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Effective population size of the critically endangered east Australian grey nurse shark *Carcharias taurus*

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ABSTRACT: Retaining genetic variation is central to species-level conservation, and knowledge of effective population sizes (N_e) can be used to predict rates at which genetic variation will be lost over generations. Here we used thousands of genome-wide single nucleotide polymorphisms to investigate N_e of the critically endangered grey nurse shark *Carcharias taurus* across 1400 km of its eastern Australian range. The eastern Australian grey nurse shark population has declined rapidly in number over the last few decades, has relatively low genetic diversity and is extremely susceptible to anthropogenic mortality. We found no evidence for any genetic structure, which is consistent with previous field observations that show widespread movement across the distribution. Estimates of the effective number of breeders (N_b) and N_e were around 400, using an approach based on proportions of siblings and another based on linkage disequilibrium. Forward simulations revealed that even if an N_e of 400 is maintained, the population will experience some loss of genetic diversity over the next 50 generations. The relatively low N_e highlights the importance of maintaining the population size of grey nurse sharks in order to retain genetic variation and therefore resilience to environmental change.

KEY WORDS: N_e · Effective number of breeders · Genetic panmixia · Shark · Conservation · Simulation · Single nucleotide polymorphisms

1. INTRODUCTION

Knowledge of effective population size (N_e) is important for assessing extinction risk and improving conservation management of endangered species (Kool et al. 2013, Ruzzante et al. 2016, Van Wyngaarden et al. 2017). N_e quantifies the level of genetic drift and inbreeding within a population, reflects past demography and can predict losses of genetic variation (Wang 2005, Husemann et al. 2016, Wang et al. 2016). N_e is defined as the number of individuals in an ideal Wright-Fisher population that would lose genetic variation at the same rate as the total population being studied (Holsinger & Weir 2009, Husemann et al. 2016). In an ideal population, N_e and census size (N_c, the total number of adult males and females in a population) would be equal, yet in real populations the number of breeding individuals is usually only a small proportion of N_c (Waples 2005). This is due to many factors, including the presence of individuals that are pre- or post-reproductive, a skewed sex ratio, the number of reproductive individuals per generation, reproductive mode and varying population size (Hedrick 2000, Trask et al. 2017). A fluctuating population size can result in N_e being larger or smaller than N_c, depending on whether the population size changes are positively or negatively autocorrelated, respectively. A harmonic N_e that is calculated over many generations of the fluctuating population may be a more informative measure in these cases (Iizuka 2010). While N_c can be difficult to infer through direct field observation, N_e estimates

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can be calculated using genetic techniques (Ferchaud et al. 2016, Ackerman et al. 2017, Pazmiño et al. 2017). Genetic estimates of connectivity, population structure and N_e are particularly useful when studying endangered marine species that are low in number or difficult to observe at the sea surface (Bilgmann et al. 2007, Andreotti et al. 2016). As such, recent studies have used genetic techniques to investigate the N_e and N_e/N_c relationship of shark populations (e.g. Blower et al. 2012, Dudgeon & Ovenden 2015, Andreotti et al. 2016, Feutry et al. 2017).

The grey nurse shark *Carcharias taurus* (Rafinesque, 1810), also known as the sand tiger shark or ragged-tooth shark (Smith et al. 2015), has a circumglobal distribution, with records from the North and South Atlantic, Indian and Western Pacific Oceans (Compagno 1984, Last & Stevens 1994, Momigliano & Jaiteh 2015). This large, coastal-dwelling shark inhabits sub-tropical to temperate waters, and typically aggregates in caves or sandy channels close to land (Stow et al. 2006, Hoschke & Whisson 2016). Sexual maturity in grey nurse sharks commences at 6–7 yr for males and 9–12 yr for females (Goldman et al. 2006, Smith et al. 2015), and they can live to approximately 34 and 40 yr of age, respectively (Passerotti et al. 2014). Grey nurse sharks exhibit low fecundity, with females giving birth to a maximum of 2 pups every 2 yr (Chapman et al. 2013).

Globally, the grey nurse shark is classified as Vulnerable under the International Union for Conservation of Nature (IUCN) (Cavanagh et al. 2003). Under the Australian Environment Protection and Biodiversity Act 1999 (EPBC Act), the east Australian population of grey nurse sharks is classified as critically endangered, and the west Australian population is listed as vulnerable (Cavanagh et al. 2003, Department of the Environment 2017a,b). Throughout their global distribution, grey nurse shark numbers are declining (Bowden et al. 2016), with the greatest threats being recreational and commercial fishing for their oil and meat (Lynch et al. 2013, Robbins et al. 2013). Since European settlement in Australia, grey nurse sharks have suffered severe population declines from fishing and targeted culling due to their aggressive appearance (Parker & Bucher 2000, Robbins et al. 2013). Despite recent conservation efforts, Australian populations have continued to suffer from accidental bycatch and from entanglement in the mesh netting and baited drumlines that are used in bather protection programmes (Lynch et al. 2013).

Genetic analyses of Australian grey nurse sharks have identified strong genetic partitioning between the west and east coast populations, showing long-term genetic isolation (Stow et al. 2006, Ahonen et al. 2009). While less research has been carried out on the west coast population, the east coast population of grey nurse sharks is relatively well studied (e.g. Bansemer & Bennett 2011, Otway & Ellis 2011, Smith et al. 2015). Genetic analysis of the east coast population has revealed relatively low genetic variation, evident through a single shared mitochondrial haplotype and low diversity at nuclear markers (Stow et al. 2006, Ahonen et al. 2009). Effective population size of the east Australian population was estimated to be 126 (95% CI: 68–474) using a small dataset of microsatellite loci (Ahonen & Stow 2009). In recent years, considerable effort has been devoted to assessing the total population size of east Australian grey nurse sharks. Photo-identification mark–recapture studies using a closed-population model have produced similar total population size estimates of 2142 (95% CI: 1465–3249) and 2049 (95% CI: 1216–2883) (Smith & Roberts 2010) to an open-population model estimate of 1893 (95% CI: 1556–2232) (Bansemer 2009). However, estimates using cattle-ear tags and mark–recapture methodology resulted in a total population size estimate of only 410–461 (95% CI: 148–766) (Otway & Burke 2004). Further research to assess the population size and status was listed as a priority in the 2014 Recovery Plan for the grey nurse shark (Department of the Environment 2014).

Throughout the range of the grey nurse shark in eastern Australian waters, aggregation sites have been identified between the states of Queensland to the north and New South Wales to the south (Otway & Ellis 2011). The sharks are known to move up and down the eastern coast, travelling distances of up to 1550 km (Otway & Ellis 2011). Movement patterns of grey nurse sharks in east Australia appear to differ depending on the maturity, sex and, for females, whether the individual is pregnant or not (Bansemer & Bennett 2011). Immature sharks show no obvious movement patterns, while mature males and females have been shown to migrate northward to mate in the late austral spring to early summer. Pregnant females tend to aggregate in southern Queensland before migrating south a few months later to pup in late winter to late spring in southern regions of east Australia (Bansemer & Bennett 2011). Although field observations reveal that grey nurse sharks are dispersing along the coast, the level of genetic connectivity among aggregation sites in east Australia is unknown. As sharks have no larval stage, population genetic structure tends to be a result of dispersal at older life stages (Chapman et al. 2015). This has been typically characterised by male-biased dis-

persal and female philopatry, as seen in the great white shark *Carcharodon carcharias* (Pardini et al. 2001) and tiger shark *Galeocerdo cuvier* (Holmes et al. 2017).

We used genome-wide single nucleotide polymorphisms (SNPs) to estimate the N_e of grey nurse sharks in eastern Australia. Based on field observations of widespread movement, we did not expect to detect genetic structure throughout the entire east Australian distribution. We estimated effective number of breeders (N_b) and N_e from all sampled individuals collectively and then used these estimates in simulations to predict future losses of genetic variation over time by drift.

2. MATERIALS AND METHODS

2.1. SNP genotype-by-sequencing and filtering

A total of 63 grey nurse shark DNA samples were collected between 1999 and 2007 from 8 locations along the east coast of Australia between Flat Rock, Queensland, to the north and Wallagoot Lake, New South Wales, to the south (Fig. 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/m610p137_supp.pdf). These samples were collected from deceased individuals and by biopsy sampling free-swimming individuals. Grey nurse shark DNA was extracted using the commercially available GenCatch™ Blood & Tissue Genomic Mini-Prep Kit (Epoch Biolabs), following the manufacturer's protocol. Extracted DNA was then stored in Multi-Core™ 1× restriction enzyme buffer (Promega). For confirmation that samples contained high-molecular-weight DNA and were not contaminated with nucleases, all DNA samples were electrophoresed on a 0.8% agarose gel, pre-stained with GelRed™. Concentration of genomic DNA after extraction was estimated using a NanoDrop. DNA concentrations ranged from around 50–100 ng μl^{-1}.

Library preparation, sequencing and SNP discovery was carried out by Diversity Arrays Technology Pty. Ltd (DArT, Canberra), following the standard DArTSeq protocol (Jaccoud et al. 2001). A combination of *Pst*I and *Sph*I restriction enzymes were used to digest 100 ng of each DNA sample, and adaptors that were complementary to cut sites were then ligated to each DNA fragment. An Illumina flow cell attachment sequence, a sequencing primer and a barcode sequence unique to each individual DNA sample were contained within the *Pst*I adaptor.

Following digestion and ligation, all samples were purified using a spin-column PCR cleanup kit (Qiagen) and amplified by PCR, using primers, and barcode sequences specific to the adaptor. The PCR conditions involved 1 min at 90°C for initial denaturation, 30 cycles of 20 s denaturation at 94°C, 30 s annealing at 58°C, 45 s extension at 72°C, followed by a final extension of 7 min at 72°C. Following PCR, equimolar amounts of all samples were pooled together, diluted and denatured using NaOH. To sequence the library, an Illumina HiSeq2500 single read platform was used. This process involved 77 cycles, resulting in equal fragment lengths of 77 bp. Later removal of barcodes resulted in sequence fragments of 69 bp. A set of technical replicates, created by running 15% of the samples back through the whole library preparation protocol and downstream analysis, were used to assess the reproducibility of SNP calls.

Illumina HiSeq2500 software converted the raw sequence data to fastq files, and individuals were separated based on the unique ligated barcodes. The quality of each read was assessed, and any containing a Phred quality score (Ewing et al. 1998) of <25 were removed. Potential contaminants were iden-

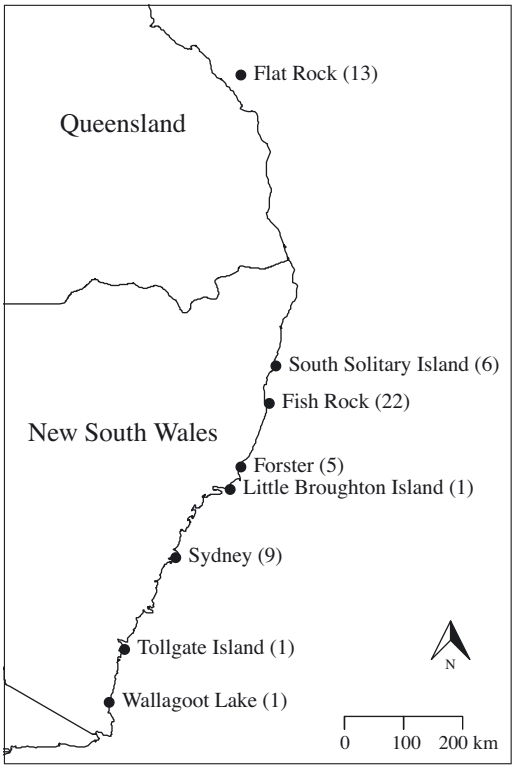

Fig. 1. Sites where grey nurse sharks *Carcharias taurus* were sampled along the east coast of Australia, with sample number in brackets. Five sharks that were sampled in New South Wales, but without precise geographic location, are excluded from this map

tified by checking all reads against GenBank bacterial, fungal and viral sequences, and the DArT database. SNPs were then identified and called, following the standard procedure in the DArT pipeline DArTSoft14™. This pipeline is very similar to the STACKS pipeline (Catchen et al. 2013), but differs in that DArTSoft14™ first calls the sequence clusters for the pooled sample, prior to each individual. As part of the DArT pipeline, all monomorphic clusters were removed and only SNPs that were present in both homozygous and heterozygous forms were called.

The DArT pipeline resulted in 8644 SNPs and an average ratio read depth of 0.72 (range: 0.30–2.98) between alleles. Following the DArT pipeline, we filtered this dataset further to only retain SNPs with 100% reproducibility and a call rate of >90, and this resulted in less than 10% missing data over the entire dataset. The number of reads for all samples ranged from 3 to 77.2, with an average read depth of 9.3. To remove any potential paralogous sequences, the dataset was filtered for a maximum read depth equal to $d + 3 \times \sigma(d)$ (Li 2014), whereby d is the average read depth and σ is the standard deviation, which resulted in a cut off at 26.09. To avoid tightly linked loci for subsequent analyses of genetic connectivity and N_e, we retained only 1 SNP per fragment. Different methods can be used to select 1 SNP per fragment, and here we consistently used the first SNP per 69 bp and removed all other SNPs on the same fragment. As there is no reference genome for the grey nurse shark (or a closely related species), sequence assembly was carried out de novo. As such, we do not know the location of fragments on chromosomes and we treated the SNPs of the filtered dataset on different fragments as independent. During exploratory stages of the data analysis, different minor allele frequency (MAF) cut offs (0.01, 0.02 and 0.05) were applied, and changing these values did not change overall results. As such, SNPs with MAF values <0.01 were considered rare and were removed to reduce the number of false heterozygotes due to sequencing errors (Mdladla et al. 2016). This resulted in a final dataset of 3087 SNPs. Some of the analyses required the removal of individuals and sample sites, and this resulted in additional monomorphic loci. These loci were also removed for the analyses.

2.2. Preliminary analyses

Observed heterozygosity (H_o), expected heterozygosity (H_e) and the fixation index $F = 1 - (H_o/H_e)$ were calculated using the software GENEPOP v. 4.6 (Rousset 2008) for all samples. Deviation from Hardy-Weinberg equilibrium (HWE) for each locus was calculated using exact test methods of Guo & Thompson (1992) in GENEPOP v. 4.6 (Rousset 2008), and using Bonferroni correction for multiple tests. A principal components analysis (PCA) and F_{ST} analysis each produced equivalent results regardless of whether the few loci deviating from HWE were included or removed, as such these loci were retained in the dataset (see Table S2). To confirm that all loci in the dataset were neutral, we conducted F_{ST} outlier tests to identify loci associated with selection using BayeScan v. 2.1 (Foll & Gaggiotti 2008). No outlier loci were detected, and we therefore used all loci for the subsequent analyses (Fig. S2 in the Supplement).

2.3. Spatial genetic structure

F_{ST} was measured to assess allelic differentiation between sampled locations that had 5 or more individuals (Flat Rock, South Solitary Island, Fish Rock, Forster and Sydney) across 3038 SNPs, following the removal of monomorphic loci. A previous simulation study suggests that this should be a sufficient sample size to estimate F_{ST} with datasets of similar properties to ours (Nazareno et al. 2017). Weir & Cockerham's (1984) method for estimating pairwise F_{ST} was carried out using the 'diffCalc' function in the R package diveRsity v. 1.9.90 (Keenan et al. 2013). Default parameters and 1000 bootstraps were used to calculate the 95% confidence intervals.

Of the 63 grey nurse shark samples, 5 were lacking precise geographic location data. Analyses on spatial genetic structure were therefore carried out with the 58 sharks for which geographic location data were available (see Table S1). To investigate spatial genotypic structure, pairwise geographic and genetic distance matrices were created in GenAlEx v. 6.5. To test for patterns of isolation-by-distance, a Mantel test using 999 permutations was then performed using the 'mantel.randtest' function in the R package 'adegenet' (Jombart 2008). To further investigate genetic structure at different spatial scales, a spatial autocorrelation analysis was performed on 7 distance class bins (0, 250, 500, 750, 1000, 1250 and 1500 km) in GenAlEx v. 6.5. These distance categories were chosen to provide sufficient sampling density while accommodating the range of distances between sampled sites, which extended to a maximum of 1386 km. Significance was assessed in GenAlEx v. 6.5 using 999 permutations to estimate the 95% confidence interval around 0 (no autocorrelation) and 1000 boot-

straps to estimate the 95% confidence intervals around the autocorrelation coefficient *r*. Heterogeneity testing for spatial autocorrelation was used in GenAlEx v. 6.5 to determine whether results were statistically credible (Smouse et al. 2008).

To test for sex-biased dispersal, juveniles (n = 6) and individuals of unknown sex (n = 5) were removed from the analyses, retaining 52 individuals (see Table S1). Juveniles were excluded by choosing only individuals >1.8 m, as males and females reach sexual maturity at around 2 and 2.2 m, respectively (Lucifora 2002, Bansemer & Bennett 2009), and individuals longer than 1.8 m but smaller than sexually mature adults are considered subadults (Lynch et al. 2013). Spatial autocorrelation analyses were conducted separately for males (n = 32) and females (n = 20) at 5 distance class bins (0, 250, 500, 750, 1100 km) in GenAlEx v. 6.5.

2.4. Relatedness

If siblings from the same age cohort were found to be sampled at the same location, this could indicate intragenerational site fidelity, while first-order relatives from different age groups (parent–offspring; sibling–sibling) sampled at the same location could indicate intergenerational site fidelity to that location (Stow et al. 2001, Reid et al. 2016). We calculated pairwise relatedness at the 5 sampling locations with sufficient numbers of individuals (n ≥ 5) with COANCESTRY v. 1.0.1.7 (Wang 2011) using the moment estimator from Queller & Goodnight (1989).

The software program Colony2 v. 2.0.6.3 (Jones & Wang 2010) was used to identify first- and second-degree relatives for all grey nurse sharks sampled. Colony2 uses multilocus genotypes to infer parentage and sibship, i.e. whether 2 individuals share 1 parent (half siblings) or 2 parents (full siblings). The program then clusters individuals into groups according to these relationships (Jones & Wang 2010). As there can be multiple configurations, the program repeats this for the dataset until the best configuration with the highest likelihood is chosen. Colony2 can accommodate for genotyping error and polygamous mating systems, and does not require information on the parental genotypes (Jones & Wang 2010, Ackerman et al. 2017).

Because the SNP dataset contained >2000 loci, the analysis was run in non-GUI mode. A comma-delimited input data file was created according to the Colony User Guide, incorporating the following parameters: analysis method = full-likelihood; likelihood precision = medium; length of run = medium; update allele frequency = no; sibship scaling = yes; number of runs = 3; random number seed = 1234; sibship prior = no prior; marker type = codominant; allelic dropout rate = 0.0000; dioecious; diploid. A conservative error rate of 0.01 was chosen, which is the equivalent of 1 error per 100 genotypes. As the error rate has little effect on the accuracy of the results (Ackerman et al. 2017), this error rate was chosen according to previous studies (Mourier et al. 2013, Pirog et al. 2017). Grey nurse sharks have multiple paternity (Chapman et al. 2013), so polygamy was chosen as the parameter to describe the mating system for both males and females. Preliminary analysis showed no significant difference in results whether inbreeding was accounted for or not (Table S3); thus, all simulations were run with no inbreeding. To validate that Colony2 was correctly identifying related individuals, we reran the analysis with artificial dyads included in the dataset.

2.5. Effective population size

Contemporary N_e was calculated for the entire east coast population of grey nurse sharks, using mature individuals (n = 57) and 2 different single-sample methods: sibship frequency and linkage disequilibrium (LD). Most methods assume closed populations with discrete generations (Kamath et al. 2015). Because grey nurse sharks on the east coast are significantly genetically divergent from grey nurse sharks in other regions (Ahonen et al. 2009), we assumed a closed population. However, N_e estimation was complicated by the fact that grey nurse sharks are iteroparous with overlapping generations, rather than the discrete generations that these methods assume. Consequently, the effective number of breeders (N_b) of the parent generation that produced the sampled cohort was estimated with this approach, rather than the N_e per generation (Ackerman et al. 2017). The juveniles (<1.8 m in length) were removed from the dataset, because this would increase the number of generations included in the analyses and upwardly bias the N_b estimate.

N_b was calculated using the sibship frequency method in Colony2 non-GUI mode, using the same parameters that were chosen for the sibling identification. If the N_b of a population is small, there is a higher probability that individuals within a random sample are related; if the N_b is large, there is a lower chance that individuals are related (Ackerman et al. 2017). The sibship assignment method is based on this premise and calculates N_b from the proportion of

full and half-sibling pairs (or first- and second-degree relatives) within the sample (Ackerman et al. 2017).

NeEstimator v. 2.1 (Do et al. 2014) was used to calculate N_b by LD on all mature individuals (n = 57). LD, the non-random association between alleles at different loci, can arise from migration, selection and genetic drift (Wang 2005). In a closed population with random mating and unlinked loci, LD would be the product of genetic drift, occurring at a rate inversely proportional to N_e (Waples et al. 2016). It is therefore possible to estimate N_e by measuring LD between loci that are inherited independently, provided that the assumption of an isolated population with random mating is met (Funk et al. 2016). In NeEstimator2.1, the random mating model under LD was chosen, and a critical value of 0.02 was selected to represent the minimum allele frequency cut off as per previous studies (O'Leary et al. 2013, Trask et al. 2017) and because critical value cut offs of 0.05, 0.02 and 0.01 produced similar N_b results. We assumed that all loci used in the analysis were unlinked, yet because there is no reference genome for the grey nurse shark and genotyping was carried out de novo, it is probable that some physically linked loci were retained. Although increased numbers of SNPs do not necessarily lead to an increase in the proportion of linked loci (Waples et al. 2016), we investigated the effect of loci number on N_e by thinning our dataset to evaluate any bias from potentially linked loci. Estimation of N_e in NeEstimator2.1 was repeated on 6 random subsets of our SNP dataset (250, 500, 750, 1000, 1500 and 2000). If our dataset of >3000 loci were biasing the N_e estimates through an increase in the proportion of linked loci, we would expect to find that estimates of N_e change as the number of loci changes. Furthermore, this allowed us to gauge the extent to which the relatively large number of SNP loci in our analysis led to a gross overestimation in the precision of our estimate. This is because inflated sample sizes resulting from pairwise comparisons, and the non-independence of these data, result in artificially narrow 95% confidence intervals (see Waples et al. 2016). While there is currently no solution to the problem of overestimating precision, it is necessary to be aware of this effect when using large numbers of loci and assuming independence for all loci (Waples at al. 2016).

Following LD estimation of N_b, the raw estimate $N_{b(LD)}$ was adjusted for bias due to overlapping generations using the formula from Waples et al. (2014). This formula incorporates 2 life history traits; adult life span (AL) and age at maturity (α). AL was calculated as ω – α + 1, where ω is maximum age and α is age at first maturity. The maximum age for this purpose was 38, as males live up to 34 yr and females live up to 40 yr (Passerotti et al. 2014). Age at first maturity was 10, as females reach sexual maturity between 9 and 12 yr (Smith et al. 2015). The equation was as follows:

$$N_{b(Adj2)} = \frac{N_{b(LD)}}{1.103 - 0.245 \times \log\left(\frac{AL}{\alpha}\right)} \quad (1)$$

Effective population size per generation $N_{e(Adj2)}$ was then calculated using the formula from Waples et al. (2014) that adjusts $N_{b(Adj2)}$ using the same 2 life history traits, AL and α, as follows:

$$N_{e(Adj2)} = \frac{N_{b(Adj2)}}{0.485 + 0.758 \times \log\left(\frac{AL}{\alpha}\right)} \quad (2)$$

Physically linked loci can lead to a downwardly biased estimate of N_e, and this bias is strongly negatively correlated with recombination rate (Waples et al. 2016). It is possible to reduce bias by correcting the N_e estimate using the number of chromosomes or genome size. We applied a formula that corrects for this bias using the number of chromosomes of the grey nurse shark (2n = 84) (Schwartz & Maddock 2002). The equation, using chromosome number (chr) = 42, was adapted from Waples et al. (2016) and is as follows:

$$N_{e(Adj3)} = \frac{N_{e(Adj2)}}{0.098 + 0.219 \times \ln(chr)} \quad (3)$$

2.6. Future genetic variation

The forward simulation program BottleSim v. 2.6 (Kuo & Janzen 2003) was used to model the possible effects of genetic drift on the current levels of genetic variation of the east Australian grey nurse shark population. Five simulations were run to explore the impact of genetic drift on different N_e. These were chosen to represent a broad spread of N_e possibilities: (1) the smallest N_e (50) previously suggested by Frankham et al. (2014) to prevent the effects of inbreeding depression; (2) the former N_e estimate (126) for this population based on microsatellite loci (Ahonen & Stow 2009); and (3) the $N_{b(sib)}$ estimate from this study (400) and its upper and lower bounds (258 and 820) rounded up to the nearest even number. The adjusted $N_{e(Adj3)}$ estimate was 416, and because of its similarity to the $N_{b(sib)}$ estimate was not included separately in the forward simulations. An input file, in the form of a multilocus genotype text file for all 63 grey nurse sharks, was created accord-

ing to the online BottleSim guidelines. All simulations were performed with the following parameters: reproduction mode = dioecy with random mating; simulation module = diploid, multilocus, constant population size; longevity of organism = 38; age at sexual maturity = 10; sex ratio = 1:1; generation overlap = maximum 100; number of years = 500; number of iterations = 500. To find out whether maintaining the current N_e would result in a loss of genetic variation over time, the population size parameter was chosen to remain constant before and during the bottleneck. However, this is a conservative estimate as it does not account for potential declines in population size over time.

3. RESULTS

3.1. Preliminary analyses

The filtering process removed 5557 SNPs, resulting in a final dataset of 3087 putatively neutral SNPs across 63 individuals. Of the 3087 loci, 377 significantly deviated from HWE across all individuals. Following Bonferroni correction, 19 loci remained significantly deviated from HWE. As there was no measurable difference in the pairwise F_{ST} results when these 19 non-HWE loci were included or removed (Table S2), they were retained in the dataset for all subsequent analyses. Across all individuals and loci, H_e was 0.2688 and H_o was 0.2591, with a non-significant global F_{IS} estimate of 0.0348. The PCA only explained 3.21% of the variation in genetic distance (Fig. S1).

3.2. Spatial genetic structure

After the removal of sampling sites with fewer than 5 individuals, 3038 SNPs were retained across 55 individuals from 5 locations (Flat Rock, South Solitary Island, Fish Rock, Forster and Sydney). Analysis of allelic frequencies among these sites provided no evidence for allelic differentiation among the sampled locations, and pairwise F_{ST} values were low and did not significantly differ from 0 (Table 1).

To investigate spatial genotypic structure, all grey nurse sharks that had geographic location data (n = 58) were used, resulting in 3076 SNPs across 8 sampling locations (Table S1). The Mantel test showed no significant linear relationship between geographic and genetic distance, and therefore no evidence for isolation-by-distance (R^2 = 0.0001537, p = 0.6135). Spatial autocorrelation analyses on all 58 individuals using unequal distance bins (0, 250, 500, 750, 1000, 1250 and 1500 km) showed no significant correlation between genetic relatedness and geographic distance (ω = 23.68, p = 0.063), demonstrating that individuals within sampling locations were no more genetically similar to each other than the genetic similarity between individuals sampled at different locations (Fig. S3a). In addition, spatial autocorrelation excluding juveniles, and carried out separately for 32 males (ω = 17.995, p = 0.096) and 20 females (ω = 14.522, p = 0.188), provided no evidence for sex-biased dispersal or philopatry (Fig. S3b,c).

Table 1. Allelic differentiation, represented by pairwise F_{ST}, between grey nurse shark *Carcharias taurus* sampling locations at Flat Rock (n = 13), South Solitary Island (n = 6), Fish Rock (n = 22), Forster (n = 5), and Sydney (n = 9), Australia

	Flat Rock	South Sol Isl	Fish Rock	Forster
South Sol Isl	0.0028	0		
Fish Rock	−0.0022	−0.0001	0	
Forster	−0.0069	0.0063	−0.0024	0
Sydney	−0.0012	−0.003	−0.0005	−0.0045

3.3. Relatedness

Pairwise relatedness was calculated for each of the 5 sampling locations with more than 5 individuals (n = 55; Table S1). Significantly high relatedness within a sample site was not found, providing no evidence for intergenerational site fidelity (Fig. S4). Sibship reconstruction in Colony2 for the total dataset (n = 63) identified 5 pairs of first-degree relatives and 8 pairs of second-degree relatives with 100% probability. The distance between related individuals ranged from 0 km (within the same location) up to 560 km for 1 pair (Table A1 in the Appendix). When rerun, Colony2 successfully identified artificial relatives with 100% probability, demonstrating that our data had sufficient power to accurately detect first- and second-order relatives.

3.4. Effective population size

N_b was estimated with 2 single sample methods, excluding juvenile grey nurse shark samples (n = 57). $N_{b(sib)}$ calculated in Colony2 using the sibship frequency method was 399 (95% CI: 257–820) assuming random mating, and 366 (95% CI: 242–698) assuming

non-random mating. These estimates were comparable to the $N_{b(LD)}$ of 316.9 (95% CI: 307.2–325.6), calculated using the LD method in NeEstimator2.1. There was no difference in the $N_{b(LD)}$ estimates calculated using random subsets of SNPs, as confidence intervals overlapped (Table S4). Bias correction of $N_{b(LD)}$ for overlapping generations gave $N_{b(Adj2)}$ of 318.29 (95% CI: 309.23–327.75), which was then adjusted to find $N_{e(Adj2)}$ of 380.95 (95% CI: 370.11–392.28). Following correction for physically linked loci, using the number of chromosomes of the grey nurse shark, $N_{e(Adj3)}$ was estimated to be 415.63 (CI: 403.81–428).

3.5. Future genetic variation

Forward simulations using BottleSim v. 2.6 predicted that over the next 50 generations (500 yr, assuming 10 yr per generation) genetic diversity will be lost through genetic drift regardless of the N_e scenario applied; however, the rate of loss differs (Fig. 2). With an N_e of 820 and 400, the population is expected to retain 98 and 96% of H_o after 50 generations, respectively. For an N_e of 258, 126 and 50, respectively, 92, 88 and 73% H_o is expected to be retained. Simulations predict that it would take around 17.5 generations (~175 yr) with an N_e of 50, and around 39 generations (~390 yr) with an N_e of 126 to lose 10% of the population's genetic diversity. The standard error ranged between 0.0023 and 0.0031 for each generation.

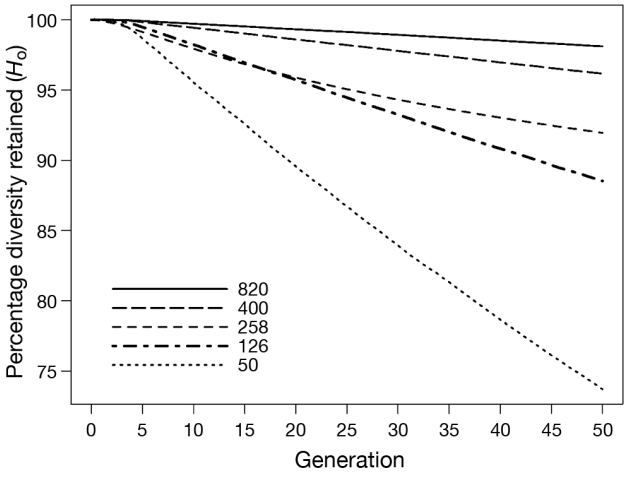

Fig. 2. Simulated loss of genetic diversity for east Australian grey nurse sharks *Carcharias taurus*, represented as the percentage of observed heterozygosity (H_o), for different effective population size (N_e) values (50, 126, 258, 400 and 820) over 50 generations. Standard error bars are not shown, as they were too small to be discernible on the graph (values ranged from 0.0023–0.0031)

4. DISCUSSION

We could not detect any significant deviation from genetic panmixia with a sample of grey nurse sharks collected at different sites along much of the east Australian range. Two single sample methods estimated N_b and N_e to be 399 (95% CI: 257–820) and 416 (CI: 403–428), respectively, and forward simulations were used to demonstrate that an N_e of 400 will result in a loss of genetic variation through genetic drift over the next 50 generations.

We tested for spatial genetic structure and confirmed that the east coast grey nurse sharks show high genetic connectivity among aggregation sites, as inferred by previous field observations (Otway & Burke 2004, Bansemer & Bennett 2011). Low F_{ST} values are not uncommon in marine organisms, due to efficient dispersal of individuals or gametes across large distances in water, and a historically large N_e for many species (White et al. 2011, Momigliano et al. 2017). Compared to F_{ST}, spatial autocorrelation analyses based on genotypic similarity can reveal genetic partitioning that has arisen over a shorter temporal scale (Epperson 2005, Banks & Peakall 2012). However, spatial autocorrelation analyses in this study provided no evidence for a relationship between geographic and genetic distance. Moreover, the presence of first-degree relatives at different sampling sites reflects dispersal among aggregation sites within their lifetime, rather than remaining together at the same location. These findings also complement previous field observations of grey nurse sharks travelling up to 1550 km along the east coast of Australia (e.g. Otway & Ellis 2011). High genetic connectivity has also been shown across substantial parts of the distribution of other shark species, including bull sharks *Carcharhinus leucas* (Karl et al. 2011), basking sharks *Cetorhinus maximus* (Hoelzel et al. 2006), blue sharks *Prionace glauca* (Bailleul et al. 2018) and great white sharks (Andreotti et al. 2016).

Separate spatial autocorrelation analyses of adult males and females provided no evidence for sex-biased dispersal or philopatry in the east Australian grey nurse sharks. In contrast, genetic structure in other shark species, including the tiger shark *Galeocerdo cuvier* (Holmes et al. 2017), great white shark *Carcharodon carcharias* (Blower et al. 2012) and scalloped hammerhead shark *Sphyrna lewini* (Guttridge et al. 2017), have been attributed to sex-biased dispersal, where the male is the disperser and the female is philopatric. Our results are consistent with previous field observations of both adult male and

female grey nurse shark movement patterns (Bansemer & Bennett 2011). These movements are in synchrony with their reproductive cycle, where adults travel north to southern Queensland (QLD) and northern New South Wales (NSW) to mate, and then pregnant females aggregate at Wolf Rock in southern QLD, before travelling to the central and southern reaches of their distribution to give birth (Bansemer & Bennett 2011). Because the samples in our study were obtained over an 8 yr period, we could not test for patterns of relatedness arising for short duration, such as those expected for natal-breeding site fidelity. Consequently, future systematic sampling of aggregation sites could potentially uncover patterns of genetic structure that were not revealed in this study.

The N_e values recommended to avoid inbreeding depression and to maintain evolutionary potential are 50 and 500, respectively (Jamieson & Allendorf 2012, Frankham et al. 2014). More recently, Frankham et al. (2014) proposed an $N_e \geq 100$ to avoid inbreeding depression in the short term and an $N_e \geq 1000$ to retain evolutionary potential. According to these more recent recommendations of N_e, the east coast grey nurse shark population (N_e ~400) is not predicted to be large enough to maintain sufficient long-term evolutionary potential. This could have negative consequences in the future, particularly with ongoing anthropogenic-related mortalities and increasing ocean temperatures (Robbins et al. 2013, Roemmich et al. 2015).

The contemporary N_b and N_e estimates from this study reflect the past few generations of grey nurse sharks in east Australia, and therefore are applicable to the time since post-European settlement. Between the 1950s and 1970s, east Australian grey nurse sharks were fished for oil, skin, flesh and fins, and targeted by fishermen due to their aggressive appearance (Department of the Environment 2014). While there are no robust historical records of grey nurse shark population sizes, a reduction in the number caught as bycatch and in shark meshing programmes since the 1930s indicates that the population has decreased substantially (Department of the Environment 2014). As such, contemporary N_e estimates can be used to gain information on the effects of recent anthropogenic-related population decline on genetic variation. However, due to the long generation time and longevity of grey nurse sharks, it is likely that we have not yet seen the effects of a recent population bottleneck on genetic variation (Stow et al. 2006). Nevertheless, forward simulations that modelled the effects of future genetic drift at different N_e values predicted that genetic diversity of the east coast population will erode over the next 50 generations.

The conservation of east Australian grey nurse sharks needs to consider the impact of some forms of recreational diving, relevant fishing practices and the use of shark nets (Bansemer & Bennett 2010, Robbins et al. 2013). Accidental capture of grey nurse sharks occurs, and aside from direct mortalities from capture and stress, many individuals are observed retaining fishing gear which could cause delayed fatality from punctured organs (Bansemer & Bennett 2010, Robbins et al. 2013). Hook and line fishing was listed as a key threatening process to grey nurse sharks in 2002 (Department of the Environment 2014). Approximately 12 individuals are killed every year through recreational fishing in south-east Australia, and it is likely that many more deaths go unreported (Otway et al. 2004, 2011). Between 2002 and 2007, 23 grey nurse sharks were killed as a consequence of commercial fishing bycatch in NSW, while between 2007 and 2012, 5 interactions were reported (Department of the Environment 2014). Due to the low fecundity, long generation time and longevity of the grey nurse shark, these mortalities could have severe repercussions for the long-term viability of this population (Otway et al. 2004).

Grey nurse shark mortalities occur despite the areas designated to protect the sharks, e.g. critical habitat areas, fishing closures and Marine National Park Zones that are spread along the east coast of Australia (Department of the Environment 2014). In part, this is because grey nurse sharks are still exposed to anthropogenic activities, and potential mortality, when moving among these protected areas and among aggregation sites (Bansemer & Bennett 2011, Otway & Ellis 2011). We have shown that the current size of the east coast population is insufficient to prevent the erosion of genetic variation, thus highlighting the need to further reduce human-induced mortality. Consequently, our work adds to the body of research that concludes that the conservation of grey nurse sharks in eastern Australia requires a further reduction of mortality (Otway et al. 2004, Stow et al. 2006, Bansemer & Bennett 2010). In this respect, we emphasise that further consideration needs to be given to threat mitigation outside of protected areas.

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Appendix. Additional data

Table A1. Results from the sibship reconstruction in Colony2, showing the location and date of sampling of the related grey nurse sharks (ID1 and ID2), whether they are classified as first- or second-degree relatives, and the approximate distance between their sampling locations. Dates are given as d-mo-yr. Dashes (–) represent missing data; QLD: Queensland; NSW: New South Wales; NA: data not available

ID1	Sampled location	Date	ID2	Sampled location	Date	Degree	Distance (km)
9	Flat Rock, QLD	10-03-01	31	Fish Rock, NSW	11-11-03	First	410
10	Flat Rock, QLD	20-08-99	45	Forster, NSW	09-06-04	First	562
60	NSW	–	62	NSW	–	First	NA
60	NSW	–	63	NSW	–	First	NA
62	NSW	–	63	NSW	–	First	NA
14	South Solitary Island, NSW	22-09-06	26	South Solitary Island, NSW	01-12-06	Second	0
32	Fish Rock, NSW	23-10-03	27	Fish Rock, NSW	01-12-06	Second	0
28	Fish Rock, NSW	01-12-06	22	Fish Rock, NSW	22-05-07	Second	0
14	South Solitary Island, NSW	22-09-06	22	Fish Rock, NSW	22-05-07	Second	80
26	South Solitary Island, NSW	01-12-06	22	Fish Rock, NSW	22-05-07	Second	80
46	Forster, NSW	19-01-02	49	Wattamolla Beach, NSW	18-12-07	Second	280
43	Forster, NSW	26-06-06	57	Tollgate Islands, NSW	16-05-02	Second	500
57	Tollgate Islands, NSW	16-05-02	61	NSW	–	Second	NA

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