995), however, in cold temperate and arctic waters, tracking has shown that some sharks move from depth to shallow waters daily (Skomal and Benz 2004; Stokesbury et al. 2005).

Wang and Yang (2004) recorded the presence of *Somniosus pacificus* in the subtropical waters of Taiwan based on their morphological characters. However, their capture much further south than previously reported in the North Pacific raised some doubts over their identification. The objectives of this study are to infer the relationship of the Taiwanese sharks to the currently recognized species and to investigate the phylogenetic relationships amongst the accepted species of the subgenus *Somniosus* through analysis of the mitochondrial *cytochrome b* (*cyt-b*) gene. *Cyt-b* has proven to be a useful marker for the phylogenetic analysis of shark and ray species (e.g., Bernardi and Powers 1992; Kitamura et al. 1996; Naylor et al. 1997; Sezaki et al. 1999; Naylor et al. 2005; Lopez et al. 2006) and is one of the markers used for species identification of unknown shark tissue samples (Heist and Gold 1999; Hoelzel 2001; Chapman et al. 2003; Chan et al. 2003). Through comparison of *cyt-b* sequences determined from samples collected at geographically separated locations, global genetic variation amongst sleeper sharks was used to examine various hypotheses regarding species relationships: H_o—there is only one widely distributed species, H_{a1}—there are three species corresponding to current taxonomy, $H_{a2–4}$—there are only two species with three combinations of pairs available.

Materials and methods

Sample collection

Seventy-five sleeper shark samples were obtained either from shark landing sites (Taiwan, Iceland), from research vessels (Antarctic) or from long line fishing through sea ice (Cumberland Sound) (Table 1). Tissue samples (skeletal muscle) were either placed directly into a 20% DMSO, 0.25 M EDTA, saturated NaCl solution and stored at room temp or 4°C until DNA extraction, or were first frozen in the field prior to long-term sample preservation in the above solution.

DNA isolation

Total DNA was extracted from all samples using a phenol/chloroform extraction (Sambrook and Russell 2001). The quality of extracted DNA was assessed through gel electrophoresis on 1% agarose gels, visualized with ethidium bromide and DNA quantity determined via UV spectrophotometry. DNA stocks were adjusted to a working concentration of 50 ng/µl in 1× TE (ph 7.5) for PCR amplification.

PCR amplification and sequencing

An 869 bp fragment of the mitochondrial *cyt-b* gene was amplified with *Somniosus* specific primers, *Somn-GLU-L1*, 5′-GAA CCA TCG TTG TTT ATT CAAC-3′, and *Somn-CYTB-H2*, 5′-GGC AAA TAG GAA ATA TCA TTC-3′. The thermal profile consisted of an initial cycle of 95°C for 5 min, 45°C for 30 s, and 72°C for 2 min, followed by 39 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 1.5 min, and finished with an elongation of the 72°C step for 6.5 min. Reaction volumes were 25 µl, consisting of 2.0 mM $MgCl_2$, 200 µM dNTPs, 1× reaction buffer, 0.2 µM of each primer, and 1 unit of hot start, Platinum *Taq* polymerase (Invitrogen Inc, Burlington, CAN). The resulting

Table 1 Sleeper shark samples, including location by ocean and nearest land mass, sample size and date collected

Species name	Sample location	Sample size	Date collected
Somniosus pacificus	North Pacific, Taiwan	28	2000–2003
	North Pacific, Alaska	15	2000
Somniosus antarcticus	Southern Ocean, Tasmania	16	2001
Somniosus microcephalus	North Atlantic, Cumberland Sound	6	1999
	North Atlantic, Iceland	10	2001–2002

product was ethanol precipitated (Sambrook and Russell 2001), resuspended in MilliQ H_2O and 200 ng was used as a template for DNA sequencing. DNA sequencing was conducted using the primers listed above with a GenomeLab DTSC quick start kit (Beckman Coulter, Mississauga, CAN). Sequences in both directions were determined on a Beckman Coulter CEQ8000 capillary sequencer and analyzed with the Sequencer (version 4.2.2, Gene Codes, Ann Arbor, USA) computer program.

Data analysis

Haplotypes were determined through visual and phylogenetic analyses using the Sequencer and MEGA, version 3 (Kumar et al. 2004) computer programs. An AMOVA analysis was conducted using the Arlequin, version 3.01 (Excoffier et al. 2005) computer program to examine hypotheses of population structure and to calculate population statistics. For the AMOVA analysis populations were grouped according to the current taxonomy: Group 1 (*S. microcephalus*)—North Atlantic, $n = 16$; Group 2 (*S. antarcticus*)—Southern Ocean, $n = 16$; and Group 3 (*S. pacificus*)—North Pacific, Alaska, $n = 15$ & North Pacific, Taiwan, $n = 28$. For pairwise comparisons, the significance of P-values was adjusted to an α value of 0.05 using a sequential Bonferroni correction (Rice 1989). Population statistics, haplotype and nucleotide diversity, estimates of θ ($\theta = 2N_f\mu$, where N_f = effective population size of females and μ = mutation rate), and probability of mismatch distribution (Table 2) were calculated by the formula implemented in Arlequin. Evidence for selection was examined by Tajima's and Fu's tests for selective neutrality (Tajima 1996; Fu 1997) as implemented in Arlequin. Estimates of migration among populations were examined by the computer program Migrate-n, version 2.1.3 (Beerli and Felsenstein 2001). Multiple runs were conducted using the default and more exhaustive search parameters to obtain estimates of θ, which was then held constant to estimate average migration rates. A network relationship among haplotypes was estimated using the TCS computer program, version 1.13 (Clement et al. 2000). In order to examine possible differences among the pacificus/antarcticus populations a nested clade analysis was conducted following Templeton (1998). Results were analyzed through standard statistical analysis of contingency tables.

Results

A region of 703 bp was compared among 75 samples collected from four sampling locations. Twenty-one haplotypes are found, distinguished by 30 variable positions (Fig. 1). The majority of changes are synonymous

Table 2 Population Statistics: n = sample size, N = number of haplotypes, h = haplotype diversity (± standard deviation, SD), π = nucleotide diversity (±SD), $\theta_{(k)}$ = Theta estimated from expect number of alleles (k) and sample size (n) (Ewens 1972), $\theta_{(\text{Hom})}$ = Theta estimated expected homozygosity (Hom) (Zouros 1979; Chakraborty and Weiss 1991), $\theta_{(S)}$ = Theta estimated from the number of segregating sites (s) and sample size (n) (Watterson 1975), $\theta_{(\pi)}$ = Theta estimated from the mean number of pairwise differences (π) (Tajima 1983), N_f = the range of female effective population sizes estimated from the previous estimates of θ. $P_{(MM)}$ = p value of a unimodal mismatch distribution estimated under sudden range expansion model matching observed distribution (Schneider and Excoffier 1999)

Species	Sample location	n	N	$h \pm$ SD	$\pi \pm$ SD	$\theta_{(k)}$ 95% CI[a]	$\theta_{(\text{Hom})} \pm$ SD	$\theta_{(S)} \pm$ SD	$\theta_{(\pi)} \pm$ SD	N_f	$P_{(MM)}$
S pacificus	Taiwan	28	8	0.7937 ± 0.0424	0.0031 ± 0.0020	3.37, 1.4–7.4	3.04 ± 0.85	2.57 ± 1.11	2.16 ± 1.38	73–47,000	0.27
	Alaska	15	7	0.8381 ± 0.0680	0.0043 ± 0.0026	4.50, 1.7–11.5	4.20 ± 2.30	3.08 ± 1.43	3.01 ± 1.86	98–65,500	0.591
S. antarcticus	Southern Ocean	16	5	0.6667 ± 0.1130	0.0023 ± 0.0015	2.10, 0.7–5.6	1.52 ± 0.80	2.11 ± 1.06	1.59 ± 1.12	46–33,000	0.353
S. microcephalus	North Atlantic	16	7	0.7750 ± 0.0876	0.0022 ± 0.0015	4.18, 1.6–10.5	2.70 ± 1.46	2.71 ± 1.28	1.53 ± 1.08	91–33,300	[b]

[a] 95% confidence interval (CI) around the estimation

[b] Model mismatch distribution and observed distribution did not converge

	10	45	72	141	143	160	231	237	261	293	306	327	333	342	369	390	396	405	414	416	417	426	458	516	525	633	654	661	667	691
H1	A	A	T	A	T	G	A	T	C	T	C	C	T	A	G	T	C	A	A	G	C	C	G	G	C	T	T	G	C	C
H2		G																				T								
H3	G									C																				
H4			C					C										G												
H5			C				T	C					C					G												
H6						A																								
H7				T											A															
H8															A															
H9															A		A													
H10															A	C	A													
H11					C																									
H12																								A						
H13			C					C										G		A										
H14			C					C										G			T									
H15					C				T		T			G					G	A			A	A		C		A	T	T
H16					C				T		T	T		G				G	G	A			A	A		C		A	T	T
H17					C				T		T		C	G				G	G	A	T		A	A	T			A		T
H18					C				T		T			G				G	G	A			A	A				A	T	T
H19					C				T		T			G				G	G	A			A	A			C	A	T	T
H20					C				T		T			G				G	G	A			A	A		C		A	T	T
H21					C				T		T			G				G	G	A			A	A		C		A	T	
					NS	NS				NS										NS			NS					NS		

Fig. 1 Mitochondrial *cytochrome-b* haplotypes of sleeper sharks. Variable positions only are recorded (relative positions are given in the top row). Differences to H1 are noted for each haplotype. Nonsynonymous substitutions (NS) are indicated in the bottom row. Genbank accession numbers for full sequences are EF090943–EF090963

resulting in six inferred changes in the cytochrome-b protein sequence. An approximately 7:1 bias toward transitional substitution is noted through examination of 143 four-fold degenerate sites; 14 transitions versus two transversions.

A minimum spanning network (Fig. 2) shows the haplotypes clustering into two discrete groups separated by 11 substitutions. The first group includes haplotypes found in the *S. pacificus* and *S. antarcticus* populations. Here the haplotypes radiate from three central haplotypes, H1, H4 and H8, which are generally found in high frequency in three of the populations sampled. A similar star-like phylogeny is noted for the *S. microcephalus* haplotypes, with high frequency haplotypes central to rarer, radiating haplotypes. The one exception is haplotype H17 that is separated from the main cluster by five substitutions.

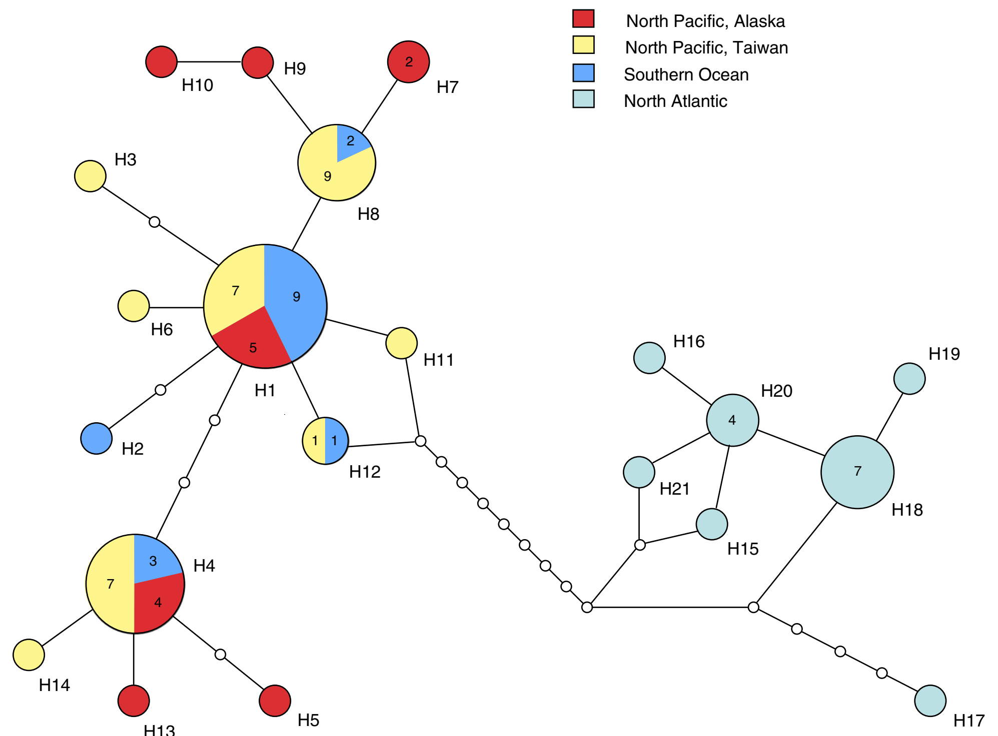

Fig. 2 Minimum Spanning Parsimony Network of sleeper shark haplotypes. The haplotype number and haplotype frequency >2 in the four sample populations are given beside and within each circle respectively. Small circles represent inferred haplotypes. Branches indicate one substitution event between extant and inferred haplotypes

Comparison of nucleotide substitutions within each sampling location and between groups reveals average nucleotide substitutions of 0.0056 ± 0.0015 and 0.0041 ± 0.0013 for the *S. pacificus/antarcticus* and *S. microcephalus* groups respectively. These values are similar to the estimates of nucleotide diversity (Table 2) but do not account for haplotype frequency. Average pairwise substitutions between the *S. pacificus*/antarcticus and *S. microcephalus* groups is 0.0205 ± 0.0048. Correcting for ancestral variation gives a distance value of 0.0157 between the groups. For the 703 nucleotides examined, this corresponds to 11 substitutions, which is the minimum number of substitutions needed to join the two haplotype groups in the network (Fig. 2).

Further examination of nucleotide substitution patterns between groups indicates strong evidence for purifying selection at this gene. The average number of synonymous changes/site is 0.0609 ± 0.0159 versus 0.0077 ± 0.0035 for nonsynonymous changes/site. The observed ratio of $\sim$ 8:1 is significantly different from the 1:1 ratio predicted under the neutral evolution of all sites. The observed polymorphism appears to be the result of neutral evolution as no evidence for selection (Tajima's and Fu's tests for selective neutrality; Tajima 1996; Fu 1997) is found at the different sampling locations.

Using the above ratio of synonymous to nonsynonymous substitutions ~9.77 synonymous substitutions have occurred at 172 synonymous sites since the divergence of the two groups. This corresponds to a neutral divergence rate of 0.056 sub/site. Few papers exist which have examined the neutral substitution rate in mitochondrial genes in sharks. Using the closing of the Isthmus of Panama as a calibration, a per site substitution rate of 0.008/Myr was recently estimated for the mitochondrial control region of hammerhead sharks (Duncan et al. 2006). This value may be used here by considering only the synonymous, i.e. neutral, substitutions between the *pacificus/antarcticus* and *microcephalus* haplotype groups to calculate a divergence time of ~7.1 Myr. Also using the closure of the Isthmus of Panama as a calibration, data reported by Martin et al. (1992) on *cyt-b* sequences from two individuals of bonnethead sharks (*Sphyrna tiburo*) sampled from either side of the Isthmus can be used to estimate a synonymous per site divergence rate of 0.0414 Myr. From this value a divergence time of ~1.35 Myr is calculated.

Similar numbers of haplotypes, 5–8, and haplotype diversity values are seen in each of the four sampling locations (Table 2). The lowest number of haplotypes and haplotype diversity values are found in the sleeper sharks collected in the southern hemisphere, however values are not statistically different from other sampling locations.

Analysis of molecular variance (AMOVA) shows evidence for genetic structure among the existing species, overall Fst = 0.7619, $P < 0.0005$. Pairwise comparisons among the four sampling locations shows significant difference between the Atlantic (*S. microcephalus*) and Pacific populations, with Fst values >0.8364, $P < 0.0005$. In contrast, no evidence for genetic structure is found among the North Pacific (*S. pacificus*) and the southern hemisphere (*S. antarcticus*) populations.

Although, no direct evidence of gene flow between *S. microcephalus* and *S. pacificus* or *S. antarcticus* is observed, average maximum likelihood estimates of migration rates among populations were estimated using the computer program Migrate-n. Estimated θ values are within the ranges estimated in Table 2. As expected, migration estimates given in terms of $2N_f m$ (N_f = effective population size of females and m = mutation rate), are high among the *S. pacificus* and *S. antarcticus* populations; Alaska—Southern Ocean, 459 (95% CI, 427–526); Taiwan—Southern Ocean, 658 (95% CI, 603–734); and Alaska—Taiwan, 790 (95% CI, 709–845). Average migration between *S. microcephalus* is low, however the 95% Confidence Interval (CI) is large, 0.045 (95% CI, 0.036–121).

In order to further explore the possible difference among the *S. pacificus* and *S. antarcticus* samples, a nested clade analysis was performed on this haplotype clade (Fig. 3). Intriguing differences are noted at the second clade step (Table 3). The *S. pacificus* samples have a roughly even distribution of haplotypes among the three clades while 69% of the *S. antarcticus* haplotypes belong to the nested clade 2-2. Unfortunately, sample size is less than five in three of nine cells making standard contingency analysis unreliable. Not surprisingly, Pearson and likelihood ratio chi-square tests do not indicate significance at the 5% level, however simply multiplying the Southern Ocean sample sizes by 2.5 to bring two of the three offending cells to five or above and thus, artificially increasing the power of the analysis, leads to P values <0.029. Therefore, if the trend in the data holds at a greater sample size then evidence for genetic structure between the *S. pacificus* and *S. antarcticus* samples would be evident.

Discussion

Two distinct clades of sleeper shark mtDNA haplotypes were found, strongly supporting the classification of *S. microcephalus* as a separate species from the Pacific and Southern Hemisphere sleeper sharks. Using recent estimates of neutral substitution rates in shark mtDNA, these two groups appear to have been separated by at least 1.35 Myr. We consider the use of a 3.5 Myr closure as a conservative date that should give minimum divergence estimates. In a detailed study of genetic distances between 15 sister taxa within a single genus of snapping shrimp

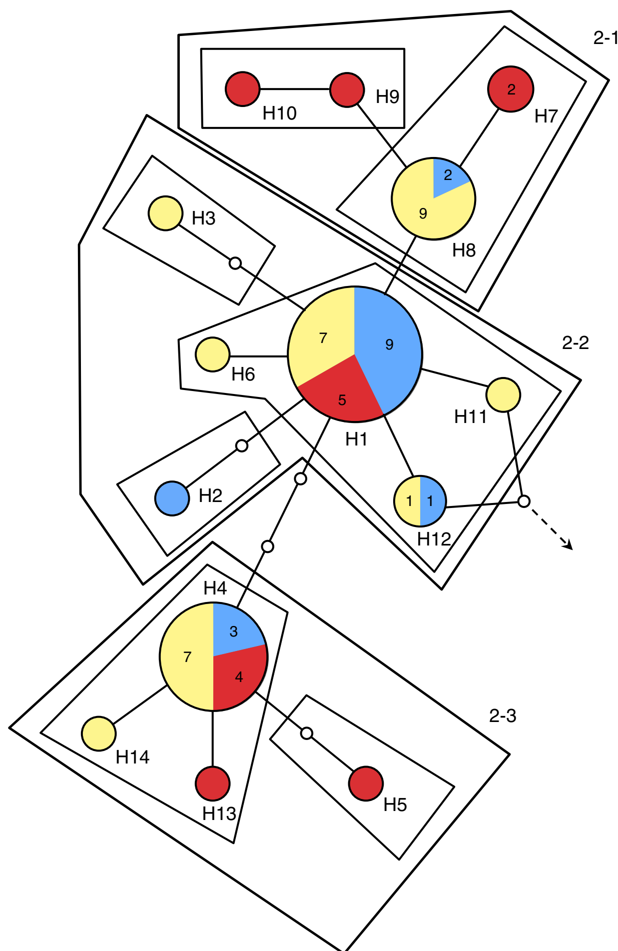

Fig. 3 Nested clade analysis of North Pacific (*Somniosus pacificus*) and Southern Ocean (*Somniosus antarcticus*) haplotypes

Table 3 Contingency table for nested clade analysis of two-step clades

	2–1	2–2	2–3
Taiwan	9	11	8
Alaska	4	5	6
Southern Ocean	2	11	3

from either side of the Isthmus, Knowlton and Weigt (1998) estimated separation times from 3 to 18 Myr. Further, as only two bonnethead shark sequences were available within population variation could not be removed from the estimate used to calculate the 1.35 Myr divergence. In contrast, the 7.1 Myr estimated from mitochondrial control region sequences of hammerhead sharks is likely too large, assuming the 3.5 Myr closure is correct. Comparison of a number of mammalian mitochondrial genomes shows that, although more variable in their rates, mitochondrial control region sequences generally have a lower substitution rate than that seen at synonymous sites (Pesole et al. 1999).

No direct evidence for gene flow between these populations was observed. Greenland sharks are well known to inhabit waters north of the Arctic Circle that are seasonally covered by sea ice (Templeman 1963; Compagno 1984; Skomal and Benz 2004). Their northern range extends from Baffin Bay in the Canadian High Arctic, eastward to Spitzbergen, Norway and the White Sea, and they have also been found in the Saguenay River, a tributary of the St. Lawrence River of eastern Canada. Pacific sleeper sharks have been noted in the Arctic Circle off the coast of Alaska in the Chukchi Sea (Benz et al. 2004). Despite any obvious barriers to shark movement through the Canadian High Arctic, given the current genetic data, maximum likelihood estimates of migration between these sharks indicate gene flow has been negligible. However, the 95% CI interval for this estimate is large and includes significant migration values at one end. Increased sampling of the populations would improve the confidence in this estimate. In addition, studies of nuclear genes should also be used to confirm migrations rates and population divergences as gene flow via males has been noted in some sharks (Pardini et al. 2001).

The separation of the North Pacific and Southern Hemisphere sleeper sharks into two species, *S. pacificus* and *S. antarcticus*, was not supported by the *cyt-b* data. The grouping of the Taiwan sharks with *S. pacificus* rather than *S. antarcticus* is not possible given the genetic data. The three populations (comprising the two species) share the most common haplotypes and no significant differences were noted in a number of tests for genetic structure. Two hypotheses may explain the lack of congruence between morphology and the mtDNA *cyt-b* sequence data.

(a) The two accepted species actually comprise a single species with a continuous distribution and the observed morphological differences are due to other biological processes such as extremes of clinal variation or a strong influence of the environment on morphology.
(b) The two accepted species are of recent origin and there hasn't been sufficient time for *cyt-b* lineage sorting to take place.

North Pacific sleeper sharks have been noted as far south as the Baja Peninsula in the Eastern Pacific (Compagno 1984), and Taiwan in the Western Pacific (Wang and Yang 2004). Sleeper sharks recorded in these northern subtropical locations are found at depth in cold water. There are, however, no records of sleeper sharks in tropical waters, supporting the second hypothesis, and indicating genetic isolation of these populations. It is possible that sharks in the Pacific have a more continuous distribution than previously thought and that sharks may be found in tropical water, but only at depth. The first hypothesis predicts that with increased sampling of deep-water tropical locations sleeper sharks will be found.

The degree of morphological similarity and the sharing of *cyt-b* haplotypes indicate that even if tropical migration between the North and South Pacific is not occurring today, it must have occurred in the recent geological past. In this respect, the analysis of these two species of sleeper shark shifts from a phylogenetic comparison of species to a phylogeographic comparison of populations. Due to its selective constraints, *cyt-b* is not a commonly used marker in this regard (Avise 2004). Analysis of mitochondrial DNA RFLP and more recently control region sequence variation have been examined to look at genetic structure within shark species (Heist et al. 1995; Heist et al. 1996a; Heist et al. 1996b; Kitamura et al. 1996; Gardner and Ward 1998; Keeney et al. 2005; Duncan et al., 2006). It is suggested that analysis of mtDNA control region variation be conducted to further examine the genetic structure of the *S. pacificus/antarcticus* group.

To our knowledge, this paper represents the largest survey of within species *cyt-b* sequence variation conducted in sharks. Intriguingly, levels of haplotype diversity and nucleotide diversity within the Pacific/Southern hemisphere populations examined in this study (Table 2) are similar to those recorded for studies examining mtDNA control region variation in populations of blacktip (*Carcharhinus limbatus*) (average $h = .805$, $\pi = 0.00214$; Keeney et al. 2005) and scalloped hammerhead (*Spyrna lewini*) sharks (average $h = 0.80$, $\pi = 0.0013$; Duncan et al. 2006). The greater amount of variation found at the relatively unconstrained mtDNA control region versus the coding gene *cyt-b* is well known. An analysis of seven bull shark (*Carcharhinus leucas*) samples from one population at both mtDNA *cyt-b* and the control regions found a greater amount of haplotypes (1 versus 5, respectively) and sequence variation (Kitamura et al. 1996). This implies that a greater amount of variation should be found in the mtDNA control region of sleeper sharks.

Among the Pacific populations, four haplotypes including three common core haplotypes are shared (Fig. 2). These may reflect the ancestral polymorphism of the founding population. Analysis of control region variation would help to resolve the true amount of haplotype sharing versus haplotype lineage sharing among the populations. Lack of structure at mtDNA *cyt-b* and control region would support ongoing gene flow. In contrast, the absence of shared haplotypes, with the noted sharing of ancestral polymorphic mitochondrial lineages, would support recent subdivision (possibly speciation).

Further examination of the genetic variation found between these populations is warranted to determine the species status. The two hypotheses presented to explain the lack of congruence between morphology and the mtDNA data can be addressed by examining more samples from the different locations and comparing morphological characters and genetic markers. Sleeper shark samples, however, are not easily obtained and the fishery that had operated in Taiwan for about a decade has apparently collapsed recently with only sporadic catches of sleeper sharks during the prime season (J. Y. Wang pers. comm. 2006). Analysis of genetic markers should include sequence analysis of the mtDNA control as well as examination of nuclear markers as discussed above. Nuclear and mitochondrial markers do not always agree (Heist et al. 1996b; Schrey and Heist 2003; Pardini et al. 2001) and would allow for increased resolution of the problem.

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