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Genetic structure and diversity of two highly vulnerable carcharhinids in Australian waters

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ABSTRACT: Molecular techniques were employed to investigate genetic structure and diversity in dusky *Carcharhinus obscurus* and sandbar *C. plumbeus* sharks in the Indo-Australian region. Tissue samples of 423 *C. obscurus* and 442 *C. plumbeus* defined 18 and 11 mtDNA ND4 haplotypes, respectively. For *C. obscurus*, weak genetic differentiation was detected between eastern and western Australian waters (pairwise Φ_{ST} = 0.04437, $p < 0.008$; pairwise F_{ST} = 0.02403, $p < 0.035$), suggesting the delineation of 2 independent populations, while patterns of gene flow between Australia and Indonesia were inconclusive. Rarefaction analysis, however, indicated that robust population comparisons in these species were reliant on sample numbers >100 at any particular location. Off Australia's temperate east coast, *C. obscurus* and *C. plumbeus* exhibited strong similarities in genetic structure, suggestive of similar evolutionary histories in the region. In addition, genetic validation revealed observers to be highly accurate in the identification of both target species in an eastern Australian shark fishery. Our findings contribute valuable information for the management and conservation of both species.

KEY WORDS: Carcharhinidae · Fisheries management · Indo-Pacific · Mitochondrial DNA · Observer accuracy · Population genetics · Stock structure

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INTRODUCTION

Topographic, biological and oceanographic limitations to dispersal result in reproductive isolation between groups of individuals. Over evolutionary time, this cessation of (or restriction to) gene exchange leads to quantifiable genetic differentiation as a result of intrinsic natural selection, genetic drift and mutation (e.g. Riginos & Nachman 2001, Hazlitt et al. 2006). In a context of increasing anthropogenic pressures, the identification of barriers to gene flow can assist with the conservation and management of a species' genetic diversity, which is an essential store of variety if a species is to successfully adapt to future environmental challenges. This is especially pertinent for taxa that are demonstrably vulnerable to human-induced population decline, e.g. elasmobranchs (Stevens et al. 2000, Field et al. 2009).

Sharks have a demonstrated susceptibility to overexploitation on the basis of their life-history traits and a vulnerability to multiple fishing gears (Cortés 2000, Stevens et al. 2000). Recent global increases in commercial fishing effort for sharks have resulted in grave population declines (Baum et al. 2003, Ferretti

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et al. 2008). While magnitudes of stock depletion are disputed (Burgess et al. 2005), there is international agreement regarding the urgent need for the effective management of shark fisheries to address issues of conservation and cascading ecological impacts catalysed by apex predator removal (Barker & Schluessel 2005, Myers et al. 2007, Ferretti et al. 2010).

The dusky shark *Carcharhinus obscurus* and the sandbar shark *C. plumbeus* are 2 large–medium carcharhinid species widely regarded as among the most vulnerable of sharks to overfishing (Smith et al. 1998). As long-lived, late-maturing species of decidedly low productivity (e.g. Simpfendorfer et al. 2002, Dudley et al. 2005, McAuley et al. 2006, Baremore & Hale 2012, Geraghty et al. 2013), demographic analyses have reported abilities to withstand only very modest levels of fishing mortality in conjunction with slow rates of population increase (Sminkey & Musick 1996, Smith et al. 1998, McAuley et al. 2007a, Romine et al. 2009).

Nevertheless, being highly sought after for their fins (Clarke et al. 2006), both species are captured in commercial and artisanal fisheries across large parts of their respective cosmopolitan ranges (e.g. Amorim et al. 1998, Castillo-Géniz et al. 1998, McVean et al. 2006, White 2007, Morgan et al. 2009), with poor records of management in some regions. In particular, *C. obscurus* and *C. plumbeus* were subject to intense targeted harvest pressure in the now-collapsed large, coastal shark fishery off the east coast of the USA, where various data sets suggest population declines of up to 64–99% in both species (Cortés et al. 2006, Myers et al. 2007, Baum & Blanchard 2010). Consequently, both sharks are globally listed by the IUCN as Vulnerable and *C. obscurus* as Endangered in the north-west Atlantic (Musick et al. 2009a,b).

C. obscurus and *C. plumbeus* are also important components of commercial shark landings in Australian waters (Simpfendorfer & Donohue 1998, Macbeth et al. 2009). Dramatic increases in catches off both the east and west coasts led to considerable concern regarding their sustainability under harvest pressure in the region (McAuley et al. 2007a, Macbeth et al. 2009), and emphasised the need for effective management input to arrest further stock decline.

Genetic techniques are useful tools for addressing shark fishery management issues. Population genetic analyses can help identify appropriate scales of management by investigating contemporary patterns of gene flow, genetic diversity and the spatial structure of stocks (Dudgeon et al. 2012). Carcharhiniformes are the most represented of the elasmobranchs in the population genetic literature, but few have been examined in any detail (Dudgeon et al. 2012). These studies have typically focused on elucidating genetic structure over broad spatial scales, consistently demonstrating large oceanic expanses to be robust barriers to mitochondrial gene flow (Duncan et al. 2006, Keeney & Heist 2006, Benavides et al. 2011a), also in *C. obscurus* (Benavides et al. 2011b) and *C. plumbeus* (Portnoy et al. 2010). Genetic subdivision on finer scales has also been reported for some shark species, raising important implications for regional fisheries management (Keeney et al. 2003, Karl et al. 2011, Tillett et al. 2012a,b, Whitney et al. 2012).

Previous investigations of genetic structure in *C. obscurus* and *C. plumbeus* in Australian and neighbouring waters have yielded a variety of results. Portnoy et al. (2010) observed genetic subdivision between eastern and western Australia in *C. plumbeus* based on mitochondrial DNA (mtDNA), while Ovenden et al. (2009) and Benavides et al. (2011b) reported evidence for genetic homogeneity between the same 2 regions in *C. obscurus*. Ovenden et al. (2009) also raised the possibility of limited dispersal across the Timor Trench in the latter species through a finding of genetic differentiation between western Australia and central Indonesia. The strength of the abovementioned findings, however, was generally limited due to small sample sizes. In light of their vulnerability to population decline, therefore, we believed that a more detailed assessment of genetic structure and diversity was warranted for these 2 species.

Using mtDNA NADH dehydrogenase subunit 4 (ND4) sequence data, we re-assessed the genetic structure of *C. obscurus* on a regional scale, testing a null hypothesis of genetic homogeneity in Indo-Australian waters, and investigated the genetic diversity of *C. plumbeus* off the east coast of Australia, permitting a comparison with *C. obscurus* in the latter region. We also applied these genetic data in establishing basic estimates of observer accuracy in an eastern Australian shark fishery, and explored the implications of our findings for the management and conservation of both species.

MATERIALS AND METHODS

Tissue sample collection

Shark tissues were collected from a range of locations in Indo-Australian waters (Fig. 1), focusing on a harvested population off Australia's east coast. Tissues were sampled from New South Wales (NSW)

waters from 2007 to 2010 from landed catch by observers on-board commercial shark-fishing vessels within the NSW Ocean Trap and Line Fishery (NSW OTLF). A small quantity (<2 g) of white muscle tissue was excised from each specimen, immediately preserved in 95% reagent grade ethanol, and stored at room temperature. Additional samples, collected from 2000 to 2012, were obtained from more distant locations, including *Carcharhinus obscurus* and *C. plumbeus* samples from waters of the Northern Territory (NT) in Australia, as well as *C. obscurus* samples from Western Australia (WA) and Indonesia. Samples from NT and WA were collected from landed catch by observers within their respective commercial shark fisheries, and preserved in 20% dimethylsulphoxide (DMSO) solution and 70% ethanol, respectively. Samples from Indonesia were collected from landed catch by a fisheries biologist at the Tanjung Luar local market in eastern Lombok, and preserved in 20% DMSO; exact capture locations were not confirmed. Additional *C. obscurus* tissues were obtained from NSW waters by sampling sharks caught in the NSW Shark Meshing (Bather Protection) Program (Reid et al. 2011). Tissues from NSW and NT were sampled from predominantly adult and sub-adult individuals, while those from WA were sampled mostly from small juveniles. Tissues from Lombok were sampled from processed trunks for which associated length measurements were unavailable.

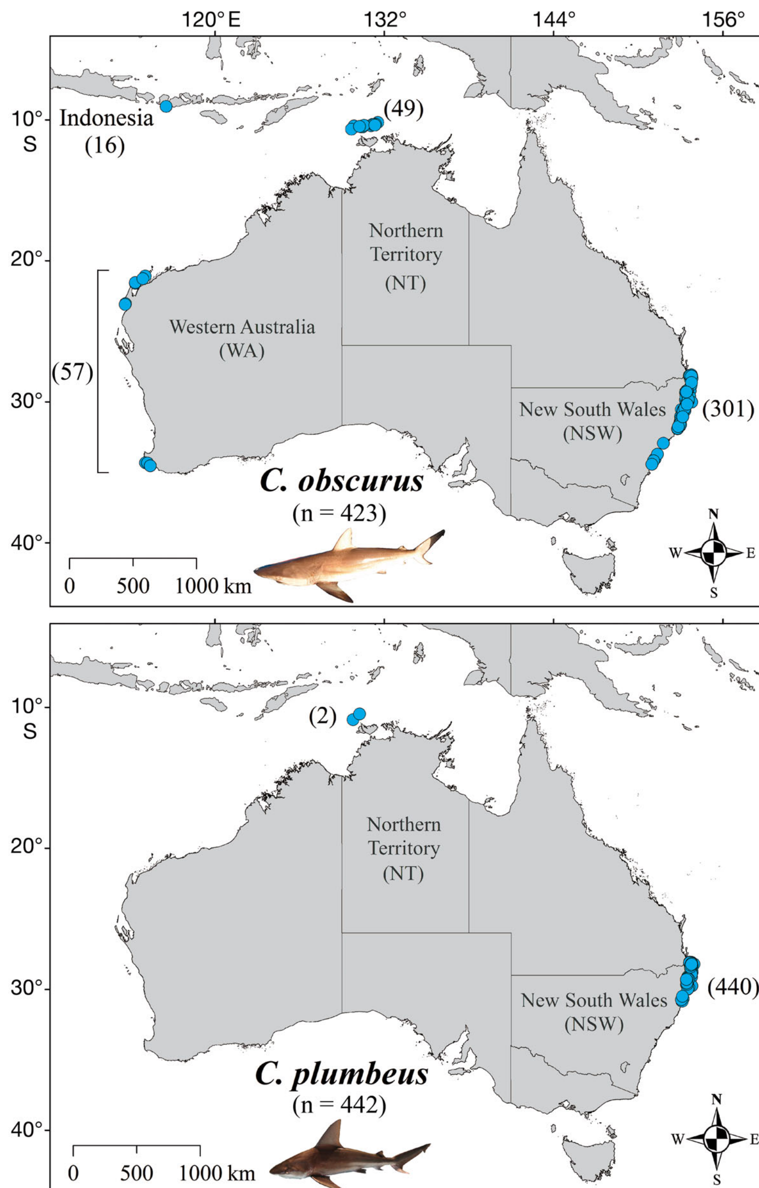

Fig. 1. Collection locations and sample sizes (in parentheses) for *Carcharhinus obscurus* and *C. plumbeus* tissues included in genetic diversity and structure analyses

DNA extraction, amplification and sequencing

To obtain mtDNA sequence data, total genomic DNA was first extracted from 5 mg of each tissue using a modified salting-out protocol (Sunnucks & Hales 1996). Samples were digested with 10 µl of Proteinase K (10 mg ml^{-1}) in 580 µl of TNES (50 mM Tris HCl, pH 7.5; 400 mM NaCl; 20 mM EDTA; and 0.5% SDS) by incubation overnight at 55°C. Proteins were precipitated by adding 170 µl of 5 M NaCl followed by microcentrifugation at 14 000 rpm for 5 min. Supernatant (600 µl) was recovered into a fresh tube and the DNA was precipitated by adding 600 µl of ice-cold 100% absolute ethanol. Tubes were stored at −20°C for approximately 1 h. DNA was then recovered by microcentrifugation at 14 000 rpm for 15 min, and the ethanol was decanted. The resulting DNA pellet was washed with 200 µl of 70% ethanol, 100 mM sodium acetate solution, and microcentrifuged at 14 000 rpm for 3 min. Following decanting, all remaining ethanol was removed using a micropipette. DNA was airdried, resuspended in 100 µl of TE buffer (10 mM Tris HCl, pH 7.6; and 1 mM EDTA) and stored at −20°C. DNA yield was checked on a 1.0% agarose TBE (90 mM Tris-borate and 2 mM EDTA; 1×) gel, run at 110 V, and stained with GelRed (Biotium).

PCR was then used to amplify the mitochondrial ND4 gene from all DNA extractions. This gene was selected for analysis following Dudgeon et al. (2009) and Ovenden et al. (2010), who demonstrated the ND4 gene to be the most polymorphic among a range of mtDNA markers (including the control region) in species related to those under study here. PCR reactions were carried out in 50 µl volumes containing 1 µl of DNA template, 1× GoTaq Colourless reaction buffer (consisting of 1.5 mM $MgCl_2$ and 200 µM deoxynucleoside triphosphates [dNTPs]) (Promega), 0.5 µl of RNase (1 mg ml^{-1}), and 0.5 µM of each of the primers ND4 (5′CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC) (Arèvalo et al. 1994) and H12293-LEU (5′TTG CAC CAA GAG TTT TTG GTT CCT AAG ACC) (Inoue et al. 2001). Amplifications were performed in an Eppendorf ep gradient S Mastercycler, using thermal cycling conditions consisting of an initial denaturation (94°C for 3 min) followed by 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 1 min, with a final extension step of 72°C for 10 min, and held at 4°C. PCR products were visualised on a 2.0% agarose TBE (1×) gel, run at 110 V, and stained as above. PCR products were purified prior to sequencing using Exosap-IT (USB Corporation). Sequencing was performed with an Applied Biosystems 3130xl Genetic Analyzer 16-array capillary sequencer (Life Technologies), with sequencing reactions and analyses carried out by the Macquarie University (MQ) DNA Sequencing Facility using Big Dye Terminator reactions and the forward PCR primer only.

Sequence alignment and ID validation

To assess observer accuracy, species identities were determined following sequence alignment. Sequences were first trimmed and edited by eye. Edited sequences were entered into Biomanager (http://web.archive.org/web/20110721184643/http://biomanager.info/) and aligned using the ClustalW (accurate) algorithm (Thompson et al. 1994). No GenBank ND4 reference sequences were available for *C. obscurus* or *C. plumbeus* prior to this study. To validate that the 2 study species had been correctly identified, and to determine the species identity of any misidentified individuals, randomly selected representatives from each separate haplotype determined from the alignment output were amplified for the mitochondrial cytochrome oxidase I (CO1) gene using the primers Fish F1 (5′TCA ACC AAC CAC AAA GAC ATT GGC AC) and Fish R1 (5′TAG ACT TCT GGG TGG CCA AAG AAT CA) (Ward et al. 2005). PCRs were carried out as above, with thermal cycling conditions consisting of an initial denaturation (95°C for 5 min), followed by 30 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 1 min, with a final extension step of 72°C for 7 min, and held at 4°C. PCR products were purified and sequenced following the same protocol outlined above for the ND4 locus. Resultant CO1 sequences were compared with reference sequences in GenBank for species recognition.

Sequence analysis

To identify and characterise mitochondrial haplotypes, aligned ND4 *C. obscurus* and *C. plumbeus* sequences were imported into Arlequin 3.5.1.2 (Excoffier & Lischer 2010). A sequence representing each haplotype was lodged in GenBank (accession codes KJ004523–KJ004551). The frequency of, and mutational steps between, haplotypes were assessed by generating statistical parsimony haplotype networks in TCS 1.21 using the default settings (Clement et al. 2000). Phylogenetic relationships among haplotypes were inferred using a maximum likelihood phylogram (or phylogenetic tree) based on the Tamura-Nei model (Tamura & Nei 1993), and generated in MEGA 5 (Tamura et al. 2011) with 1000 bootstrap replicates. The best-fitting model of nucleotide substitution, as offered by MEGA 5, was determined by likelihood ratio tests and calculations of Akaike and Bayesian information criteria performed in jModelTest 2.1.1 (Darriba et al. 2012). To assess the ability of the ND4 region to differentiate between carcharhinids, the phylogram was rooted with a range of morphologically similar species, as well as with 2 sphyrnid species as outgroups. Genetic diversity indices were also obtained with Arlequin using the Tamura-Nei substitution model (Tamura & Nei 1993), and included polymorphism statistics, number of haplotypes (n_H), haplotype diversity (h) and nucleotide diversity (π).

Rarefaction exact curve analysis

To determine whether sample sizes adequately represented population genetic variation, rarefaction exact curves were generated to qualitatively assess the proportion of haplotypic diversity sampled at each location for both *C. obscurus* and *C. plumbeus*. The expected number of haplotypes found for a given sample number was calculated using the rarefaction formula of Hurlbert (1971), and executed in the statistical package R (R Development Core Team 2010).

Genetic diversity and structuring

For *C. plumbeus*, a restricted sample distribution permitted the investigation of genetic diversity on a fine scale in NSW waters only. For *C. obscurus*, however, a suitable sample distribution was available to examine both genetic diversity and population structuring on a regional scale. For this latter species, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was implemented in Arlequin to test a null hypothesis of panmixia (genetic homogeneity) across Indo-Australian waters. To evaluate the overall extent of genetic subdivision between sampling locations, we employed 2 *F*-statistic metrics of genetic divergence: Φ_{ST} (Excoffier et al. 1992) and F_{ST} (Wright 1965). While Φ_{ST} has been regarded as the superior metric on the basis of its incorporation of a measure of genetic distance between haplotypes, frequency-based F_{ST} has been proposed as a more appropriate measure of genetic differentiation among locations where migration is theoretically occurring at a faster rate than mutation (Bird et al. 2011). Φ_{ST} was calculated via the computing of a distance matrix using the Tamura-Nei model (Tamura & Nei 1993) for estimation of genetic distance between sequences, while F_{ST} used haplotype frequencies only. AMOVA partitioned genetic variance among, and within, populations and calculated Φ_{ST} and F_{ST} fixation indices. Genetic differentiation between sample locations was also measured by calculating pairwise Φ_{ST} and F_{ST} estimates. Statistical significance was determined following 10 000 permutations of the sequence data and, in the case of pairwise Φ_{ST} and F_{ST}, assessed at an initial critical significance level of $\alpha = 0.0083$ (adjusted from $\alpha = 0.05$) following sequential Bonferroni correction for 6 simultaneous comparisons (Holm 1979). The AMOVA structure consisted of 1 group made up of the following 4 putative populations: NSW (n = 301), NT (n = 49), WA (n = 57) and Indonesia (n = 16; Fig. 1). The analysis outlined above is henceforth referred to as the 'original analysis'.

C. obscurus sample sizes were strongly biased towards NSW, where sample numbers were an order of magnitude greater than at the remaining 3 locations (Fig. 1). We evaluated the influence of this sampling bias on the *F*-statistics of pairwise population comparisons involving NSW via random re-sampling simulations. Ten thousand replicate random sample sets of n = 100, n = 50 and n = 16 (for comparison with Indonesia only) were selected without replacement from the NSW population, while NT, WA and Indonesian sample sizes were kept unchanged. Population pairwise Φ_{ST} and associated p-values were generated for each replicate random sample set in Arlequin using the batch processing function and permutation settings as outlined above. Resultant Φ_{ST} and p-value distributions were plotted, and the likelihood of producing a contradictory result to that of the original analysis was calculated as the percentage of p-values either ≤ 0.05 or >0.05, depending on the outcome of the original analysis.

RESULTS

Observer accuracy off Australia's NSW coast

The ND4 gene region proved to be an excellent marker for carcharhinid species recognition (Fig. 2), as also shown by Tillett et al. (2012c), hence confirming its suitability for use in the present study.

Genetic validation was possible for a total of 296 sharks visually identified by scientific observers as *Carcharhinus obscurus* in the NSW OTLF from 2007 to 2010. Of these, 286 were genetically confirmed to be *C. obscurus*, translating to an observer-accuracy estimate of 96.6 % for the identification of this species in the fishery (Table 1). Misidentified individuals (n = 10) were all of adult size and represented 6 different carcharhinid species (Table 1).

Genetic validation was possible for a total of 487 sharks visually identified by scientific observers as *C. plumbeus* in this same fishery over the same temporal period. Of these, 484 were genetically confirmed to be *C. plumbeus*, translating to an observer-accuracy estimate of 99.4 % for the identification of this species in the NSW OTLF (Table 1). Misidentifications (n = 3) once again were all of adult size and comprised 3 different carcharhinid species (Table 1). Overall observer accuracy was estimated at 98.3 % for the identification of these 2 target species combined.

Genetic diversity and summary statistics

Carcharhinus obscurus

An 857 bp mtDNA ND4 sequence was obtained for 423 *C. obscurus* individuals collected from Australian and Indonesian waters (Fig. 1). A total of 18 haplotypes were defined, characterised by 18 polymorphic sites composed of 15 transitions and 3 transversions (see Table A1 in the Appendix). Phylogenetic analysis placed these haplotypes into 2 shallow clades (Fig. 2). Two haplotypes (DS9 and, to a lesser degree, DS15) dominated the sample set, and were common at all 4

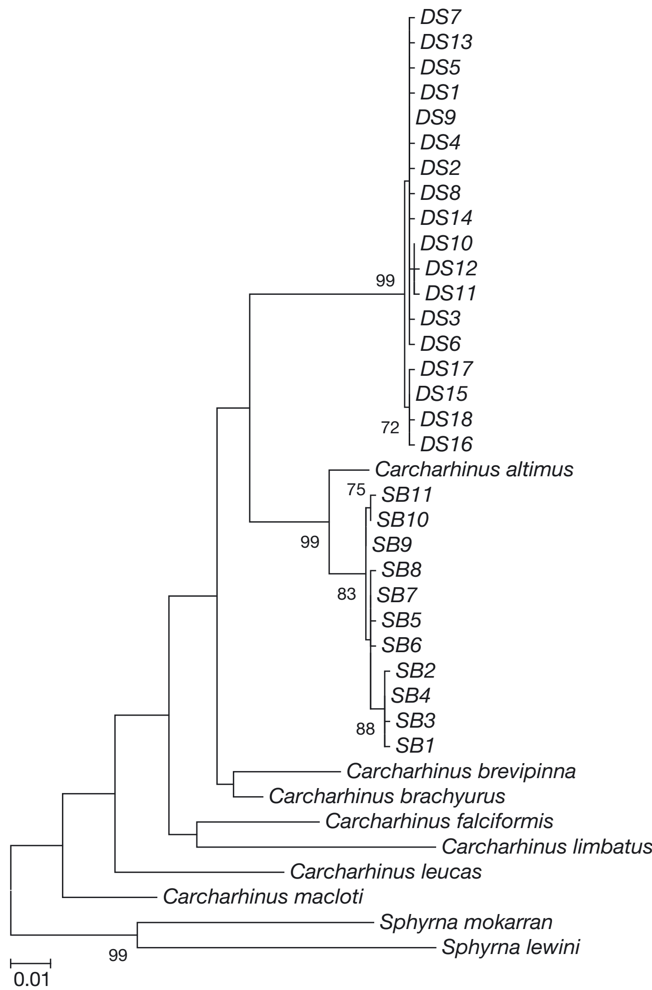

Fig. 2. Inferred phylogenetic maximum likelihood tree for *Carcharhinus obscurus* (DS1–18) and *C. plumbeus* (SB1–11) mtDNA ND4 haplotypes from Australian and Indonesian waters. Nodal bootstrap support is displayed where ≥70%. Scale represents the proportion of polymorphic sites between haplotypes

locations (Table 2a). Overall haplotype (*h*) and nucleotide (π) diversities were moderate and low, respectively (h = 0.5150, π = 0.0012; Table 3). Notwithstanding differences in sample size, the greatest number of haplotypes (n = 12) was found in NSW waters, of which 5 were unique to the area (Table 3). Ten haplotypes were found in WA waters, 3 of which were unique, and 5 haplotypes were found in both NT and Indonesia, each exhibiting 1 unique haplotype. Haplotype and nucleotide diversities ranged across the putative populations; Indonesia displayed the highest diversity values (h = 0.7500, π = 0.0016) and NT the lowest (h = 0.3520, π = 0.0008). Standard deviation estimates, however, rendered differences in diversity between the locations impossible to discern (Table 3).

Carcharhinus plumbeus

An 857 bp mtDNA ND4 sequence was obtained for 442 *C. plumbeus* individuals collected from eastern and northern Australian waters (Fig. 1). A total of 11 haplotypes were defined, characterised by 12 polymorphic sites composed exclusively of transitions (see Table A2 in Appendix 1). Phylogenetic analysis placed these haplotypes into 2 shallow clades (Fig. 2). Two haplotypes (SB4 and, to a far lesser degree, SB7) dominated the sample set (Table 2b). Overall haplotype and nucleotide diversities were low for *C. plumbeus*, at 0.2814 and 0.0009, respectively (Table 3). No unique haplotypes were found amongst the 2 NT samples, with both being the most common haplotype SB4 (Table 2b). Given the low sample size from NT, this location was henceforth excluded from further analyses, with detailed investigations focusing exclusively on eastern Australian (NSW) waters.

Table 1. Percentage of individuals identified by observers as *Carcharhinus obscurus* and *C. plumbeus* for each genetically identified shark species in the New South Wales Ocean Trap and Line Fishery. The number of individuals is given in parentheses; **bold** print shows percentage of correctly identified *C. obscurus* and *C. plumbeus*. Total lengths (TL; cm) for misidentified individuals are displayed; na: not applicable

Genetic identification	Observer identified as *C. obscurus*	TL of misidentified ind.	Observer identified as *C. plumbeus*	TL of misidentified ind.
C. obscurus	**96.6** (286)	na	0.2 (1)	302
C. plumbeus	0.3 (1)	210	**99.4** (484)	na
C. falciformis	1.0 (3)	235, 242, 256	0.2 (1)	214
C. leucas	0.7 (2)	220, 293	0	na
C. limbatus	0.7 (2)	252, 254	0.2 (1)	208
C. brevipinna	0.3 (1)	276	0	na
C. altimus	0.3 (1)	269	0	na
Total	296		487	

Rarefaction exact curves

Rarefaction exact curves indicated trends towards asymptotic relationships for NSW waters in both *Carcharhinus obscurus* and *C. plumbeus* (Fig. 3), suggesting that the majority of the available haplotypic diversities were likely sampled at this location in both species and that more intensive sampling was likely to yield few additional haplotypes. Steep slopes, however, were observed for the remaining 3 *C. obscurus* sample locations (Fig. 3), indicating that a proportion of

Table 2. Mitochondrial DNA ND4 haplotype relative frequencies observed from putative populations in Indo-Australian waters for (a) *Carcharhinus obscurus* and (b) *C. plumbeus*. NSW: New South Wales; NT: Northern Territory; WA: Western Australia; (–) haplotype not found

(a) Haplotype	Relative frequency NSW (n = 301)	NT (n = 49)	WA (n = 57)	Indonesia (n = 16)	GenBank accession code
DS1	–	–	0.018	–	KJ004534
DS2	–	–	0.018	–	KJ004535
DS3	–	–	0.053	–	KJ004536
DS4	–	–	–	0.063	KJ004537
DS5	–	0.020	–	0.063	KJ004538
DS6	–	0.020	–	–	KJ004539
DS7	0.003	–	0.018	–	KJ004540
DS8	0.010	–	0.018	–	KJ004541
DS9	0.648	0.796	0.702	0.438	KJ004542
DS10	0.040	–	0.053	0.188	KJ004543
DS11	0.007	–	0.018	–	KJ004544
DS12	0.003	–	–	–	KJ004545
DS13	0.003	–	–	–	KJ004546
DS14	0.020	–	–	–	KJ004547
DS15	0.239	0.143	0.070	0.250	KJ004548
DS16	0.003	–	–	–	KJ004549
DS17	0.013	0.020	0.035	–	KJ004550
DS18	0.010	–	–	–	KJ004551

(b) Haplotype	Relative frequency NSW (n = 440)	NT (n = 2)	GenBank accession code
SB1	0.011	–	KJ004523
SB2	0.014	–	KJ004524
SB3	0.005	–	KJ004525
SB4	0.841	1.000	KJ004526
SB5	0.005	–	KJ004527
SB6	0.002	–	KJ004528
SB7	0.102	–	KJ004529
SB8	0.009	–	KJ004530
SB9	0.005	–	KJ004531
SB10	0.005	–	KJ004532
SB11	0.002	–	KJ004533

Table 3. Genetic diversity indices observed in the mitochondrial DNA ND4 region for *Carcharhinus obscurus* and *C. plumbeus* sample locations from Australian and Indonesian waters. (Location abbreviations as in Table 2.) Values in parentheses represent standard deviations. n: sample size; n_H: number of haplotypes; n_{Hq}: number of unique haplotypes; *h*: haplotype diversity; π: nucleotide diversity. na: not applicable

Location	n	n_H	n_{Hq}	*h*	π
C. obscurus					
NSW	301	12	5	0.5224 (±0.027)	0.0012 (±0.0009)
NT	49	5	1	0.3520 (±0.080)	0.0008 (±0.0007)
WA	57	10	3	0.5031 (±0.080)	0.0010 (±0.0008)
Indonesia	16	5	1	0.7500 (±0.078)	0.0016 (±0.0012)
Pooled	423	18	na	0.5150 (±0.025)	0.0012 (±0.0009)
C. plumbeus					
NSW	440	11	na	0.2826 (±0.027)	0.0009 (±0.0008)
NT[a]	2	1	na	na	na
Pooled	442	11	na	0.2814 (±0.027)	0.0009 (±0.0008)

[a]Diversity indices not available for *C. plumbeus* from NT; both samples were the same haplotype

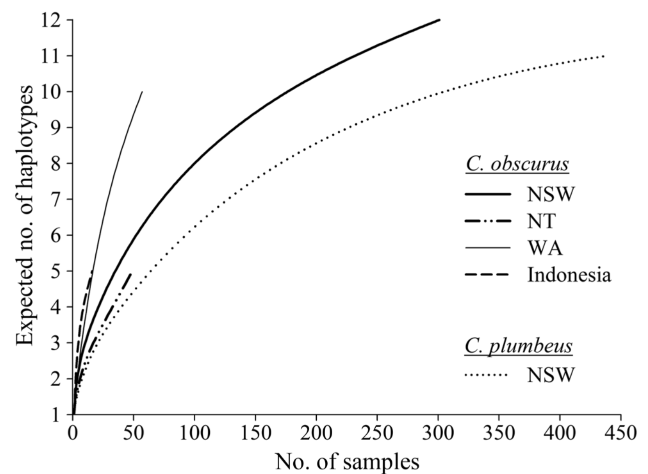

Fig. 3. Rarefaction exact curves for *Carcharhinus obscurus* and *C. plumbeus* collection locations in Australian and Indonesian waters. NSW: New South Wales; NT: Northern Territory; WA: Western Australia

the available genetic diversities were unsampled. These analyses suggest that adequate representations of levels of genetic variation in *C. obscurus* or *C. plumbeus* in Indo-Australian waters are reliant on sample sizes in excess of 100 from any given location.

Carcharhinus obscurus: regional genetic structure

A haplotype network incorporating the 4 putative populations of *C. obscurus* demonstrated the presence of 2 shallow clades centred on the 2 most common haplotypes, DS9 and DS15, both of which were shared between all 4 sample locations (Fig. 4). Low-frequency variants shared between, and unique to, locations were also present. Nevertheless, AMOVA fixation indices detected significant levels of differentiation between putative populations for both *F*-statistic metrics (Φ_{ST} = 0.02462, p < 0.03; F_{ST} = 0.02723, p < 0.01; Table 4). We therefore rejected the null hypothesis that *C. obscurus* are panmictic in Indo-Australian waters. Pairwise comparisons revealed weak genetic subdivision between eastern and western Australia, significant after sequential Bonferroni adjustment for Φ_{ST} only (NSW versus WA; Φ_{ST} = 0.04437, p < 0.008; F_{ST} = 0.02403, p < 0.05; Table 5). Evidence for weak differentiation between NT and Indonesia (F_{ST} = 0.13925, p < 0.05) and between WA

Table 4. AMOVA analyses of spatial genetic variation of mitochondrial DNA ND4 sequences for *Carcharhinus obscurus* from Australian and Indonesian waters

Source of variation	df	Test statistic	SS	Variance components	Variation (%)
Among populations	3	Φ_{ST}	3.875	0.01235	2.46
		F_{ST}	2.149	0.00712	2.72
Within populations	419	Φ_{ST}	205.056	0.48939	97.54
		F_{ST}	106.517	0.25422	97.28
Fixation indices	Φ_{ST} = 0.02462; p = 0.02143 (±0.00099)				
	F_{ST} = 0.02723; p = 0.00999 (±0.00069)				

Table 5. Mitochondrial DNA ND4 population pairwise Φ_{ST} (below diagonal) and F_{ST} (above diagonal) estimates of genetic divergence for *Carcharhinus obscurus* collected from Indo-Australian waters. **Bold** indicates that the pairwise value is significant after sequential Bonferroni correction (initial α = 0.0083); * denotes values significant at the p ≤ 0.05 level. (Location abbreviations as in Table 2)

	NSW (n = 301)	NT (n = 49)	WA (n = 57)	Indonesia (n = 16)
NSW		0.02208	0.02403*	0.03592
NT	0.01362		0.00668	0.13925*
WA	**0.04437**	0.00285		0.07440*
Indonesia	−0.00597	0.02476	0.03010	

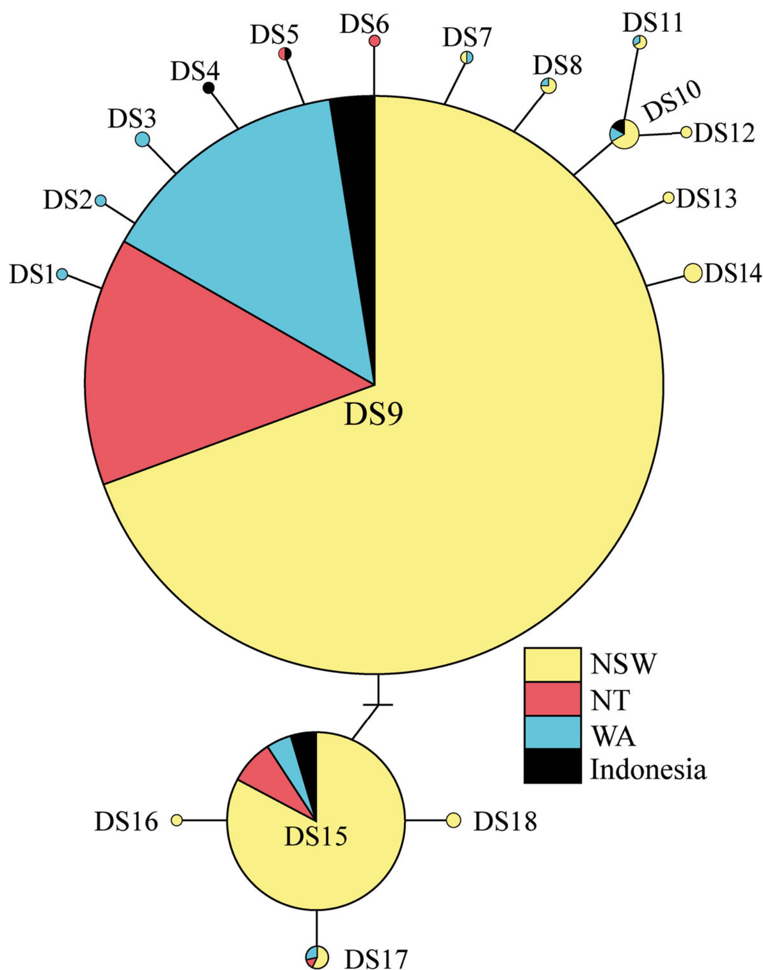

Fig. 4. Mitochondrial DNA ND4 haplotype network for *Carcharhinus obscurus* (n = 423) from Australian and Indonesian waters. Sizes of circles correspond to the number of individuals displaying each haplotype. Colours indicate the proportion observed from each of the 4 putative populations. (–) mutational step/missing haplotype. NSW: New South Wales; NT: Northern Territory; WA: Western Australia

and Indonesia (F_{ST} = 0.07440, p < 0.05) was also detected based on haplotype frequencies, with neither comparison significant after Bonferroni correction.

Random re-sampling simulations, however, demonstrated an increasing likelihood of finding a non-significant pairwise result between NSW and WA with decreasing NSW sample size (Fig. 5). More specifically, 14.18% of replicate comparisons where sample size was set to 100 for NSW (and left at 57 for WA) did not provide statistical support for the original analysis, where sample size was 57 for WA and 301 for NSW. This increased to 36.8% when the NSW sample size was reduced to 50. In addition, pairwise Φ_{ST} distributions displayed stable mean Φ_{ST} values (despite increased variation) but increasing mean p-values relative to the output of the original analysis as random NSW sample-set size decreased (Fig. 6). Simulations involving random NSW sample sets of n = 100 returned pairwise Φ_{ST} values normally distributed around a mode (and mean) very near the Φ_{ST} produced by the original analysis, and a mean p-value <0.05 (Fig. 6a). Simulations involving random NSW sample sets of n = 50, despite a more variable and skewed distribution, once again returned a mean Φ_{ST} very near that produced by the original analysis, but in contrast returned a non-significant mean p-value (>0.05; Fig. 6b). Replicate pairwise comparisons between NSW and NT and Indonesia, in contrast, displayed little change in the likelihood of returning a contradictory

result to the original analysis as random NSW sample size was altered (Fig. 5).

Species comparison off the NSW coast

There was a marked similarity in mtDNA features between *C. obscurus* and *C. plumbeus* samples collected from NSW waters. Large sample sets revealed similar numbers of haplotypes for *C. obscurus* (n_H = 12, n = 301) and *C. plumbeus* (n_H = 11, n = 440) (Table 3). Comparative haplotype networks revealed strikingly similar topologies for the 2 species, with both networks being shallow and suggestive of the presence of 2 distinct, yet closely related, clades separated by 1 to 2 mutation steps (Fig. 7). A difference between the 2 species, however, was observed in their diversity indices, where *C. obscurus* exhibited moderate (h = 0.5224), and *C. plumbeus* low (h = 0.2826), genetic diversity (Table 3).

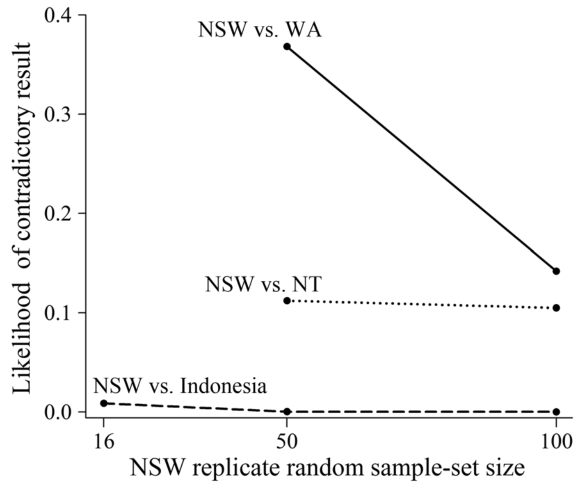

Fig. 5. Likelihood of generating a contradictory pairwise result to that of the original analysis given 10 000 replicate random re-samples of the New South Wales (NSW) *Carcharhinus obscurus* population at varying sample sizes. NT: Northern Territory; WA: Western Australia

DISCUSSION

Observer accuracy in a NSW shark fishery

Genetic validation revealed high observer accuracy in the identification of *Carcharhinus obscurus* and *C. plumbeus* in the NSW OTLF. This was not unexpected given the morphological distinctions coupled with a large modal size-at-capture within the fishery; the vast majority of the shark catch in the NSW OTLF is landed as mature, adult individuals (Macbeth et al. 2009). While morphologically similar to one another, and to a range of other species, at smaller sizes, *C. obscurus* and *C. plumbeus* are characterised by diagnostic traits that become increasingly discernible as the individuals grow larger (Last & Stevens 2009).

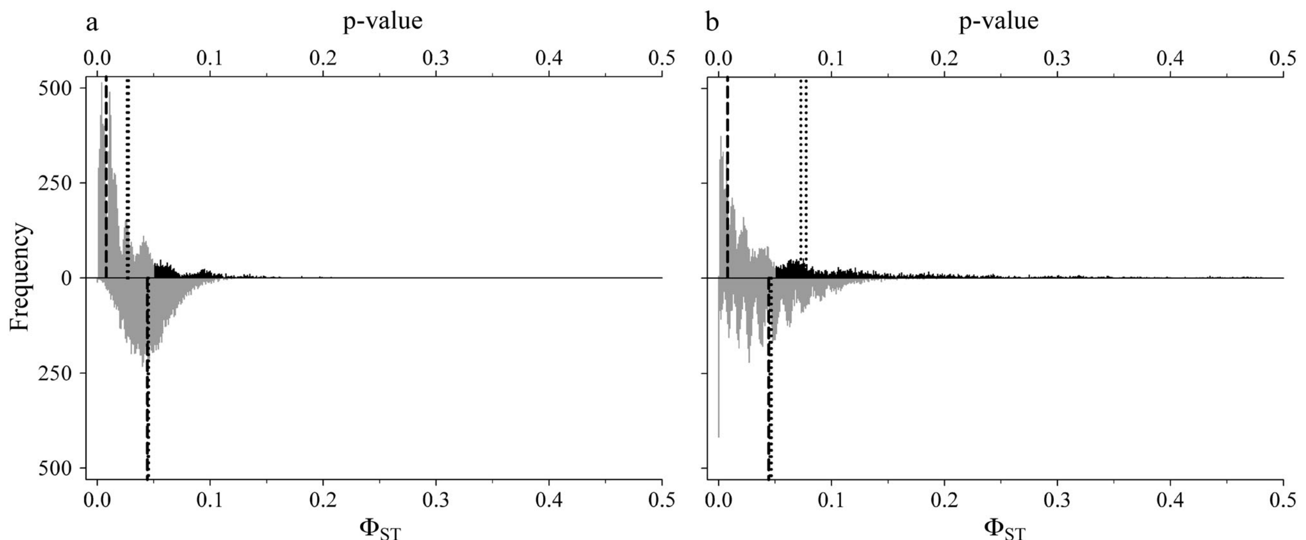

Fig. 6. New South Wales versus Western Australia pairwise Φ_{ST} (see 'Materials and methods: Genetic diversity and structuring' for details) and p-value distributions following 10 000 replicate random re-samples of the NSW *Carcharhinus obscurus* population at (a) n = 100 and (b) n = 50. Grey and black zones on simulated p-value distributions represent $p \leq 0.05$ and $p > 0.05$, respectively. Dotted lines denote upper and lower 95 % confidence intervals around simulated means (upper and lower intervals are difficult to discern due to their proximity). Dashed lines indicate the pairwise Φ_{ST} and p-value generated by the original analysis

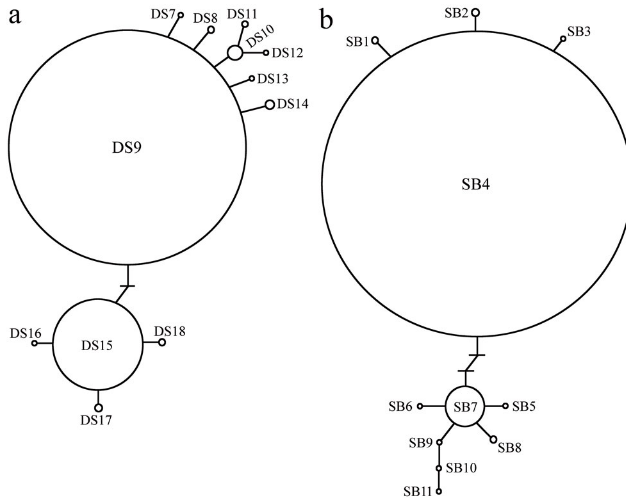

Fig. 7. Comparative ND4 haplotype networks for (a) *Carcharhinus obscurus* (n = 301) and (b) *C. plumbeus* (n = 440) in NSW waters. Sizes of circles correspond to the relative frequency of each haplotype. (–), mutational steps/missing haplotypes

Our estimates of observer accuracy were markedly higher than those reported from the NT ONLF by Tillett et al. (2012c), who estimated overall observer accuracy at 80% compared with 98.3% in the present study. Also, species-specific identification accuracy ranged from 70 to 92.7% in northern Australia (Tillett et al. 2012c), compared with 96.6 to 99.4% off the east coast as presented here. Lower observer accuracy in the NT ONLF can be attributed to the targeting of morphologically similar species (e.g. Australian blacktip *C. tilstoni* and *C. limbatus*; *C. leucas* and *C. amboinensis*) at predominantly neonate and small juvenile life stages. The NSW OTLF, therefore, is less vulnerable to observer-based catch-data inaccuracies than the northern Australian shark fishery.

Regional gene flow in *Carcharhinus obscurus*

This study represents a re-assessment of genetic structure in *C. obscurus* from Indo-Australian waters, following on from Ovenden et al. (2009). Using a different mtDNA marker, higher sample numbers and the addition of northern Australian samples, we detected weak genetic subdivision between eastern and western Australia. We observed genetic homogeneity, however, between northern Australia and both eastern and western Australia. In considering the Indonesian population, the application of 2 *F*-statistic metrics (Φ_{ST} and F_{ST}) produced contrasting results, with some evidence for differentiation between Indonesia and Australia based on haplotype frequencies. Discrepancies between these 2 metrics can arise due to their differing methods of calculation, and typically occur when genetic subdivision is at the margins of statistical significance (Broderick et al. 2011).

Our finding of unencumbered gene flow between northern Australia and more southern regions (NSW and WA) was not surprising from a point of view of dispersal potential. *C. obscurus* attains a large size (Last & Stevens 2009) and is suspected of undergoing long-range temperature-driven migrations on a seasonal basis, with tagging studies revealing an ability to travel considerable distances (Hussey et al. 2009, Rogers et al. 2013). Our findings of genetic subdivision between eastern and western Australia, however, challenge those of Ovenden et al. (2009) and also Benavides et al. (2011b), who failed to detect genetic differentiation between these same 2 locations using control region sequence data. We also provide evidence for and against the findings of Ovenden et al. (2009) relating to genetic subdivision between Australia and Indonesia. While these disparities may be accounted for by the examining of different genetic markers, the conclusions drawn by the above-mentioned authors were suitably circumspect given the generally low sample numbers upon which their comparisons were based.

Despite the comparatively robust sample numbers used in the present study, we, too, have reason to be circumspect in our findings. Random-resampling simulations offered some evidence that our detection of significant genetic differentiation between NSW and WA was driven, in part, by the strong bias in sample sizes between the 2 locations. Replicate pairwise comparisons indicated an increasing likelihood of finding a non-significant result between the 2 regions as the NSW sample size was decreased towards a more balanced analysis. These simulations either highlight the weak nature of genetic subdivision between Australia's east and west coasts or draw its actual existence into question. Conversely, replicate pairwise comparisons between NSW and NT and Indonesia appeared to be unaffected by a balancing of the NSW sample size, suggestive that the outcomes of the original analysis were robust to biased sample sizes in these instances.

Rarefaction analysis emphasised an additional limitation of our study. NSW was demonstrated as the only location at which an adequate proportion of the available genetic variability was likely sampled, with much of the available diversity appearing to have remained unsampled from NT and Indonesia, and possibly also WA. The rarefaction exact curves suggested that sample sizes in excess of 100 (and even

up to 150) may be required to accurately represent levels of genetic diversity, and hence to confidently discern haplotype relative frequencies at any given location. It is important to consider, however, that these particular results pertain specifically to the ND4 region and should not be applied to other mitochondrial genes. We would anticipate rarefaction curve trajectory, and therefore optimum sample size estimates, to be heavily reliant on the degree of polymorphism of the mtDNA region employed. Nevertheless, these findings serve as a cautionary note for studies investigating genetic structure over fine and regional spatial scales, where signals of genetic differentiation are unlikely to be strong, and reiterate that conclusions based on small sample sizes should be treated with considerable caution. For this reason, and given the contradicting metric results coupled with our inability to confirm that the samples were actually collected from Indonesian waters, we have henceforth placed little emphasis on results involving the Indonesian location.

Notwithstanding the above-mentioned limitations, evidence for regionally restricted gene flow between eastern and western regions of Australia, as presented in this study for *C. obscurus*, is consistent with mtDNA research on a range of other shark species representing a broad spectrum of different ecologies and life histories: scalloped hammerhead *Sphyrna lewini* (Duncan et al. 2006), grey nurse *Carcharias taurus* (Ahonen et al. 2009), *C. plumbeus* (Portnoy et al. 2010), pigeye *Carcharhinus amboinensis* (Tillett et al. 2012a) and great white *Carcharodon carcharias* (Blower et al. 2012). On comparable geographic scales, genetic subdivision was detected in bull *Carcharhinus leucas* and common blacktip *Carcharhinus limbatus* sharks between Gulf of Mexico and north-western Atlantic waters (Keeney et al. 2005, Karl et al. 2011).

Regional and fine-scale genetic subdivision in sharks based on mtDNA is often attributed to reproductive philopatry, a sex-biased behavioural trait widely documented in this taxon (Hueter et al. 2005, Portnoy & Heist 2012). Discerning reproductive philopatry in a justifiable manner, however, requires a stringent experimental design (Keeney et al. 2005, Dudgeon et al. 2012), which the present study lacked; tissue collection was both spatially and temporally opportunistic, with the exception of WA, where small individuals were sampled over consecutive days. While it is possible that our finding of regional subdivision reflects signs of philopatry, this study is unable to provide an informative test of this hypothesis.

Alternatively, the shallow divergence observed between eastern and western Australian regions may have resulted from repeated periods of isolation associated with the rise and fall of the Torres Strait land-bridge during the Pleistocene epoch, as is hypothesised for *Carcharhinus amboinensis* by Tillett et al. (2012a). However, unlike *C. obscurus*, *C. amboinensis* exhibits a distribution restricted to northern areas in Australian waters (Last & Stevens 2009). Given the former species' Australia-wide distribution, genetic divergence between eastern and western regions based on this historic, northern physical boundary is difficult to reconcile for *C. obscurus*, and assumes restricted gene flow across southern Australia, which we can neither refute nor support. Furthermore, under this hypothesis, one would expect similar levels of divergence between NSW and NT, which we did not observe.

Species comparison in NSW waters

C. obscurus and *C. plumbeus* exhibited strong similarities in their patterns of genetic diversity in NSW waters. Rarefaction curves from this region suggested that our sample sets had likely captured the majority of the respective genetic diversities available in both species, and hence were accurate representations of each species' genetic structure in the area. The haplotype-network topologies for both species, resolved thus through highly robust sample numbers, were very similar, suggestive that *C. obscurus* and *C. plumbeus* populations have experienced related evolutionary histories off Australia's east coast. In light of this, given our finding of weak genetic differentiation between the east and west coast in *C. obscurus*, Portnoy et al.'s (2010) similar result for *C. plumbeus* is perhaps not unexpected. These similarities suggest that *C. obscurus* may, to some degree, be a suitable proxy for patterns of gene flow in *C. plumbeus* around Australia, excluding southern waters where the latter species is not found.

However, while comparable levels of diversity were found off the east coast based on haplotype numbers, diversity indices indicated low haplotypic diversity in *C. plumbeus* compared with moderate haplotypic diversity in *C. obscurus*. This low apparent diversity in *C. plumbeus* in NSW waters may be accounted for by the exclusive sampling of the species' southern-most distribution limit (Last & Stevens 2009). Extreme and/or unstable environmental conditions are associated with distribution boundaries, and have been hypothesised to result in low population density and increased genetic drift and inbreeding in peripheral populations (e.g. Arnaud-Haond et

al. 2006, Lind et al. 2007). If this is indeed the case, one would anticipate the sampling of core Australian populations to reveal increased genetic diversity in *C. plumbeus*. Given also that this study is focused primarily on impacted populations, it is equally important to consider the potential impact of past anthropogenic pressure on the current observed diversities of both commercially targeted species.

Management implications and further work

Notwithstanding the limitations discussed earlier, our results tentatively support restricted gene flow in *C. obscurus* between eastern and western Australia. This suggests the allocation of 2 management units for *C. obscurus* in Australian waters: eastern and western regions. Under this scenario, stock recovery from a population collapse in the east would rely on reproduction by surviving local individuals and replenishment by immigrants from northern Australia. While the apparent genetic homogeneity involving northern Australia renders the most suitable boundary between these 2 management units uncertain, our results nevertheless support a more integrated approach to management between adjacent Australian states in this species.

The closely related genetic structures observed here in *C. obscurus* and *C. plumbeus* in NSW waters, resulting presumably from similar evolutionary histories, raise important implications for the management and conservation of these species. Given that both species appear to have responded similarly to evolutionary influences over time, and given also that both exhibit related biological traits in Australian waters (Simpfendorfer et al. 2002, McAuley et al. 2006, 2007b, Geraghty et al. 2013), it is likely that contemporary environmental and/or anthropogenic pressures will impact the 2 species' populations in a similar manner. Of concern, therefore, is that the majority of both species' genetic diversities in NSW waters is present as low-frequency haplotypes, suggestive of a vulnerability to rapid loss of genetic diversity under intense fishing pressure in the region.

High observer accuracy in the NSW OTLF, however, augurs well for the management of these species and the fishery. Scientifically sound catch-composition information is a valuable means of recognising fishing-induced ecosystem consequences such as species-specific shifts in abundance, size at capture and/or catch per unit effort (Burgess et al. 2005, Field et al. 2009). The maintenance of such high observer accuracy, however, is somewhat dependent on the fishery maintaining its focus on the more easily identified adults; identification success rate would presumably drop should effort shift to juveniles.

The use of only one mitochondrial marker limited the resolution of the present study, as did the exclusive use of mitochondrial sequence data. We were unable, therefore, to test a null hypothesis that gene flow between the putative populations is equal between males and females. Conflicting genetic structures between mitochondrial and bi-parentally inherited nuclear data (or mito-nuclear discordance) is a widely identified phenomenon in sharks (Portnoy & Heist 2012). Researchers have typically hypothesised male-biased dispersal (e.g. Pardini et al. 2001, Daly-Engel et al. 2012), also in *C. plumbeus* between eastern and western Australia (Portnoy et al. 2010), which implies persistent male dispersal despite constrained female gene flow. Patterns of male-mediated gene flow, therefore, can have significant implications with respect to interpretations of genetic subdivision and, in turn, the allocation of appropriate management units (Toews & Brelsford 2012).

Southern Australian waters were unsampled in this study, highlighting a lack of knowledge regarding gene flow in this region. A recent satellite-tagging study by Rogers et al. (2013) demonstrated the mixing of *C. obscurus* between southern and south-western Australian waters, but not between southern and eastern waters. Their findings, however, were based on data from only 3 individuals tagged in the same location. Given, therefore, that definitive information pertaining to movement (or lack of) between eastern and western Australia is not currently available, genetic sampling of southern waters would greatly improve interpretations of the current data.

With the shortcomings of this study in mind, we recommend this work not be viewed as a definitive template for management policy but rather as a foundation for future studies. We strongly encourage further work aimed at achieving greater genetic structure resolution for *C. obscurus* and *C. plumbeus* in Australian and neighbouring waters via more extensive sampling and the use of more and varied genetic markers. We also urge evaluations of connectivity in these species around Australia, particularly between the east and west coasts. For *C. obscurus*, we suggest a focus on southern Australian waters. More robust assessments of contemporary gene flow, as well as physical tagging and tracking, would greatly assist the effective management of these species in Indo-Australian waters through the appropriate allocation of management units.

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Appendix 1

Table A1. Polymorphic sites for mitochondrial DNA ND4 haplotypes defined from Australian and Indonesian waters for the dusky shark *Carcharhinus obscurus*. (.) indicates the same nucleotide as in haplotype DS1

Haplotype	Nucleotide polymorphism position (1–857)																	
	21	34	90	109	124	189	192	199	289	360	400	421	423	453	594	648	649	822
DS1	A	G	T	G	G	T	T	G	G	C	G	T	G	T	T	A	C	G
DS2	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.
DS3	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.
DS4	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
DS5	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.
DS6	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.
DS7	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.
DS8	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
DS9	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
DS10	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.
DS11	.	.	.	.	.	A	.	.	.	.	.	C	A	.	.	.	.	.
DS12	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	T	.	.
DS13	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.
DS14	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A
DS15	.	.	.	.	.	.	C	.	.	.	.	.	A	C	.	.	.	.
DS16	.	.	.	.	.	.	C	A	.	.	.	.	A	C	.	.	.	.
DS17	.	.	.	.	.	.	C	.	.	.	.	.	A	C	C	.	.	.
DS18	.	A	.	.	.	.	C	.	.	.	.	.	A	C	.	.	.	.

Table A2. Polymorphic sites for mitochondrial DNA ND4 haplotypes defined from Australian waters for the sandbar shark *Carcharhinus plumbeus*. (.) indicates the same nucleotide as in haplotype SB1

Haplotype	Nucleotide polymorphism position (1–857)											
	9	72	120	160	186	199	209	327	531	600	650	655
SB1	G	T	T	T	A	G	T	C	T	C	T	T
SB2	.	.	C	.	.	.	C	.	.	.	.	.
SB3	.	.	.	.	G	.	C	.	.	.	.	.
SB4	.	.	.	.	.	.	C	.	.	.	.	.
SB5	A	.	.	.	.	.	C	T	.	T	.	C
SB6	A	.	.	C	.	.	C	T	.	.	.	C
SB7	A	.	.	.	.	.	C	T	.	.	.	C
SB8	A	C	.	.	.	.	C	T	.	.	.	C
SB9	A	.	.	.	.	.	C	T	C	.	.	C
SB10	A	.	.	.	.	.	C	T	C	.	C	C
SB11	A	.	.	.	.	A	C	T	C	.	C	C

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