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Evidence for reproductive philopatry in the bull shark *Carcharhinus leucas*

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Reproductive philopatry in bull sharks *Carcharhinus leucas* was investigated by comparing mitochondrial (NADH dehydrogenase subunit 4, 797 base pairs and control region genes 837 base pairs) and nuclear (three microsatellite loci) DNA of juveniles sampled from 13 river systems across northern Australia. High mitochondrial and low microsatellite genetic diversity among juveniles sampled from different rivers (mitochondrial $\phi_{ST} = 0{\cdot}0767$, $P < 0{\cdot}05$; microsatellite $F_{ST} = -0{\cdot}0022$, $P > 0{\cdot}05$) supported female reproductive philopatry. Genetic structure was not further influenced by geographic distance ($P > 0{\cdot}05$) or long-shore barriers to movement ($P > 0{\cdot}05$). Additionally, results suggest that *C. leucas* in northern Australia has a long-term effective population size of 11 000–13 000 females and has undergone population bottlenecks and expansions that coincide with the timing of the last ice-ages.

Key words: estuary; microsatellites; mitochondria; northern Australia population; predator; structure.

INTRODUCTION

Philopatry occurs when an animal stays in or returns to a specific location (Mayr, 1963; Heist, 2004; Duncan *et al*., 2006). There are many types of this behaviour in sharks, although reproductive philopatry may be considered as one of the most important behaviours (Speed *et al*., 2010). This occurs when adults return to specific nurseries to either mate or give birth (Feldheim *et al*., 2004; Hueter *et al*., 2005). Philopatry in sharks has challenged the assumption that populations lack genetic subdivision due to high mobility and distances travelled by adults (Hueter *et al*., 2005);

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however, at present field, evidence for this behaviour is very limited (Chapman *et al*., 2009; Speed *et al*., 2010).

Reproductive philopatry has significant implications for species management. If local extinctions of philopatric species occur, the chance of recovery is greatly reduced as the likelihood of an individual re-utilizing an area is not random (Hueter *et al*., 2005). For this reason, each region must be managed as a unique population in order to ensure that habitat degradation or fishing (artisanal, recreational and commercial) does not significantly affect local biodiversity. Furthermore, restricted gene flow increases the genetic diversity of the meta-population; local extinctions that reduce this diversity can weaken the adaptive potential of a species (Avise *et al*., 1987). Where such diversity is created by reproductive philopatry, the need to manage a species at relatively small spatial scales may not be immediately evident, as adults can be distributed ubiquitously outside natal areas, giving a false impression of abundance and genetic connectivity.

This study investigates reproductive philopatry in the bull shark *Carcharhinus leucas* (Müller & Henle 1839) across northern Australia. The species is a large (>3400 mm total length, L_T) apex predator, common in shallow tropical and subtropical waters globally (Hueter *et al*., 2005; Last & Stevens, 2009). Despite their affinity for shallow water, adults are capable of large-scale movements and have been tracked on migrations of over 1500 km in the Gulf of Mexico (Brunnschweiler *et al*., 2010; Carlson *et al*., 2010). For this reason, it has been assumed that there is little likelihood of genetic structuring within populations (Kitamura *et al*., 1996; Ward, 2000; Karl *et al*., 2011). Karl *et al*. (2011), however, recently identified restricted habitat use (philopatry, not reproductive philopatry) in females in the western Atlantic Ocean confirming complex patterns of habitat use in *C. leucas*.

Carcharhinus leucas are designated as near threatened by the IUCN due to the close proximity of critical habitats (estuaries and rivers) to anthropogenic influences (IUCN, 2010). Northern Australia is an ideal study location to resolve fine-scale population structure in this species, as the region has very little urbanization of coastlines and river systems including low levels of fishing pressure. This provides an opportunity to study a shark population likely to be far less disturbed by anthropogenic influences than in most other places within its range.

Little is known about the reproductive biology of *C. leucas*, but catch data and anecdotal evidence suggest that gestation requires *c*. 10–11 months and litters range in size from six to 12 pups (Last & Stevens, 2009). The frequency of reproductive cycles is still unknown. Females return to freshwater and estuarine nursery grounds to pup (Last & Stevens, 2009). Mature *C. leucas* (>2 m L_T) found in rivers are rarely males (Montoya & Thorson, 1982; Snelson *et al*., 1984; Last & Stevens, 2009), suggesting females use this part of their range for parturition. After pupping, rivers are used as nurseries for *C. leucas* (Thorburn & Rowland, 2008). Juveniles reside in these areas for *c*. 4 years (Thorburn & Rowland, 2008) and the repeated use of these sites across multiple years are consistent with the definition of nurseries proposed by Heupel *et al*. (2007). Research to date has not addressed whether the use of nurseries over multiple breeding cycles generates population genetic structure in *C. leucas* (reproductive philopatry).

The extent of reproductive philopatry of *C. leucas* was tested by comparing the diversity of two genetic markers (mitochondrial DNA and microsatellites) in juveniles sampled from nurseries within rivers and their associated estuarine systems

across northern Australia. With an appropriate sampling strategy of nurseries, comparisons of genetic structure identified in these two types of DNA can be used to investigate sex-specific patterns of habitat use within the population. For example, genetic population structure identified in mitochondrial (mt) DNA (maternally inherited) that is not present in microsatellites (bi-parentally inherited) can indicate female philopatry suggesting male-mediated dispersal. The aims were to determine: (1) whether population structure exists between closely located nurseries and if this structure is shaped by sex-specific movement patterns, (2) whether this structure is influenced by either geographic distance or long-shore barriers to movement such as changes in substrate (phylogeographic patterns), (3) whether populations across northern Australia have been influenced by sea-level changes in the Pleistocene epoch and (4) estimate long-term effective population size of *C. leucas* in northern Australia.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PRESERVATION

Tissue samples of *C. leucas* were obtained from commercial fishers and on-board scientific observers operating along the Northern Territory, Australia, coastline in 2009, and during fishery-independent surveys across northern Australia between 2002 and 2009. Samples were collected from 13 northern Australian river systems including the Fitzroy, Robinson, Mitchell and Ord Rivers from Western Australia; the Daly, East Alligator, Roper, Towns, Limmen and Robinson Rivers from the Northern Territory and from the Mitchell, Mission and Wenlock Rivers in north Queensland. The Department of Fisheries of the Northern Territory provided additional samples collected from Blue Mud Bay and to the north of Tiwi Islands in the Northern Territory (Fig. 1). All capture locations, apart from samples collected to the north of the Tiwi Islands (Darwin coastal), were nursery areas. All individuals caught within rivers were juveniles (<1200 mm L_T) with a mean ± S.D. size of 900 ± 155 mm (*c.* 3 years of age) (Thorburn & Rowland, 2008; Tillett *et al.*, 2011). Each sample consisted of *c.* 5 g of white muscle tissue that was preserved in either 95% ethanol solution or 10% DMSO (dimethylsulphoxide in saturated 5 M NaCl solution). Sample sizes and locations are shown in Fig. 1.

Due to the physical similarities among many *Carcharhinus* spp., and the presence of numerous congener species in the region, mtDNA *nd4* and control region sequences were compared with reference samples and where possible type specimens obtained from the Northern Territory Museum and laboratory collections. Sequences from this study were compared against known sequences of the sandbar *Carcharhinus plumbeus* (Nardo 1827), whitecheek *Carcharhinus dussumieri* (Müller & Henle 1839), bignose *Carcharhinus altimus* (Springer 1950), common blacktip *Carcharhinus limbatus* (Müller & Henle 1839), Australian blacktip *Carcharhinus tilstoni* (Whitley 1950), graceful *Carcharhinus amblyrhychoides* (Whitley 1934), pig-eye *Carcharhinus amboinensis* (Müller & Henle 1839), spinner *Carcharhinus brevipinna* (Müller & Henle 1839) and spot-tail sharks *Carcharhinus sorrah* (Müller & Henle 1839). Where possible, species identifications were verified by comparison to sequences on GenBank.

GENOMIC DNA EXTRACTION

Total genomic DNA was extracted from 50 mg of preserved tissue using the Chelex method (Walsh *et al.*, 1991; Estoup *et al.*, 1996). Tissue was placed in a small vial containing a 200 µl solution of 10% Chelex 100 (Bio-Rad Inc.; http://www.bio-rad.com/webmaster/pdfs/9184_Chelex.PDF) in TE buffer (5 mM Tris-HCl pH 8·0 with 0·5 mM EDTA). The enzyme proteinase K (100 ng) was then added (5 µl) to the vial producing a final concentration of 2·4 ng,

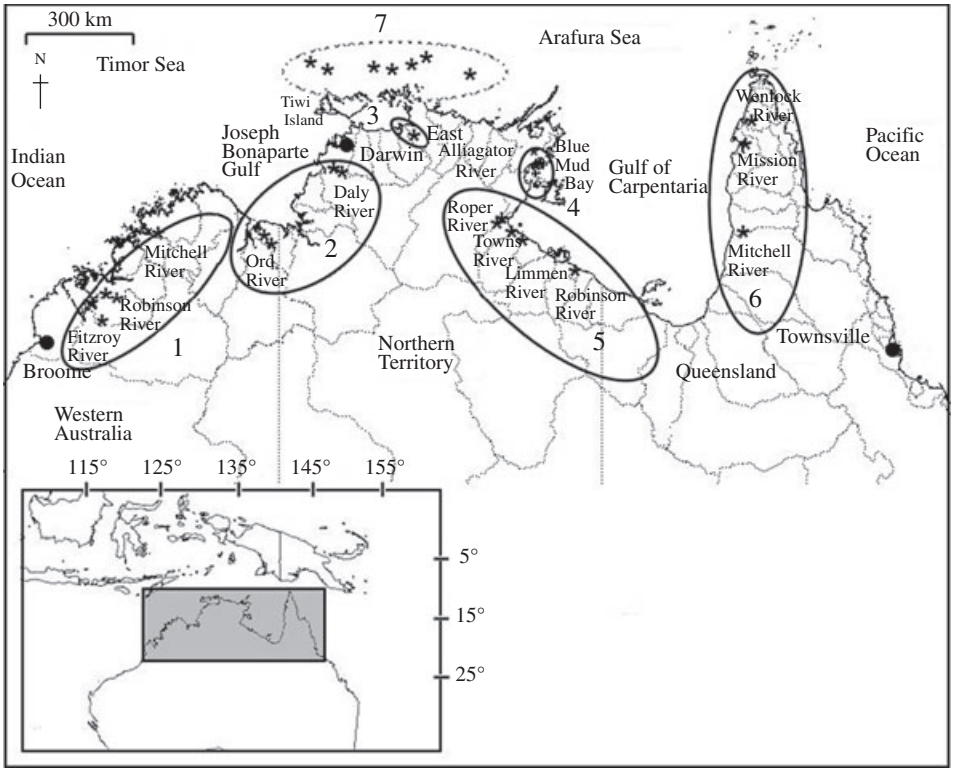

FIG. 1. *Carcharhinus leucas* ($n = 169$) capture locations: group 1, Fitzroy, Robinson and Mitchell Rivers, Western Australia (WA) ($n = 15$); group 2, Ord River, WA, and Daly River, Northern Territory (NT) ($n = 44$); group 3, East Alligator River, NT ($n = 22$); group 4, Blue Mud Bay, NT ($n = 18$); group 5, Roper, Towns, Limmen and Robinson Rivers ($n = 27$), NT; group 6, Mitchell, Wenlock and Mission Rivers, Queensland ($n = 17$); group 7, Darwin coastal ($n = 26$).

and heated at 55° C for 3 h on a shaking platform to facilitate tissue digestion. The mixture was then boiled for 8 min and centrifuged at 13 000 *g* for 5 min to precipitate the Chelex resin and bind polyvalent metal ions from the denatured DNA in solution. The supernatant containing the extracted DNA was transferred to a fresh vial for manipulation and storage.

AMPLIFICATION AND SEQUENCING OF MTDNA

The mitochondrial control region and nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (*nd4*) genes were amplified from 169 individual *C. leucas* using polymerase chain reaction (PCR) and sequenced. The 5′ end of the *nd4* gene was amplified and sequenced using the forward primer, *nd4* (CACCTATGACTACCAAAAGCTCATGTAGAAGC) (Arevalo *et al*., 1994), and the reverse primer, H12293-LEU (TTGCACCAAGAGTTTTTGGTTCCTAAGACC) (Inoue *et al*., 2001). Amplification reactions were performed using 20 µl PCR reaction mixtures containing 11·85 µl of demineralized water, 2 µl of 10× PCR reaction buffer containing 15 mM $MgCl_2$, 2 µl of 2·5 mM deoxynucleotide triphosphate (dNTP) mix, 1 µl of each 10 µM primer, 0·75 units of *Taq* DNA polymerase (Sigma Aldrich; www.sigmaaldrich.com) and 2 µl of DNA template. Thermocycling conditions included an initial denaturation step of 94° C for 1 min followed by 30 cycles of a denaturing step at 94° C for 30 s, an annealing step at 50° C for 30 s and an extension step at 72° C for 30 s. A final extension step of 5 min at 72° C completed the thermocycling.

PCR products were purified using commercial QIAquick PCR purification kits (Qiagen; www.qiagen.com) and viewed on a 1·5% agarose TAE (containing Tris base, acetic acid and EDTA) gel stained with ethidium bromide. Cycle sequencing reactions used ABI Big Dye Terminator v3.1® (www.appliedbiosystems.com). Fragment separation was carried out by capillary electrophoresis (Applied Biosystems 3130xl; www.appliedbiosystems.com) under conditions recommended by the manufacturer producing 797 base pairs of sequence.

The 5′ end of the control region was amplified using the forward primer GWF (CTGCCCTTGGCTCCCAAAGC) (Pardini *et al*., 2001) and a reverse primer that was designed from preliminary *C. leucas* sequence CL2 (GGAAAAATATACGTCGGCCCTCG). The primer was designed using PRIMER3 v0.4.0 (Rozen & Skaletsky, 2000). Amplification reactions consisted of 20 µl PCR reaction mixtures containing 11·77 µl of demineralized water, 1·28 µl of 2·5 mM dNTP mix, 2 µl of 10× PCR reaction buffer containing 15 mM $MgCl_2$, 0·6 µl of each 10 µM primer, 1·6 µl of 25 mM $MgCl_2$, 0·75 units of *Taq* DNA polymerase (Sigma Aldrich) and 2 µl of DNA template. Thermocycling conditions consisted of an initial denaturation step of 94° C for 1 min and 30 s followed by 35 cycles of a denaturation step at 94° C for 10 s, an annealing step at 59° C for 30 s and an extension step at 72° C for 1 min. A final extension step of 5 min at 72° C completed the thermocycling. PCR products were purified following the same protocol used for the *nd4* gene. Cycle sequencing reactions and fragment separation also followed the same procedures as the *nd4* gene, although *C. leucas* control regions were sequenced with the designed internal reverse primer CLR4 (ATTTCTTTCCAAACTGGGGGAGTC). Again, this primer was designed using PRIMER3 v0.4.0 (Rozen & Skaletsky, 2000). A fragment of 837 base pairs was produced.

AMPLIFICATION AND GENOTYPING OF MICROSATELLITES

Samples were screened for 14 and genotyped for three microsatellite loci developed for shark species other than *C. leucas* (Ovenden *et al*., 2006; Portnoy *et al*., 2007). Loci were selected based on their successful cross-species amplification (Ovenden *et al*., 2006) and the highest number of polymorphic alleles between distant phylogenetic clades. Amplification was achieved using PCR methods. Reaction mixtures (total volume of 6 µl) contained 1·18 µl of milli-Q water; 3 µl of 2× Qiagen Multiplex PCR Master Mix containing a pre-optimized mix of *Taq* DNA polymerase, dNTPs and providing a final concentration of 6 mM $MgCl_2$; 0·02 µl of 10 µM forward primer with an M13 extension (Schuelke, 2000); 0·2 µl of 10 µM reverse primer; 0·1 µl of fluoro-labelled M13 primer; 1 µl of DNA template (12–40 ng) and 0·6 µl of 5× Q-solution (Qiagen). The DNA template and reaction mix were initially denatured at 95° C for 15 min and then underwent 37 cycles of a denature period at 94° C for 30 s, an annealing period with loci-specific temperatures of 50, 52 and 58° C for loci *CPL-90*, *CS-08* and *CPL-166*, respectively, for 45 s and an extension time of 72° C for 1 min and 30 s. The thermocycling was completed with a final extension time of 72° C for 45 min. Loci were individually amplified but subsequently combined for fragment separation according to label colour and fragment size. Microsatellite fragment separation and scoring were performed using capillary electrophoresis (ABI3130xl; Applied Biosystems). The size of microsatellite amplicons (in base pairs) was calculated to two decimal places and amplicons were allocated to a group that represented the mean allele size.

POPULATION STRUCTURE AND PHILOPATRY

Population structure and the influence of sex-specific movement patterns were determined by: (1) comparing the genetic differences identified in mtDNA and microsatellite (ms) DNA and (2) comparing the relatedness of juveniles within and between each nursery.

mtDNA control region and *nd4* sequences were aligned and edited individually using the software Geneious v4.65 (Drummond *et al*., 2009). No premature stop codons were identified in the protein coding *nd4* gene. Identical sequences were condensed into unique haplotypes by eye and then confirmed using Arlequin v3.11 (Excoffier *et al*., 2005) and MEGA 4.0 (Kumar *et al*., 2008) softwares. Diversity indices for each capture location were estimated and compared. These included haplotype diversity (h) (likelihood of randomly choosing two different haplotypes from the one population), nucleotide diversity (π) (likelihood that two

homologous base positions from two different haplotypes from the same population were different) and the number of polymorphic sites (Tajima, 1983; Nei, 1987). The best fit model of nucleotide substitution, and its associated gamma shape used to estimate the molecular evolution of gene regions, was determined by performing hierarchical likelihood ratio test and by calculating approximate Akaike Information Criteria (AIC) using MrModelTest v2.2 (Posada & Crandall, 1998) implemented in PAUP 4.0b10 (Swofford, 2000).

Connectivity between capture locations was subsequently assessed using F-statistics by a series of pair-wise comparisons and analysis of molecular variance (AMOVA) using Arlequin v3.11 (Excoffier *et al*., 2005). To avoid possible skewing of genetic distance estimates due to the different molecular evolution of some haplotypes within rivers, traditional F_{ST} measures that do not incorporate molecular evolution of haplotypes were used (Wright, 1984).

To increase haplotype diversity within capture locations, both genes were concatenated. This was supported by the total linkage of the genes due to their common origin within the mtDNA genome. Darwin coastal ($n = 26$), north of the Tiwi Islands, was the only capture location that was not a juvenile nursery (solely adults captured), and as such was omitted from population structure assessment, but was included in phylogeographic reconstructions as a unique haplotype was identified in this location, and relatedness estimates. Juveniles were assumed not to move between rivers as has been documented in tracking studies in Calaoosahatchee River, south-west Florida, U.S.A. (Heupel & Simpfendorfer, 2008; Heupel *et al*., 2010). Thus, it is assumed that any identified structure is influenced by female movements to pupping areas rather than juvenile dispersal.

Sample sizes in some capture locations were low. To ensure that results were not confounded by this, F-statistics were initially compared between individual capture locations then those locations with low sample sizes were pooled. Two types of pooling strategies were used: (1) by state divisions (Western Australia, Northern Territory and Queensland) to coincide with fisheries jurisdictions and (2) by geographical proximities (calculated as km by sea between rivers) and genetic similarities (based on unpooled pair-wise F_{ST} comparison) ensuring genetic relationships between capture locations with larger sample sizes were not altered. F-statistics confirmed which grouping supported unpooled genetic structure. AMOVA then tested the hierarchical contribution of variance and that no significant genetic differences existed within groups. This grouped Fitzroy, Robinson and Mitchell Rivers (group 1) all opening to the Indian Ocean along the Western Australian coastline; the Ord and Daly Rivers (group 2) within the Joseph Bonaparte Gulf; the East Alligator River (group 3) in the Van Diemen Gulf; Blue Mud Bay (group 4) was genetically distinct from the East Alligator River and rivers to the east and as such not pooled; Roper, Towns, Limmen and Robinson Rivers (group 5) on the western side of the Gulf of Carpentaria; Mitchell, Mission and Wenlock Rivers (group 6) on the eastern side of the Gulf of Carpentaria and the adult population, Darwin coastal (group 7).

The null hypothesis of Hardy–Weinberg equilibrium (HWE) in msDNA was tested using Arlequin v3.11 (Excoffier *et al*., 2005) and GenAlEx v6.1 (Peakall & Smouse, 2005). In addition, the software, Microchecker v2.2.3 (van Oosterhout *et al*., 2004) was implemented to identify possible causes for any deviations from HWE. Microsatellite genetic diversity was characterized by the number of alleles per locus (N_a), expected (H_e) and unbiased (H_{ue}) heterozygosity, observed heterozygosity (H_o) and fixation index (F) using Arlequin v3.11 (Excoffier *et al*., 2005) and GenAlEx v6.1 (Peakall & Smouse, 2005). The probability of rejecting the null hypothesis of genotypic disequilibrium between pairs of loci across populations was estimated by Arlequin v3.11 (Excoffier *et al*., 2005). Population structure identified with mtDNA was tested by pair-wise comparisons and AMOVA following above protocols.

If females are mating elsewhere and returning to the same location following each gestation cycle to pup, then juveniles from the same location should be more closely related to each other than with individuals from surrounding areas. Relatedness among individuals within and between capture locations was estimated using the software M-L relate (Kalinoswiki *et al*., 2006). This software calculates the likelihood that each pair of individuals are full-siblings, half-siblings, parent-offspring or unrelated and then reports the relationship that has the highest likelihood. The average per cent of each category within and between each pooled nurseries was then calculated and compared.

INFLUENCE OF GEOGRAPHIC DISTANCE AND LONG-SHORE BARRIERS TO MOVEMENT ON POPULATION STRUCTURE

Observed genetic divergence among haplotypes present in Western Australia and north Queensland raised the question whether genetic structure was driven by isolation by distance. This was tested by correlating the genetic distance (F_{ST}) between all un-pooled capture locations with the geographical distance (km) by sea. Genetic distances were calculated using Arlequin v3.11 (Excoffier *et al*., 2005).

Long-shore barriers impeding the return of females to nursery areas, such as areas of sub-optimal habitats, could also create genetically distinct sub-groups (Frankham *et al*., 2002). Diverse inshore environments exist across northern Australia with the north-west (the coastline of tropical Western Australia) characterized by large areas of continental shelf and slope, highly variable tidal regimes and influenced by complex ocean currents (Australian Government Department of the Environment, 2008*a*). The coastline of the Northern Territory and Gulf of Carpentaria is characterized by shallow tropical ecosystems with water depths generally <70 m (Australian Government Department of the Environment, 2008*b*). The influence of the substratum as defined by the Australian Government Department of the Environment was tested by comparing the phylogenetic relationship between nurseries with similar substrata and AMOVA pooling nurseries by substratum type.

Phylogeographic patterns were investigated by reconstructing intraspecific phylogenies among unique mtDNA haplotypes and relating these to geographic locations. Both character-based (neighbour-joining and maximum parsimony) and model-based (maximum likelihood and Bayesian inference) methods were used. All analyses were performed on each gene region individually and then with the two gene regions concatenated. Mutations were unweighted and indels were treated as a fifth state. Outgroups were selected based on the availability of both *nd4* gene and control region sequences, interspecific genetic similarities and the robustness of topological alternate combinations of outgroups. Maximum likelihood and maximum parsimony analysis were performed using the software PAUP 4.0b10 (Swofford, 2000) and Bayesian inference was performed using the software MrBayes v3.1 (Huelsenbeck & Ronquist, 2001). Concatenated sequences were partitioned for Bayesian inferences accommodating different models of evolution for each gene region. Priors for maximum likelihood and Bayesian inference were determined by performing hierarchical likelihood ratio test and by calculating AIC using the software MrModelTest v2.2 (Posada & Crandall, 1998). Heuristic tree searches were performed with 1000 random addition replications and the statistical support for nodes was determined *via* 1000 non-parametric bootstrap replicates. A majority-rule consensus tree was also constructed based on the 1000 bootstrap replicates. Bayesian inference was run using the Metropolis-coupled Markov Chain Monte-Carlo (MCMC) algorithm from randomly generated starting trees for 3×10^6 generations, sampling trees every 1000 generations. Two simultaneous runs were performed with three heated chains and one cold chain each with a temperature parameter of 0·2. The S.D. of split frequencies was used as a convergence diagnostics to determine when posterior probability distribution had reached stationarity. The burn-in was set to discard the initial 25% of samples following guidelines outlined in the manual. Only Bayesian trees are presented. In addition to conventional phylogenetic reconstructions, statistical parsimony networks (TCS) were also generated (Clement *et al*., 2000). Unlike traditional methods, parsimony networks assume that the ancestral haplotype is present in the current sample, incorporates homoplasy and is not limited to bifurcation at branch nodes. Gaps were again treated as a fifth state and the connection limit was set to 95%.

INFLUENCE OF PLEISTOCENE SEA-LEVEL CHANGES ON POPULATION STRUCTURE

Phylogenetic reconstructions demonstrated a single clade across northern Australia; therefore, influences of Pleistocene sea-level changes, such as regional population expansion, were determined by calculating Tajima's D and Fu's F_S on all capture locations pooled as one population using Arlequin v3.11 (Excoffier *et al*., 2005). Tajima's D compares estimates of the mutational parameter (θ) based on the number of polymorphic sites and the mean

number of pair-wise differences (Tajima, 1983, 1996). Significant differences in these estimates confirm deviation of population equilibrium. Whereas Fu's F_S calculates the probability of observing k or less alleles in a neutral population based on the observed average number of pair-wise differences (Fu, 1997). Significant positive and negative values for both statistics may be indicative of population bottlenecks or expansions, respectively (Ramos-Onsins & Rozas, 2002).

ESTIMATE OF LONG-TERM EFFECTIVE POPULATION SIZE

The mismatch distribution was also estimated under the sudden expansion model and used to estimate τ and θ (Schneider & Excoffier, 1999). These values were subsequently used to roughly estimate time since expansion assuming a divergence rate of 0·67–0·80% (10^6 years^{-1}), which was calculated from molecular clock estimates for the control region of other carcharhinids (Duncan *et al*., 2006; Schultz *et al*., 2008); and the long-term effective population size (Gaggiotti & Excoffier, 2000) defined as the number of individuals that would give rise to a loss in genetic diversity at the same rate as the actual population (Frankham *et al*., 2002).

RESULTS

POPULATION STRUCTURE AND PHILOPATRY

Six mitochondrial *nd4* and 13 control region haplotypes were described across northern Australia. Models of nucleotide substitution were generalised time reversible model (GTR) and Hasegawa, Kishino and Yano (HKY)+I for *nd4* and the control region, respectively. Neither of these genes required gamma corrections. Concatenating these two regions increased the number of haplotypes to 18 (Table I). (see Tables SI–IV, Supporting Information for haplotype summary tables for individual gene regions).

Significant mtDNA population genetic structure [adjusted for multiple comparisons by the false discovery rate method (Narum, 2006)] existed between individual nurseries ($\phi_{ST} = 0{\cdot}0767,\ P < 0{\cdot}001$), but not within (see Supporting Information for pair-wise F_{ST} comparisons between individual rivers). Genetic differences were not congruent with fisheries jurisdictions ($\phi_{ST} = -0{\cdot}0602,\ P > 0{\cdot}05$). F-statistics validated the pooling strategy.

Haplotype frequencies differed among pooled nurseries (Table I). Haplotype CLEU_CN01 was dominant in all locations representing 30–90% of haplotype diversity. Conversely, most other haplotypes were only present in one or two locations mostly due to their relative rarity. Both nucleotide and haplotype diversity varied between pooled nurseries measuring the lowest in group 4 (mean ± S.E. $h = 0{\cdot}1111 \pm 0{\cdot}0964$, $\pi = 0{\cdot}0068 \pm 0{\cdot}0129$) and highest in group 2 (mean ± S.E. $h = 0{\cdot}8531 \pm 0{\cdot}0402$, $\pi = 0{\cdot}1514 \pm 0{\cdot}093$). Differences in genetic diversity between nurseries indicated by significant F_{ST} values (adjusted for multiple comparisons by the false discovery rate method) were recorded between pair-wise comparisons (Table II). The greatest pair-wise F_{ST} value was measured between groups 4 and 2 ($F_{ST} = 0{\cdot}217$). The lowest significant F_{ST} value was recorded between groups 5 and 2 ($F_{ST} = 0{\cdot}044$). The most similar nurseries were groups 4 and 1, although groups 1 and 3 and groups 6 and 3 were also not distinctly different (Table II).

The unbiased heterozygosity in msDNA was $0{\cdot}796 \pm 0{\cdot}0434$. The mean ± S.E. number of alleles was $4{\cdot}833 \pm 0{\cdot}307$ for locus *CPL-166*, $18{\cdot}333 \pm 1{\cdot}801$ for *CPL-90*

TABLE I. Concatenated mtDNA nicotinamide adenine dinucleotide (NADH) dehydrogenase 4 (*nd4*) (797 bases) and control region haplotypes (837 bases) total $n = 169$, total bases = 1634; including numbered polymorphic sites, haplotype frequencies, shared haplotypes and indices of population diversity for *Carcharhinus leucas* from seven sampling areas in northern Australia. Nurseries (excluding group 7) are pooled by geographic distances and genetic similarities; sample sizes for each grouping are given*

Haplotypes	2 7	4 0 8	4 1 2	4 2 3	5 9 7	6 2 1	6 2 4	6 5 1	9 6 4	9 9 6	1 0 6 8	1 1 9	1 1 6 8	1 2 0 5	1 2 2 8	1 2 4 2	1 3 9 9	1 5 0 0	1 5 4 5	1 5 6 2	Group 1 (Fitzroy, Robinson and Mitchell Rivers, $n = 15$)	Group 2 (Daly and Ord Rivers, $n = 44$)	Group 3 (East Alligator River, $n = 22$)	Group 4 (Blue Mud Bay, $n = 18$)	Group 5 (Roper, Towns, Limmen and Robinson Rivers, $n = 27$)	Group 6 (Mitchell, Mission and Wenlock Rivers, $n = 17$)	Group 7 (Darwin coastal, NT, $n = 26$)
CLEU_CN01	C	T	T	T	T	C	C	T	G	T	G	T	T	C	A	T	G	A	G	A	0·867	0·341	0·682	0·944	0·556	0·647	0·692
CLEU_CN02	T	C	*	C	C	*	T	C	*	*	*	*	*	*	*	*	A	*	*	*	0·067	—	—	—	—	—	—
CLEU_CN03	*	*	C	*	*	*	*	*	*	*	A	*	*	*	*	*	A	*	*	*	0·067	0·114	0·227	—	—	—	0·039
CLEU_CN04	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A	C	*	*	—	0·023	—	—	0·037	—	—
CLEU_CN05	*	*	C	*	*	*	*	*	A	C	*	*	G	*	G	*	A	C	*	*	—	0·068	0·046	—	0·037	—	—
CLEU_CN06	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	—	0·114	0·046	—	—	0·294	0·077
CLEU_CN07	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	—	0·091	—	0·056	0·037	—	0·115
CLEU_CN08	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	—	0·046	—	—	—	—	—
CLEU_CN09	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	A	*	*	*	—	0·023	—	—	—	—	—
CLEU_CN10	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A	T	*	*	—	0·023	—	—	—	—	—
CLEU_CN11	*	*	C	*	*	*	*	*	*	C	A	*	*	*	*	*	A	*	*	*	—	0·068	—	—	—	—	—
CLEU_CN12	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	—	0·023	—	—	—	—	—
CLEU_CN13	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	—	—	—	—	—	0·059	0·039
CLEU_CN14	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	—	0·046	—	—	0·222	—	—
CLEU_CN15	?	*	C	*	*	*	*	*	A	C	*	*	G	*	G	C	A	C	*	*	—	—	—	—	0·037	—	—
CLEU_CN16	*	*	*	*	*	*	*	*	A	*	*	*	*	*	G	*	*	*	A	*	—	—	—	—	0·037	—	—

TABLE I. Continued

Haplotypes	2 7	4 0 8	4 1 2	4 2 3	5 9 7	6 2 1	6 2 4	6 5 1	9 6 4	9 9 6	1 0 6 8	1 1 1 9	1 1 6 8	1 2 0 5	1 2 2 8	1 2 4 2	1 3 9 9	1 5 0 0	1 5 4 5	1 5 6 2	Group 1 (Fitzroy, Robinson and Mitchell Rivers, $n = 15$)	Group 2 (Daly and Ord Rivers, $n = 44$)	Group 3 (East Alligator River, $n = 22$)	Group 4 (Blue Mud Bay, $n = 18$)	Group 5 (Roper, Towns, Limmen and Robinson Rivers, $n = 27$)	Group 6 (Mitchell, Mission and Wenlock Rivers, $n = 17$)	Group 7 (Darwin coastal, NT, $n = 26$)
CLEU_CN17	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	—	0·023	—	—	0·037	—	—
CLEU_CN18	*	*	*	*	*	*	T	*	*	*	*	*	*	*	G	*	*	*	*	*	—	—	—	—	—	—	0·039
Number of haplotypes																					3	13	4	2	8	3	6
Number of polymorphic sites																					9	12	9	1	12	3	7
Nucleotide diversity per location (within population, %) (mean ± S.E.)																					0·082 ± 0·060	0·151 ± 0·093	0·111 ± 0·075	0·007 ± 0·013	0·122 ± 0·079	0·061 ± 0·049	0·055 ± 0·044
Haplotype diversity per location (within populations) (mean ± S.E.)																					0·257 ± 0·142	0·853 ± 0·040	0·502 ± 0·105	0·111 ± 0·096	0·658 ± 0·087	0·522 ± 0·101	0·517 ± 0·113

*Note: CLEU_CN18 was only present in low frequencies in the adult population, Darwin coastal, as such it is only included in phylogenetic reconstructions. See Supporting Information for haplotype GenBank accession numbers.

TABLE II. mtDNA pair-wise F_{ST} values between pooled nurseries for *Carcharhinus leucas* from seven sampling areas in northern Australia (total $N = 143$). Nurseries are pooled based on geographic distances and genetic similarities; sample sizes for each grouping are given

	Group 1 (Fitzroy, Robinson and Mitchell Rivers, $n = 15$)	Group 2 (Ord and Daly Rivers, $n = 44$)	Group 3 (East Alligator River, $n = 22$)	Group 4 (Blue Mud Bay, $n = 18$)	Group 5 (Roper, Towns, Limmen and Robinson Rivers, $n = 27$)	Group 6 (Mitchell, Mission and Wenlock Rivers, $n = 17$)
Group 1		0·154	0·028	−0·010	0·096	0·109
Group 2	<0·01		0·066	0·217	0·044	0·064
Group 3	>0·05	<0·05		0·127	0·062	0·062
Group 4	>0·05	<0·001	<0·05		0·159	0·190
Group 5	<0·05	<0·05	<0·05	<0·01		0·075
Group 6	>0·05	<0·05	>0·05	<0·05	<0·05	

*F_{ST} values are above the diagonal and P-values are below the diagonal. Significant values corrected for multiple comparison by false discovery rate are indicated in bold.

and $17{\cdot}167 \pm 1{\cdot}956$ for *CS-08* (Table III). None of the capture locations deviated from HWE or showed evidence of non-random association of alleles. The population structure evident in pooled nursery groups from their mtDNA was not present in msDNA (overall individual $F_{ST} = -0{\cdot}0022$, $P > 0{\cdot}05$; overall pooled $F_{ST} = 0{\cdot}0056$, $P > 0{\cdot}05$) or evident in any loci individually (*CPL-166*: $F_{ST} = -0{\cdot}008$, $P > 0{\cdot}05$; *CPL-90*: $F_{ST} = 0{\cdot}0125$; *CS08*: $F_{ST} = 0{\cdot}0062$, $P > 0{\cdot}05$). Relatedness between juveniles indicated by the maximum likelihood that two individuals were unrelated, half-siblings, full-siblings or parent-offspring was no greater within than between pooled locations (see Supporting Information).

INFLUENCE OF GEOGRAPHIC AND LONG-SHORE BARRIERS TO MOVEMENT ON POPULATION STRUCTURE

There was no evidence of isolation by distance between nurseries ($P > 0{\cdot}05$). Phylogenetic reconstruction based on the *nd4* gene grouped all six haplotypes within one clade (see Supporting Information). Reconstruction based on the control region did not produce well-supported clades (see Supporting Information). Concatenating sequences slightly improved resolution, although again did not produce well-supported clades (see Supporting Information). The 95% statistical parsimony network supported one clade structure indicated by one main haplotype and the occurrence of numerous subsequent haplotypes that are only one or a few mutational events apart (Fig. 2). Two lineages are present represented by haplotype CLEU_CN02 separated by five mutational events and haplotypes CLEU_CN05 and CLEU_CN15 separated by four mutational events. There was no evidence of phylogeographic structure which would be indicated by all haplotypes from that substratum type forming tight clusters away from other river systems (see Supporting Information for the 95% statistical parsimony network of unpooled capture locations). Pooling nurseries by substratum type did not produce significant genetic structure ($P > 0{\cdot}05$).

TABLE III. The pooled nurseries, sample size (n), number of microsatellite alleles per locus (N_a), average observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased heterozygosity (H_{ue}), fixation index (F) for six *Carcharhinus leucas* nurseries sampled in northern Australia (total $N = 146$). Nurseries are pooled based on geographic distances and genetic similarities; sample sizes for each grouping are given

	Locus	n	N_a	H_o	H_e	H_{ue}	F
Group 1 (Fitzroy, Robinson and Mitchell Rivers, $n = 15$)	CPL-166	12	4	0·333	0·462	0·482	0·278
	CPL-90	11	11	0·545	0·855	0·896	0·362
	CS-08	12	11	0·833	0·813	0·848	−0·026
Group 2 (Daly and Ord Rivers, $n = 44$)	*CPL-166*	44	6	0·523	0·463	0·468	−0·129
	CPL-90	42	22	0·857	0·922	0·933	0·071
	CS-08	44	22	0·977	0·929	0·940	−0·052
Group 3 (East Alligator River, $n = 22$)	*CPL-166*	24	5	0·625	0·563	0·575	−0·109
	CPL-90	24	23	1·000	0·943	0·963	−0·061
	CS-08	24	19	1·000	0·929	0·949	−0·077
Group 4 (Blue Mud Bay, $n = 18$)	*CPL-166*	19	5	0·526	0·630	0·647	0·165
	CPL-90	18	16	0·944	0·907	0·933	−0·041
	CS-08	19	12	0·947	0·888	0·912	−0·067
Group 5 (Towns, Roper, Limmen and Robinson Rivers, $n = 27$)	*CPL-166*	26	4	0·500	0·510	0·520	0·019
	CPL-90	26	20	0·885	0·889	0·906	0·005
	CS-08	26	22	0·962	0·939	0·958	−0·024
Group 6 (Mitchell, Mission and Wenlock Rivers, $n = 17$)	*CPL-166*	21	5	0·619	0·503	0·516	−0·230
	CPL-90	19	18	0·842	0·909	0·933	0·073
	CS-08	21	17	0·952	0·925	0·948	−0·029

INFLUENCE OF PLEISTOCENE SEA-LEVEL CHANGE ON POPULATION STRUCTURE AND ESTIMATES OF LONG-TERM EFFECTIVE POPULATION SIZE

Population expansion was evident across northern Australia (Tajima's $D = -1{\cdot}469$, $P < 0{\cdot}05$; Fu's F_S test $= -7{\cdot}490$, $P < 0{\cdot}05$). The mismatch distribution was unimodal (Fig. 3), which closely matched the expected distributions under the sudden expansion model (Harpending raggedness index $= 0{\cdot}0739$, $P > 0{\cdot}05$). The τ-value (1·986) roughly estimated 75 000–90 000 years since expansion. The θ-value (0·293) roughly estimated a female, long-term effective population size of 11 000–13 000.

DISCUSSION

This study demonstrates that population structure in mtDNA exists among juveniles sampled from different freshwater nurseries (mean ± S.D. pair-wise distance by sea between grouped nurseries = 1083 ± 556 km). In contrast, no structure in microsatellite markers of juveniles was found at the equivalent spatial scale. These results, combined with the directed sampling of nurseries and the residence of juveniles within these habitats for *c*. 4 years (Simpfendorfer *et al*., 2005; Heupel & Simpfendorfer, 2008; Yeiser *et al*., 2008; Heupel *et al*., 2010), support the hypothesis

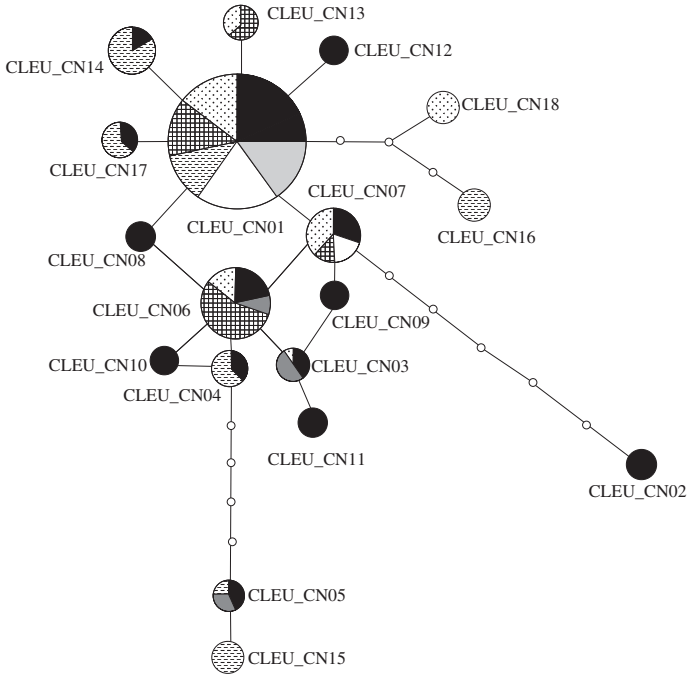

FIG. 2. Statistical parsimony network of concatenated nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (*nd4*) and control region genes of *Carcharhinus leucas* from seven areas sampled in northern Australia ($n = 169$). The size of each circle represents the relative frequency of each haplotype and the colour composition depicts the capture locations in which haplotypes were identified. Each circle represents one mutational event; small colourless checks indicate unidentified haplotypes. Group 1 (■): Fitzroy, Robinson and Mitchell Rivers, Western Australia (WA) ($n = 15$); group 2 (■): Ord River, WA and Daly River, Northern Territory (NT) ($n = 44$); group 3 (□): East Alligator River, NT ($n = 22$); group 4 (□): Blue Mud Bay, NT ($n = 18$); group 5 (⊟): Roper, Towns, Limmen, and Robinson Rivers ($n = 27$), NT; group 6 (⊞): Mitchell, Wenlock and Mission Rivers, Queensland ($n = 17$); group 7 (⊡): Darwin coastal ($n = 26$).

that identified population structure is due, at the very least, to female movement patterns, strongly suggesting reproductive philopatry. Conclusions are further supported by the absence of mature males in freshwater and estuaries nurseries (Montoya & Thorson, 1982; Snelson *et al*., 1984; McCord & Lamberth, 2009). These results are similar to studies on *C. leucas* in the western Atlantic Ocean that found restricted patterns of female habitat use, although Karl *et al*. (2011) did not directly sample nurseries and therefore could only conclude philopatry, not reproductive philopatry. Female reproductive philopatry also appears to be the case in other similar species of carcharhinid sharks such as lemon *Negaprion brevirostris* (Poey 1868) (Chapman *et al.,* 2009) and *C. plumbeus* (Portnoy *et al*., 2010). There have been very few studies on this phenomenon and if widespread among species, it will have important implications for the management of shark populations. Thus, studies on reproductive philopatry in other coastal sharks must be a priority for future research.

The lack of population genetic structure in msDNA that was present in mtDNA suggests that males display different patterns of habitat use than female conspecifics, although the low number of microsatellites assayed in this study may have reduced

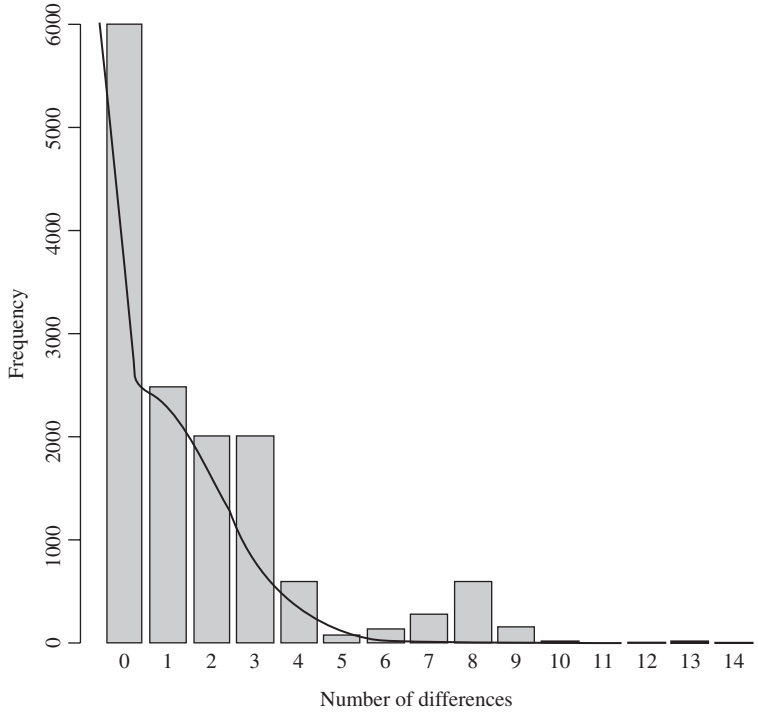

FIG. 3. The observed pair-wise difference and the expected mismatch distribution (——) under the sudden expansion model of concatenated NADH dehydrogenase subunit 4 (*nd4*) and control region haplotypes for all locations pooled as one population for *Carcharhinus leucas* from seven areas sampled in northern Australia ($n = 169$).

power despite comparable sample size (n) and the number of alleles (N_a) with other studies (Feldheim *et al*., 2001; Keeney *et al*., 2005; Ovenden *et al*., 2009). Equal relatedness within and between nurseries is expected if msDNA shows no genetic structure. Loci selected in this study, however, might not be appropriate for discerning population genetic structure. An increase in the number of loci assayed (10–15) by future studies would enhance information on male movement patterns (dispersal potential). For example, additional information will help discriminate between males and females both migrating long distances to find a mate, but females returning to specific freshwater and estuarine nurseries to pup (similar dispersal potential between sexes) or whether females are truly remaining in closer proximity to nurseries and males are travelling greater distances to mate indicative of male-mediated dispersal.

In addition to reproductive philopatry, the influence of isolation by distance on the genetic structure of *C. leucas* across the coast of northern Australia was investigated. If such effects occurred and females were commonly straying to nearby rivers when they returned to pup, then *C. leucas* from neighbouring rivers should have been more similar genetically than those in distant rivers (Wright, 1946). Genetic and geographic distances, however, were not correlated, suggesting that straying to nearby rivers by females does not occur frequently enough to increase genetic similarities (Keeney *et al*., 2005).

Long-shore barriers were also shown not to play any role in the genetic structure of *C. leucas*, with a lack of clusters of closely related haplotypes among geologically similar locations in the 95% statistical parsimony network (Fig. 3) and other phylogenetic trees (see Supporting Information). Results suggest that the presence of different habitats in coastal environments does not limit movements of *C. leucas* sufficiently to structure populations.

The occurrence of two lineages within the TCS network (Fig. 3), (haplotype CLEU_CN02 separated from the main clade by five mutational events and haplotypes CLEU_CN05 and CLEU_CN15 separated by four events) was consistent with the possibility of evolution of haplotypes in geographically isolated populations that have now become contiguous. These genetic differences among populations probably have a historical element, reflecting changes in coastal environments that occurred during ice-ages in the Pleistocene epoch when a land bridge connected Cape York and Papua New Guinea. This isolated populations on the east coast from those in northern and Western Australia (Voris, 2000). These populations became reconnected once sea levels rose at the end of the epoch.

The relatively low long-term effective population size (N_e) of 11 000–13 000 females calculated from the mismatch distribution (assuming constant mutation rates between *N. brevirostris* and *C. leucas*) supports the idea of previous constrictions of population size due to changes in coastal habitats. Significant Tajima's D and Fu' F_S statistics (Tajima's $D = -1{\cdot}469$, $P < 0{\cdot}05$; Fu's F_S test $= -7{\cdot}490$, $P < 0{\cdot}05$) indicate that the population underwent expansion *c*. 75 000–90 000 years ago (also based on the mismatch distribution and assuming equal mutation rates between *N. brevirostris* and *C. lecuas*). Both bottlenecks and expansions occurred during the Pleistocene, probably reflecting changes in the north Australian coastline during the last ice age (Voris, 2000).

This work uses a simple genetic approach for the analysis of reproductive philopatry in *C. leucas*. By sampling genetic variation in juveniles resident in nurseries, that may or may not be connected by dispersal, rather than including multiple locations separated by vicariant events, it was possible to remove the effect of ancient geological history as a casual factor for observed population genetic structure. Similarly, as conclusions of female reproductive philopatry are based on genetic structure between juveniles residing in nurseries following parturition, and not solely derived from different population genetic structure present in markers, the likelihood of falsely concluding reproductive philopatry due to differences in marker evolution is reduced.

In conclusion, population genetic structure exists between juveniles residing in closely located nurseries. This heterogeneity in genetic diversity is not attributable to geological events or isolation by distance, and combined with known life-history variables (movements of pregnant females into freshwater and estuarine habitats, utilization of these areas by juveniles until *c*. 4 years old and limited juvenile movement between nurseries) strongly supports the prolonged utilization of specific nurseries by female *C. leucas* in multiple breeding events (reproductive philopatry). Furthermore, historical changes in population dynamics indicate that *C. leucas* are susceptible to changes in coastal environments, although barriers of sub-optimal habitat are not enough to restrict movement. Results support growing evidence for the complex behaviours of *C. leucas*, although additional research is needed to confirm whether these sex-specific differences in behaviour correlate to differences in dispersal and evolutionary potential.

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SUPPORTING INFORMATION

Supporting Information may be found in the online version of this paper:

Fig. S1. Statistical parsimony network of concatenated nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (*nd4*) and control region genes ($n = 169$). The size of each circle represents the relative frequency of each haplotype and the colour composition depicts the capture locations in which haplotypes were identified. Each circle represents one mutational event; small colourless checks indicate unidentified haplotypes.

Fig. S2. Inferred phylogeny of nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (*nd4*) gene reconstructed using Bayesian inference ($n = 169$).

Fig. S3. Inferred phylogeny of the control region reconstructed using Bayesian inference. Nodal support is given as Bayesian probabilities ($n = 169$).

Fig. S4. Inferred phylogeny of concatenated nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4 (*nd4*) and control region genes calculated using Bayesian inference partitioned by gene. Nodal support is given as Bayesian probabilities ($n = 169$).

Table SI. Pair-wise F_{ST} values between un-pooled rivers. F_{ST} values are above diagonal, P-values are below diagonal; Significance is indicated in bold (significance level $P < 0{\cdot}016$ corrected for multiple comparisons by FDR method); total $n = 169$

Table SII. Nicotinamide adenine dinucleotide (NADH) dehydrogenase 4 (*nd4*) haplotypes (797 bases) total $n = 169$; including numbered polymorphic sites, haplotype frequencies, shared haplotypes and indices of population diversity; sample sizes for each group are given*

Table SIII. Control region haplotypes (837 bases) total $n = 169$; including numbered polymorphic sites, haplotype frequencies, shared haplotypes and indices of population diversity; sample sizes for each group are given*

Table SIV. Relatedness (%) within and between pooled nurseries and adult population for *Carcharhinus leucas* (total $n = 169$)* Nurseries are pooled based on geographic distances and genetic similarities

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