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RESEARCH ARTICLE

Genetic structure of leopard shark (*Triakis semifasciata*) populations in California waters

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Abstract The leopard shark (*Triakis semifasciata*) is an important predator in coastal marine ecosystems of California, targeted by recreational and commercial fishermen and of specific interest in fisheries management. From October 2003 to August 2006, 169 leopard sharks were collected from the coast of California (between 40.750°N and 32.678°N) and analyzed for mitochondrial and nuclear genetic structure. Analyses of mtDNA control region sequences revealed relatively low levels of genetic variation (five haplotypes, average pairwise divergence $\pi = 0.0067$). In contrast, leopard sharks were highly polymorphic for inter simple sequence repeats (ISSRs), which characterize a broad range of the nuclear genome. The null hypothesis of panmixia in California waters was rejected for both genetic markers, and ISSRs displayed a statistically significant pattern of isolation by distance (IBD) across the species range ($P = 0.002$). A variety of analyses showed that divergence is most pronounced in the northernmost population of Humboldt Bay. Natal philopatry in *T. semifasciata* was tested using Siegel-Tukey tests on data partitioned by breeding site status, and sex-specific philopatry was tested by comparing IBD plots between sexes. Although there was some evidence for natal philopatry in leopard sharks ($P = 0.038$), and population divergence may be related to the proximity of breeding sites ($P = 0.064$), we found no support for sex-specific philopatry. In addition to identifying a novel set of highly variable genetic markers for use in shark population studies, these results may be used to better inform management decisions for leopard sharks in California.

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Introduction

Sharks are an ancient lineage of fishes that have considerable ecological and economic value worldwide due to their predatory trophic status and highly sought fins (Cortes 1999; Clarke et al. 2006; Myers et al. 2007). Many sharks are vulnerable to overexploitation due to their low fecundity and slow maturation (Musick et al. 2000). As in other migratory species, an understanding of their genetic population structure is essential for effective management (Hueter et al. 2005). Several factors hinder the progress of shark population genetics, including slow mutation rates in mitochondrial DNA (mtDNA) that lead to low genetic variation (Martin et al. 1992), difficulty in sampling individuals from an entire species range, and unclear dispersal patterns as a result of poorly defined barriers in the marine environment (Palumbi 1994; reviewed by Heist 2004). Philopatric behavior (i.e., natal homing: Hueter et al. 2005) and the lack of pelagic larvae may further affect patterns of genetic population structure when compared to fishes with highly dispersive larvae. However, newer molecular techniques have made the study of shark population genetics more feasible.

The leopard shark (*Triakis semifasciata*) is one of approximately 40 cartilaginous fishes found in coastal California waters (Ebert 2003). This species is important to

both recreational and commercial fisheries in California, yet is vulnerable to overexploitation throughout its historic range from central Oregon to Mazatlan, Mexico (Miller and Lea 1972; Smith and Abramson 1990; Cailliet 1992; Smith 2001). The current status of leopard shark populations in Mexican waters is unknown (Compagno et al. 2005). The genetic structure of this species has not been studied, and very little is known about individual movement or potential for philopatric behavior.

Philopatry, defined as the successive use of native habitat throughout the life cycle of an organism, has been observed in numerous terrestrial and marine taxa, including sharks (Hueter et al. 2005). The tendency to return to a breeding locality in successive years (i.e., natal philopatry) has been observed in at least seven species of sharks using tagging, tracking, and genetic methods (reviewed by Hueter et al. 2005). Differences in patterns of genetic variation between males and females have provided evidence for sex-specific philopatry in species that include white sharks (Pardini et al. 2001), lemon sharks (Feldheim et al. 2002), blacktip sharks (Keeney et al. 2003) and shortfin mako sharks (Schrey and Heist 2003). Females of these species often remain closer to their home territories, while males roam farther away. In California, nine locations are suspected breeding sites for leopard sharks: Humboldt Bay, Tomales Bay, Bodega Bay, San Francisco Bay, Elkhorn Slough, Morro Bay, Santa Monica Bay (Los Angeles), Catalina Harbor (Santa Catalina Island), and San Diego Bay (Talent 1985; Monaco et al. 1990; Smith and Abramson 1990; Ebert and Ebert 2005; Fowler et al. 2005; Smith 2005; G. Cailliet, personal communication; D. Ebert, personal communication; S. Smith, personal communication). Six of these breeding locations were sampled during this study and this information was later used to partition data for tests of philopatry. Despite large aggregations occurring periodically in coastal California (primarily during the spring), the degree of philopatric behavior in leopard sharks and the potential for sex-specific patterns are largely unknown.

In this study, we use sequence data from the mtDNA control region coupled with genotypic data from inter-simple sequence repeats (ISSRs) to assess variation among leopard shark populations. The control region is commonly used in studies of vertebrates as it is usually highly variable and hypothesized to be selectively neutral (Meyer 1993). Developed in 1994, ISSRs constitute the amplification of DNA fragments between simple sequence repeats through the use of a single, anchored primer that binds to the tandem repeat motif (Gupta et al. 1994; Zietkiewics et al. 1994). ISSRs are used to assess variation at numerous loci throughout the nuclear genome. To date, ISSRs have not been used to study the genetics of sharks, but they typically yield large numbers of polymorphic loci in taxa such as plants, fungi, invertebrates (Wolfe 2005) and at least one teleost fish (Bay et al. 2006). ISSRs tend to be more reproducible than some other dominant molecular markers, require little prior genetic information on the species of interest, and are time efficient and cost effective (Wolfe 2005).

We specifically focused on the following questions: (1) Do leopard sharks function as one panmictic unit in California waters? (2) Which populations warrant specific attention, based on patterns of genetic structure? (3) Is there genetic evidence for philopatric behavior in this species? Answers to these questions will contribute to our current understanding of the leopard shark fishery in California and aid in the future assessment and management of similar fisheries.

Materials and methods

Sampling

From October 2003 to August 2005, tissues from 169 leopard sharks were obtained from 10 sites throughout California (20 individuals per site where possible). Tissue samples from the northern portion of the study range were donated, and samples from the Southern Bight were collected by the first author with others (see Acknowledgements). Size (total length), sex, and location (GPS coordinates) were recorded for each individual when possible. Sampling sites (~25 km in diameter) were chosen by access and availability. Fin clips were taken from the free rear tip region of the first dorsal fin to prevent re-sampling and permanent injury to released sharks. Wounds on leopard sharks heal over the course of approximately 4 months (Reif 1978). To verify the safety and effectiveness of the fin clipping technique, three control individuals were kept in an aquarium at Scripps Institute of Oceanography (La Jolla, CA, USA) and monitored for 10 months from 2004 to 2005. Scar tissues healed and remained visible on these sharks. After collection, tissues were stored in 95% ethanol for transport, transferred into a 1.5 ml tube, and stored at −80°C. All DNA extractions were performed using a DNeasy kit (Qiagen), which yielded 4,000–40,000 ng of DNA per fin clip.

MtDNA methods

Prior to amplifying mtDNA, we designed novel primers to target the entire control region of leopard sharks by aligning the mitochondrial genomes of six Chondrichthyan fishes and one teleost from Genbank (NIH) using Sequencher 4.1.2 (Gene Codes Corporation); *Scyliorhinus canicula* (NC_001950), *Mustelus manazo* (NC_000890), *Heterodontus francisci* (NC_003137), *Chimaera monstrosa*

(NC_003136), *Squalus acanthias* (NC_002012), *Raja radiata* (NC_000893) and the teleost *Thunnus alalunga* (NC_005317). Our forward primer, CR1 (5′-CCTGCCC TTGGCTCCCAAAGCCAAGATTC-3′) and reverse primer CR2 (5′-TTACAATTAARACTAAGGCRAGGACCAA A-3′) successfully amplified approximately 1,050 base pairs of the control region gene in leopard sharks.

PCR amplifications were performed with a final volume of 25 µl as follows: 1 × Taq Polymerase Buffer (No $MgCl_2$), 0.25 µM of each dNTP, 2.3 µM $MgCl_2$, 0.48 µM Primer "CR1", 0.48 µM of Primer "CR2", 0.6 µM BSA (Invitrogen), 0.75 units of *Platinum Taq* Polymerase (Invitrogen), and 10–100 ng of DNA. Amplifications were performed under the following conditions: 94°C for 2 min, 94°C for 30 s, 72°C for 1.5 min, followed by 39 cycles of 94°C for 30 s, 72°C for 1.5 min, and a final extension of 2 min at 72°C. Successful mtDNA amplifications were cleaned with a Qiaquick kit (Qiagen) and cycle sequenced using BigDye (Applied Biosystems) on an ABI 377 automated sequencer. Sequence alignments were performed using Sequencher 4.1.2 (Gene Codes Corporation).

ISSR methods

Sixty-five di-repeat ISSR primers with a single nucleotide anchor at the 3′ end (University of British Columbia Nucleic Acid-Protein Service Unit, Primer Set #9) were tested using relaxed PCR conditions of 2.8 µM $MgCl_2$ and 50°C annealing temperatures. Thirty-eight of these primers successfully amplified leopard shark DNA. After optimization of PCR conditions, three primers reliably produced polymorphic bands visible with agarose gel (2%) electrophoresis. Primers 808 (5′-$(AG)_8$C -3′), 811 (5′-$(GA)_8$C -3′), and 818 (5′-$(CA)_8$G -3′) were optimized for PCR reagents and annealing temperature to the nearest 0.5°C using unlabeled primers, and subsequently amplified in separate reactions with fluorescent dye-labeled primers compatible with the *Rox* dye set (ABI). Primer 808 was labeled with NED (yellow), primer 811 with VIC (green), and primer 818 with 6FAM (blue). All ISSR PCR amplifications were performed with a final volume of 25 µl using 1 × Taq Polymerase Buffer (No $MgCl_2$), 0.25 µM of each dNTP, 1.48 µM of $MgCl_2$, 0.9 µM of unlabeled primer or 0.3 µM of dye-labeled primer, 1.25 units of *Platinum Taq* Polymerase (Invitrogen), and 5–50 ng of DNA. All ISSR PCR amplifications were performed under the following conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 59.2°C for 30 s (64°C for 818), 72°C for 2 min and a final extension at 72°C for 7 min. Positive and negative controls were performed for each set of PCR amplifications.

ISSR genotyping was performed on an ABI 3100 capillary machine, with 1.0 µl of each dye-labeled PCR product added to 11 µl of formamide and 0.75 µl of *Rox 1000* size standard (ABI). The presence or absence of a locus (defined as an observable band of dye on the chromatogram generated by the capillary machine) was determined using GeneMapper 3.7 (ABI). To ensure accuracy and repeatability, three positive control samples were genotyped five times each for primers 808 and 811, and two positive control samples ten times each for primer 818. Any discrepancies among positive controls for the presence or absence of amplified DNA at a given locus resulted in our removing that locus from the study. Based on estimates of precision from these replicates and others, we were able to unambiguously size fragments to the nearest 1 bp. We converted and combined genotype data for all individuals, primers, and loci into binary code with "0" representing absence and "1" representing the presence of amplified DNA.

Analyses of population structure

For mtDNA data, we quantified genetic structure among sites as Φ_{ST} (an analog of F_{ST}) using analysis of molecular variance (AMOVA). Calculations were conducted in Arlequin 3.0 (Excoffier et al. 2005), with the uncorrected number of pairwise differences between sequences as the distance matrix and statistical significance assessed using 10,000 permutations. Because ISSRs are dominant markers, it is not possible to distinguish between homozygous dominant and heterozygous individuals, and therefore calculate allele frequencies directly. As a result, we utilized the null model of Hardy-Weinberg (H-W) random mating genotype frequencies for ISSRs, as is commonly assumed for dominant nuclear markers in diploid organisms. No prior information exists that would justify rejecting the null hypothesis of H-W frequencies in leopard sharks. Similar species, such as *Squatina californica* (Gaida 1997), *Mustelus antarcticus* (Gardner and Ward 1998), and four other *Mustelus* spp. (Gardner and Ward 2002) do not depart statistically from H-W assumptions with codominant markers over spatial scales similar to the present study. Even if a low degree of local preferential inbreeding occurs in leopard sharks (i.e., if $F_{IS} > 0$), we do not expect to see a dramatic bias in estimates of population subdivision if the pattern is consistent among sampling sites. We also assumed that the use of a large number of ISSR loci distributed throughout the nuclear genome would overwhelm the effect of selection on any particular locus, if selection is present. Thus, we accepted the assumption of random mating H-W genotype frequencies for the ISSR data for the analyses that required this assumption.

To estimate ISSR variation within sampling sites, we used Popgene 1.31 (Yeh et al. 1999) to calculate basic summary statistics including number of loci (number of bands present in at least one individual), percent polymorphic

loci, and allele frequencies (presence or absence of bands). Because population (i.e., gene pool) boundaries for leopard sharks are not clearly defined by environmental features, we quantified population structure using a variety of methods with different assumptions. Nei's (1972; 1978) unbiased D was calculated between each pair of sites under the assumption of H-W frequencies. We tested for patterns of isolation by distance (IBD) by assessing the relationship between (1) Nei's unbiased D (ISSR data) versus geographic distance, and (2) Φ_{ST} (mtDNA data) versus geographic distance using Mantel tests with the software IBDWS 2.6 (Jensen et al. 2005; 10,000 randomizations). Statistical significance in the IBD relationship is commonly assessed with Mantel Tests, but slope and intercept values are rarely compared between species or sexes. We compared slopes and intercepts from the IBD reduced major axis regression by jackknifing over populations in the IBDWS program. (Note that although *pairs* of populations are used when plotting the relationship, the unit of independence for generating confidence intervals and hypothesis testing is the population.) Residuals from the IBD regressions were also examined to determine whether one or a few sites had undue influence; we repeated the analysis excluding Humboldt Bay because it was genetically and geographically the most divergent sample site. Additional analyses of genetic population structure performed using two other genetic distances (Roger's distance and chord distance) were qualitatively similar to Nei's unbiased D and are not presented here.

Overall ISSR divergence among all sites was quantified as Θ^{β}, an estimate of F_{ST}, using Hickory 1.0 (Holsinger et al. 2002). This program uses a Bayesian algorithm to estimate heterozygosity within each sampling site (Table 1), which is subsequently used for estimates of Θ^{β}. We performed our analyses using the F-free model in Hickory, which chooses inbreeding coefficients randomly from a non-informative prior during sampling, and thus requires no assumptions regarding mating. We performed Bayesian assignment tests using Bayesian Analyses of Population Structure 3.2 (BAPS) to see how individuals cluster without an a priori assumption of population membership (Corander et al. 2005). A cluster is defined as a grouping of individuals based on genetic similarity; under model assumptions of H-W genotype frequencies and linkage equilibrium, a cluster is equivalent to a population or gene pool. We specified the maximum number of clusters (k) as 169, our total number of samples, as we had no reason a priori to assume any particular smaller number. Preliminary analyses with a similar program, STRUCTURE 2.1 (Pritchard et al. 2000) yielded results that were qualitatively similar in terms of individual cluster membership. However, because likelihood values for $k > 7$ clusters failed to approach a well-defined asymptote in STRUCTURE, we only present here the results of the BAPS analysis.

Finally, we used Alleles in Space (AIS) (Miller 2005) to visualize spatial patterns of ISSR variation among individuals. Using geographic data (projected in Universal Transverse Mercator) and the genetic distance scores calculated in AIS, we created a three-dimensional landscape with genetic distance in the z dimension. (The x and y axes simply correspond to cardinal directions.) Because AIS interpolates using *residuals* from an IBD regression across individuals, a positive peak on the z axis will be found in a geographic area where genetic divergence is unusually high, after IBD is accounted for.

Tests for philopatry

To investigate the possibility of philopatry in leopard sharks, we partitioned our genotype data by breeding site

Table 1 Leopard shark sampling sites, GPS coordinates of sampling sites (decimal degrees), sampling dates, breeding site status as indicated by scientific literature, the number of males and females collected at each sampling site, and the total number of leopard sharks collected per site

Sampling site	Latitude	Longitude	Date	Breeding site?	No. of males	No. of females	No. of sex unknown	Sample size
Humboldt Bay	40.750	−124.210	N/A	Yes	N/A	N/A	24	24
Tomales Bay	38.155	−122.949	6/06	Yes	1	2	0	3
San Francisco Bay	37.708	−122.279	3/04 to 8/05	Yes	4	10	6	20
Elkhorn Slough	36.800	−121.901	3/04 to 6/05	Yes	3	17	0	20
Santa Barbara	34.404	−119.864	5/04 to 6/04	No	2	3	0	5
Ventura	34.309	−119.362	10/03 to 6/04	No	12	1	4	17
Santa Catalina Island	33.317	−118.427	4/04 to 6/04	Yes	4	16	0	20
Los Angeles	33.793	−118.412	4/04 to 8/04	Yes	13	6	1	20
Carlsbad	33.144	−117.339	4/04 to 8/04	No	6	6	6	18
San Diego	32.678	−117.260	2/04 to 8/04	Yes	11	10	1	22
Totals					56	71	42	169

status (to test for natal philopatry) and by sex (to test for sex-specific philopatry). Individual leopard sharks that were captured within 25 km of known breeding sites were considered more likely contributors to the local gene pool than individuals captured near other breeding sites or >25 km away from any known breeding site. For these analyses, we calculated the average genetic divergence of each sampling site, estimated as the average pairwise Nei's unbiased distance to all other sampling sites. Within each site, expected heterozygosity was used as a measure of overall genetic variation.

For both average genetic divergence and overall genetic variation, we used Siegel–Tukey tests (Neave and Worthington 1988) to test for differences in variance between breeding sites and non-breeding sites. Breeding sites would be expected to have higher variance than non-breeding sites if individuals at breeding sites primarily represent one gene pool (strict philopatry), and individuals at non-breeding sites represent transients from many gene pools. In the case of strict philopatry, we also expected the mean estimate of genetic divergence to be higher for breeding sites than for non-breeding sites, since individuals at non-breeding sites are more likely to represent multiple gene pools.

When the ISSR genotype data were partitioned by sex, 127 individuals were included from nine sampling sites, with both sexes represented at these sites (total of 56 males, 71 females). We tested for differences among males and females in IBD slopes and intercepts and the Bayesian estimates of differentiation Θ^{β} by comparing 95% confidence intervals between the sexes. IBD confidence intervals were estimated by jackknifing over populations in IBDWS (Jensen et al. 2005), and CIs for Θ^{β} were calculated in Hickory (Holsinger et al. 2002). In the case of strict sex-specific philopatry, sedentary females should display greater genetic divergence, as measured by pairwise genetic distance, overall Θ^{β} and IBD slope and/or intercept.

Results

Mitochondrial sequences in the forward direction yielded 600 bp after alignment and trimming of ambiguous ends. Five haplotypes ($k = 5$) and four polymorphic nucleotide sites ($s = 4$) were found. G/A substitutions occurred in 11 individuals and T insertions occurred in three individuals. The number of substitutions per site was very low ($\pi = 0.0067$), and one rare haplotype occurred only in Humboldt Bay (Haplotype A, Table 2). The greatest number of haplotypes found in leopard sharks from one sampling site was four, which occurred in Los Angeles.

For the ISSRs, primer 808 produced 49 polymorphic loci (i.e., fragments of DNA) ranging from 57 to 493 bp in length, and no monomorphic loci. Primer 811 produced 20 polymorphic loci and 1 monomorphic locus ranging from 89 to 447 bp. Primer 818 produced 22 polymorphic loci from 69 to 495 bp in length and no monomorphic loci. Our three ISSR primers produced a total of 91 polymorphic loci and one monomorphic locus (98.9% polymorphism). We excluded 14 of the 91 polymorphic loci from analyses due to discrepancies among one or more positive control replicates. The frequency of DNA amplified at a given locus ranged from 0.003 to 0.9585 for all individuals at all loci. The number of loci and expected heterozygosity were highest in Los Angeles and Humboldt Bay (Fig. 1).

Population structure

For both sets of molecular markers, populations showed significant structure [mtDNA $\Phi_{ST} = 0.069$ ($P = 0.01$) and ISSR $\Theta^{\beta} = 0.110$ (95% CI: 0.088, 0.139)]. Patterns of mtDNA IBD were not statistically significant, as Φ_{ST} was not correlated with geographic distance across population pairs ($P = 0.54$). This may have been due to low levels of mtDNA variation. For the ISSRs, Nei's unbiased genetic

Table 2 Summary statistics from ISSR and control region (mtDNA) data by sampling site. Heterozygosity estimates with standard deviation and BAPS cluster assignments found in leopard sharks from each sampling site as calculated using ISSR data. mtDNA haplotype letters correspond to unique haplotypes and the number of leopard sharks per sampling site with each haplotype in parentheses

Sampling site	Sample size	ISSR data			Contol region (mtDNA)
		H_e	SD	Cluster (BAPS)	Haplotype (s)
Humboldt Bay	24	0.129	0.007	1, 3	A (1), E (23)
Tomales Bay	3	0.113	0.009	2, 4 ,5	E (3)
San Francisco Bay	20	0.115	0.006	1, 2, 3, 5	E (20)
Elkhorn Slough	20	0.093	0.007	2, 3, 4, 5	E (20)
Santa Barbara	5	0.104	0.008	3, 5	E (5)
Ventura	17	0.099	0.006	3, 4, 5, 6	D (1), E (16)
Santa Catalina Island	20	0.104	0.006	3, 4, 5	B (2), D (1), E (17)
Los Angeles	20	0.125	0.006	3, 4, 5, 6, 7	B (1), C (3), D (3), E (13)
Carlsbad	18	0.106	0.006	3, 4, 5	C (1), E (17)
San Diego	22	0.098	0.005	2, 3, 4, 5	D (1), E (21)
Average H_e	169	0.109	0.003		

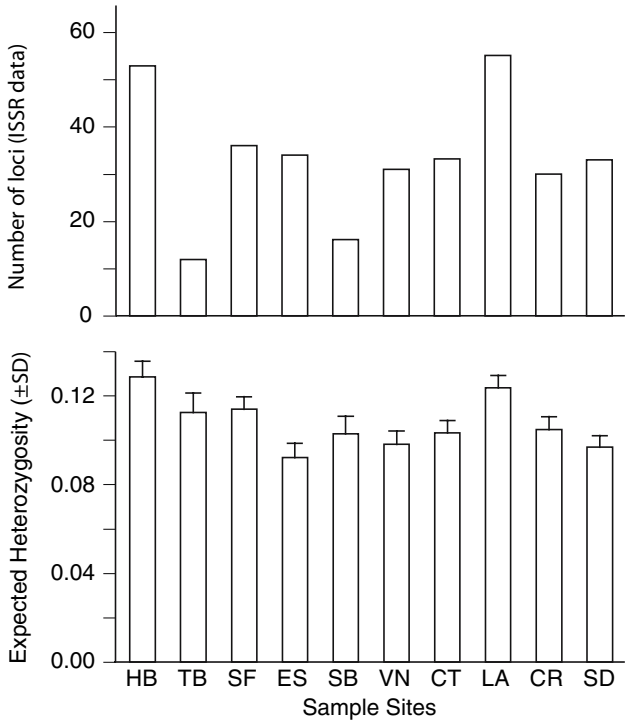

Fig. 1 ISSR summary statistics by sampling site. **a** Number of ISSR loci. **b** Expected heterozygosity (ISSR data) + 1 SD. Sampling sites are abbreviated from North to South using the following abbreviations: *HB* = Humboldt Bay, *TB* = Tomales Bay, *SF* = San Francisco, *ES* = Elkhorn Slough, *SB* = Santa Barbara, *VN* = Ventura, *CT* = Santa Catalina Island, *LA* = Los Angeles, *CR* = Carlsbad, *SD* = San Diego

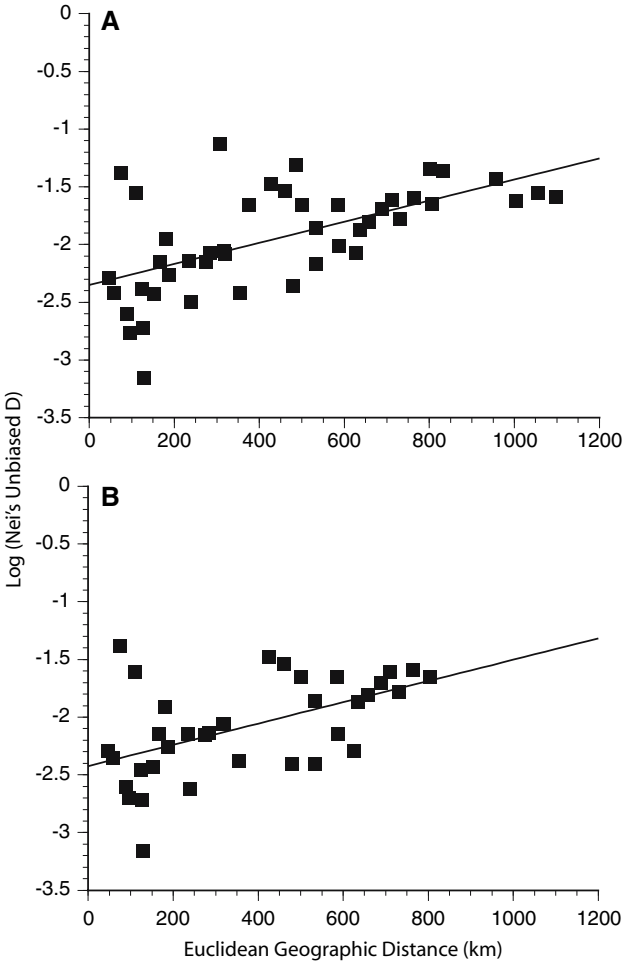

Fig. 2 Isolation by distance (IBD) plots for ISSR data. **a** Log genetic distance (Nei's unbiased D) versus Euclidean geographic distance including all sampled individuals. **b** Log genetic distance (Nei's unbiased D) versus Euclidean geographic distance excluding all individuals sampled in Humboldt Bay, CA, USA

distance ranged from 0.0006 to 0.076 between site pairs, and log (Nei's unbiased D) increased with the geographic distance between sites [($r = 0.18$), $P = 0.002$; $y = 0.0015x - 2.62$; Fig. 2a]. These results did not change qualitatively when the most divergent sampling site (Humboldt Bay) was removed [($r = 0.15$), $P = 0.005$; $y = 0.0018x - 2.73$; Fig. 2b]. The average residuals from the log-transformed IBD relationship were highest for the contrast between San Francisco and Tomales Bay, indicating that these populations are unusually divergent. The lowest residuals were for the San Diego versus Los Angeles contrast, indicating atypically high genetic similarity (Fig. 3).

Patterns of ISSR population structure were explored further using the AIS interpolation surface, with positive and negative peaks reflecting genetic divergences that were unusually high or low, after correcting for IBD (Fig. 4). The most prominent positive peak was observed between Humboldt Bay and Tomales Bay in northern California (Fig. 4a). Leopard sharks sampled from San Francisco Bay were more similar genetically to those from Elkhorn Slough than Tomales Bay (Fig. 4b). Further south on the genetic landscape, a series of three negative peaks were observed (Fig. 4c). These peaks corresponded to unusually high connectivity between Elkhorn Slough and three

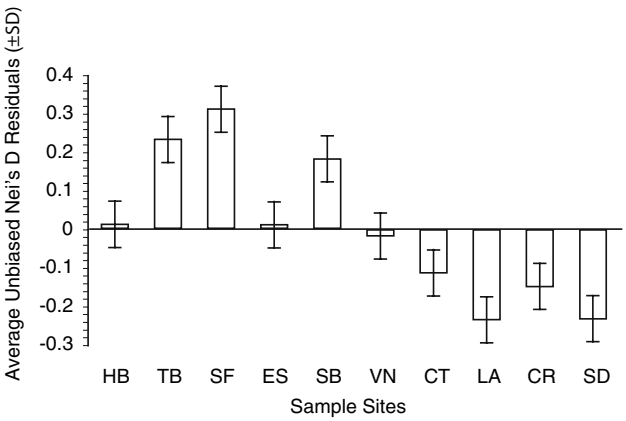

Fig. 3 Average pairwise genetic distances between individual leopard sharks from different sampling sites. These distances are unbiased Nei's distance residuals from the log-transformed IBD relationship of ISSR data. Sampling sites are abbreviated as in Fig. 1

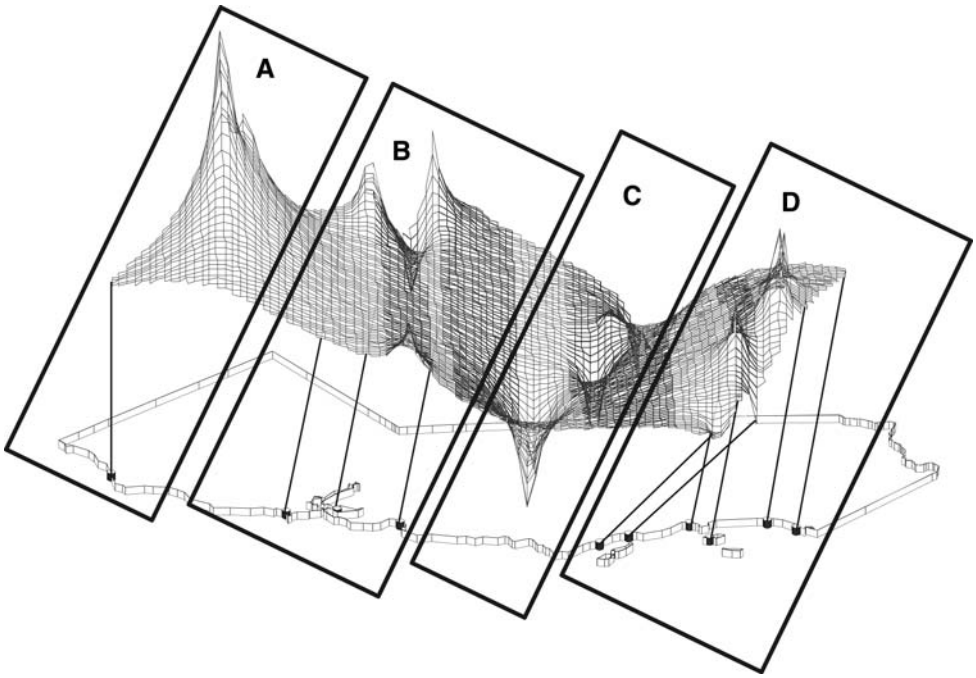

Fig. 4 Alleles In Space interpolation plot for ISSR data. UTM (northing) is on the *y* axis, UTM (easting) is on the *x* axis and residual genetic distance (Nei's unbiased D) on the *Z* axis. Positive "peaks" represent high genetic discontinuities and negative peaks represent high genetic similarities. Letters *A* through *D* correspond to regions of specific interest (see text)

sampling sites in southern California. Several positive peaks occurring within southern California were a result of genetic discontinuity between Santa Catalina Island and three other sampling sites: Santa Barbara, Los Angeles, and Carlsbad (Fig. 4d).

The BAPS analysis assigned individuals to seven genetic clusters without the use of sample site information ($-\ln k(7) = 5246$, $P > 0.999$ compared with other values of k). Clear patterns were observed when individuals were subsequently sorted by cluster and sampling site (Fig. 5). For example, cluster 1 was comprised only of leopard sharks collected north of San Francisco Bay (Fig. 5a). Individuals in cluster 2 were found north of Elkhorn Slough, except for one individual in San Diego which may have represented a transient individual (Fig. 5b, d). Clusters 3, 4 and 5 were relatively evenly distributed among sample sites. In contrast to northern California, sample sites south of Ventura tended to contain individuals that represented more gene pools. In addition, clusters 6 and 7 were unique to Los Angeles and Ventura (Fig. 5c). Los Angeles contained the highest number of ISSR-defined gene pools, which paralleled its relatively high diversity of mtDNA haplotypes.

Philopatry

For ISSR data, the average pairwise estimate of Nei's unbiased distance was more variable among breeding sites than among non-breeding sites (Siegel-Tukey test, $U = 0$, $P \leq 0.02$). This was consistent with the hypothesis that samples from non-breeding sites represented more than one gene pool. Within-site heterozygosity was also more variable among breeding sites than non-breeding sites, although the contrast was not quite significant (Siegel-Tukey test, $U = 4$, $P > 0.10$).

Nei's unbiased D showed a significant pattern of IBD for males [Fig. 6a: ($r = 0.279$), $P = 0.008$, $y = 0.0011x - 2.15$], but the IBD slope for females was not significantly different from 0 [Fig. 6b: ($r = 0.05$), $P < 0.10$, $y = 0.000254x - 1.71$]. It is possible that the IBD slopes or intercepts could differ between sexes, even if the slope of the female relationship is not significant. However, no aspect of the IBD relationship differed between the sexes. For example, the IBD plot for males had a slope of 0.00253 (95% CI: 0.00127, 0.00378) and an intercept of −2.69 (95% CI: −3.28, −2.10), whereas the IBD plot for females had a slope of 0.00113 (95% CI: 0.00083, 0.00143) and an intercept of −2.03 (95% CI: −2.20, −1.86). The Bayesian estimate of overall genetic divergence (ignoring possible IBD) was twice as high in females ($\Theta^{\beta} = 0.109$) as in males ($\Theta^{\beta} = 0.050$). However, this contrast was not statistically significant either (95% CI: 0.082, 0.161 and 0.028, 0.092, respectively).

Discussion and conclusions

Both traditional and newer types of population genetic analyses demonstrate that multiple, significantly structured populations of leopard sharks exist in California waters. In this first application of ISSRs to cartilaginous fishes, these

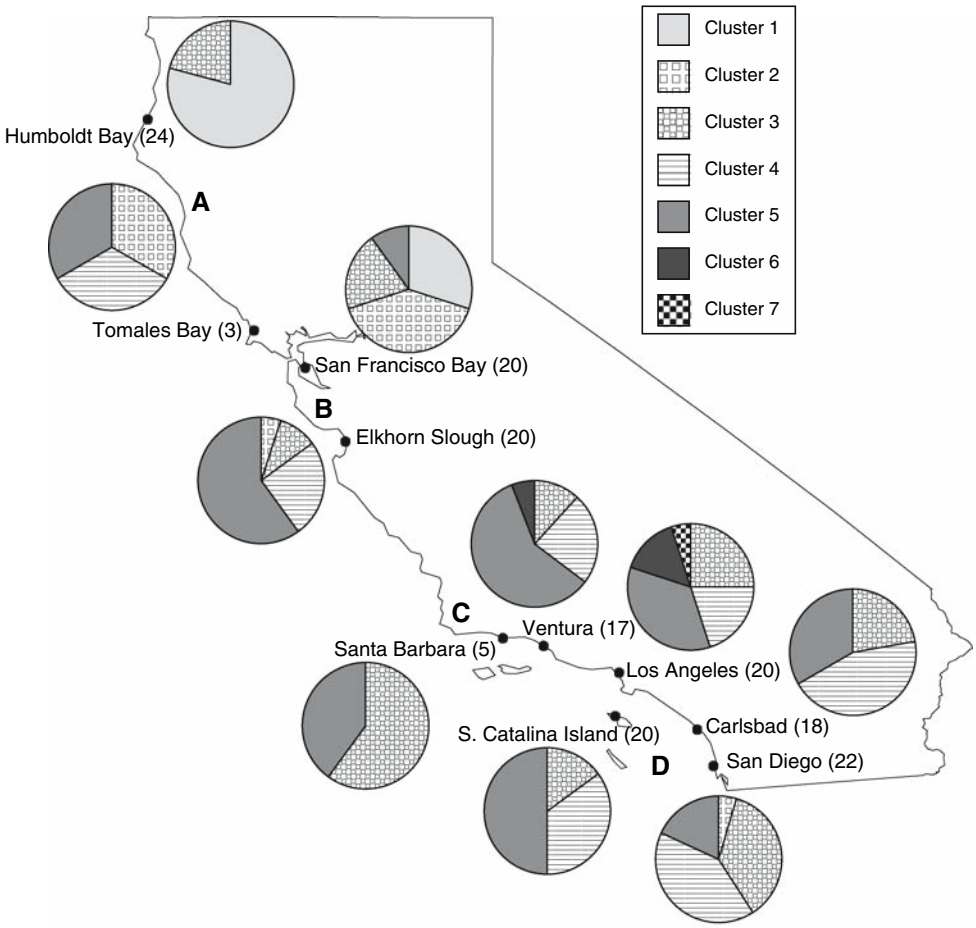

Fig. 5 BAPS cluster percentages by sampling site with all sampled individuals included. Letters *A* through *D* correspond with regions of specific interest (see Fig. 4 and text)

genetic markers proved useful in quantifying population structure. We rejected the null hypothesis that leopard sharks form one panmictic population in California waters, and found evidence that both spatially limited gene flow and proximity to breeding sites may contribute to this structure. Thus, dispersal and gene flow in this species are limited in comparison to the scale of this study. The isolated gene pools that we have identified will likely warrant separate attention from a fisheries management perspective.

Estimates of population subdivision were comparable in the two types of genetic markers (mtDNA $\Phi_{ST} = 0.069$, ISSR $\Theta^{\beta} = 0.110$), and comparable to sympatric species such as the Pacific angel shark (mtDNA $F_{ST} = 0.09$: Gaida 1997) and shovelnose guitarfish (mtDNA $F_{ST} = 0.23$: Sandoval-Castillo et al. 2004). Specific comparisons among genetic markers from different studies depend on inheritance patterns, mutation rates and sampling schemes (e.g., mtDNA has particularly low mutation rates in most sharks: Martin et al. 1992). Although these studies provide very limited information regarding the range of individual sharks for activities other than breeding, this growing body of evidence suggests that elasmobranch gene pools are not necessarily panmictic along the Pacific coast of North America.

In addition to general patterns of IBD, the landscape genetics visualization highlights potential dispersal barriers along the coast of California. Although the precise location of many potential barriers cannot be known without additional sampling focused in these areas, both this analysis and the assignment test suggest the presence of seven gene pools between Humboldt Bay and San Diego, CA, USA. All analyses agreed that leopard sharks in Humboldt Bay are particularly isolated. Humboldt Bay is near or at the northern extent of the leopard shark's range, potentially limiting its accessibility to dispersing individuals. In addition, leopard sharks from Humboldt Bay may exhibit life history traits different from leopard sharks in other populations. For example, Webber (2003) suggested that female leopard sharks in Humboldt Bay mature at a larger size and have lower fecundity than leopard sharks elsewhere. This would change the effective population size and overall generation time within the population, and possibly create reproductive or developmental incompatibilities with other populations. Together with our genetic data, this study suggests that Humboldt Bay may represent a model for future studies of incipient peripatric speciation in elasmobranchs (sensu Mayr 1954) or as an example of local adaptation. Further study of the underlying processes will be needed to

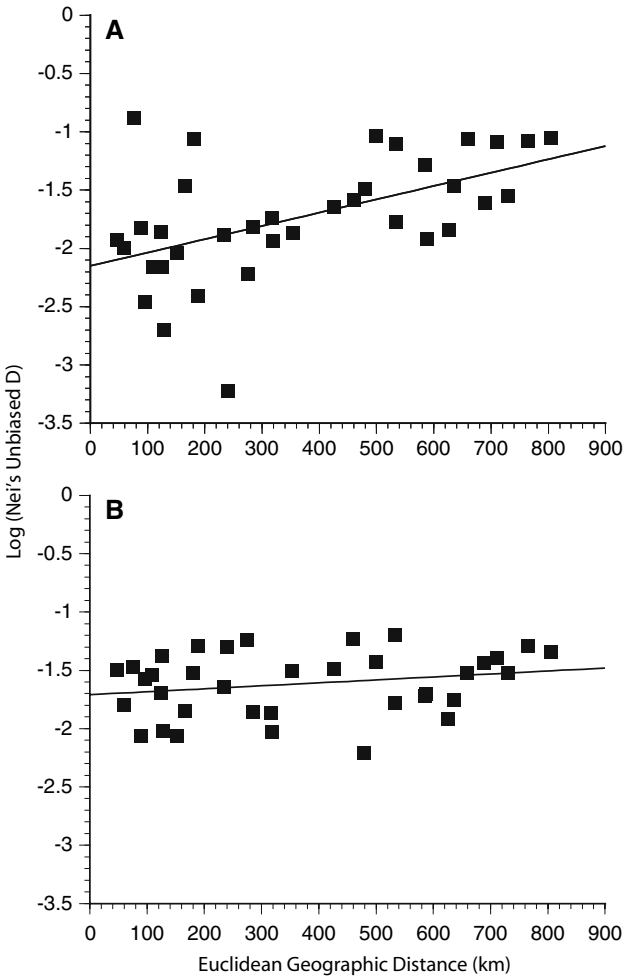

Fig. 6 Log genetic distance (Nei's unbiased D) versus Euclidean geographic distance between pairs of sample sites. **a** IBD plot for males. **b** IBD plot for females

determine whether the unique aspects of Humboldt Bay leopard sharks represent a natural, long-term phenomenon or whether management plans should attempt to reverse relatively recent isolation of this population.

In contrast to Humboldt Bay, the Los Angeles site appears to represent a region of transition for leopard sharks. Leopard sharks found in this area need to travel relatively short distances to known mating grounds. The average IBD residuals are very low in comparison to most other sampling sites, indicating high genetic connectivity which is best explained by the sampling of transient individuals from many gene pools. Further support for this hypothesis is provided by relatively high ISSR expected heterozygosity estimates in Los Angeles, a large number of ISSR clusters, and a large number of mtDNA haplotypes.

Although this study found only limited support for natal philopatry, breeding site status and proximity to other breeding sites does appear to structure patterns of genetic variation in leopard sharks. Precise interpretations are hampered by a limited knowledge of historical breeding sites from throughout the range of this species, and the possibility that some of these sites have been extirpated. We cannot separate the effects of natal philopatry (limited gene flow) from other microevolutionary processes (e.g., genetic drift, selection), even though it is clear that non-breeding sites are more genetically homogeneous than breeding sites. We did not find statistically significant different patterns of philopatry among sexes. However, a better understanding of individual movement patterns and reproduction cycles from future studies (e.g., tagging and tracking data) would facilitate more refined hypothesis testing using genetic data (Hueter et al. 2005).

Through implementation of a molecular marker that is novel for sharks, the results of our study provide answers to several basic questions regarding the genetic population structure of *T. semifasciata*. Despite the inherent challenges of working in a habitat without clearly defined boundaries, and a slowly evolving species that is highly mobile (Smith 1984; Hopkins and Cech 2003; Smith et al. 2003; B. Zeigler, personal communication; A. Carlisle, personal communication), we were able to determine that multiple leopard shark gene pools exist in California. Humboldt Bay is a site of special interest due to its genetic and likely ecological isolation. In contrast, Los Angeles appears to be a site of transition among gene pools, emphasizing that population boundaries can be diffuse in areas where breeding does not take place. We suggest that leopard shark populations be managed as multiple regional management units, with special attention given to the preservation of breeding sites for both population replenishment and to prevent population fragmentation. Because larger sharks generally have a higher fecundity than smaller sharks (Ebert and Ebert 2005), it is possible that genetically isolated populations of leopard sharks could benefit from the protection of larger individuals. This may be achieved, for example, by increasing the minimum length requirement or decreasing the bag limits permitted to commercial and recreational fishermen. As a caveat to these recommendations, we note that it is currently unclear what proportions of population subdivision are historical rather than a result of recent population declines and extirpations. The utility of mtDNA to infer recent versus prehistoric processes in some types of genetic analyses (e.g., Templeton 1998) is limited in this species by low mutation rates. In addition, an increased sample size may result in increased resolution for some analyses.

Logically, a next step would be to identify all historical breeding sites throughout the entire species range from Oregon to Mexico. If possible, the genetic analysis of historical samples would help to accurately interpret contemporary genetic structure and the current scarcity of leopard sharks from the range boundaries. In addition, the specific mechanisms by which leopard shark populations are

structured should be investigated further, as these mechanisms are likely to affect other elasmobranchs and a suite of other marine species. For example, Duncan et al. (2006) recently discovered that populations of scalloped hammerhead sharks are significantly structured with regard to ocean basin, with some indications of philopatric behavior. Continued research on shark population structure at a variety of scales will help to ensure the persistence of this fragile component of the world's fisheries.

Elasmobranch populations are declining at an unprecedented rate worldwide, which necessitates the development of cost and time effective methods for answering basic questions about the population structure of these fishes. The techniques described here meet these goals and set the stage for future efforts in elasmobranch population genetics, and by extension global fisheries management.

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