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Historical Biogeography and Genetic Status of the Enigmatic Pig-Nosed Turtle (*Carettochelys insculpta*) Within the Australo-Papuan Region

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Keywords: evolutionarily significant units | genetic rescue | historical demography | management units

ABSTRACT

Aim: We examine the phylogeographic genetic structure of the endangered pig-nosed turtle *Carettochelys insculpta*, the last remaining member of a once globally widespread family, now restricted to northern Australia and southern New Guinea, a region with a complex geological and eustatic history. We examine their historical biogeography, demographic history and genetic status of threatened populations.

Location: Northern Australia, Southern New Guinea.

Methods: We reconstruct phylogenetic relationships and patterns of genetic diversity using a genome-wide dataset of 15,081 single nucleotide polymorphisms and two mitochondrial loci from samples spanning the full species' range.

Results: The Australian, Papua New Guinea and Indonesian Papua turtles are recovered as three distinct lineages; the Australian lineage diverged from the New Guinea lineages *ca* 660 Kya, while the Papua New Guinea and Indonesian Papua Province lineages diverged *ca* 564 Kya. Although the fossil record shows that *C. insculpta* has been a long-standing representative of the Australia and New Guinea fauna (since at least the Miocene), extant lineages diverged later in the Middle Pleistocene. Both the Australian and Papua New Guinea lineages were likely shaped by bottlenecks, isolation and genetic drift, which in the Australian lineage greatly reduced effective population sizes to 48–88.

Main Conclusions: The contemporary genetic structure of *C. insculpta* is most consistent with a vicariance model whereby a large interchanging population occupying northern Australia and New Guinea came to be fragmented and diverged into Australian, Papua New Guinea and Indonesian Papua lineages. Subsequent dispersal via paleodrainages of the submerged continental shelf under the influence of Pleistocene sea-level change is thought to have been impeded by the isolation of the Akimeugah and Arafura Basins. All populations of the Australian lineage show low genetic diversity without contemporary gene flow, suggesting they are vulnerable to inbreeding and reduced fitness, requiring the consideration of genetic rescue.

1 | Introduction

The highly diverse and endemic biota of Australia and New Guinea (Mittermeier and Mittermeier 1997) have been shaped by a history of complex geological, climatic and eustatic events that have influenced dispersal, vicariance and speciation over evolutionary timescales. This has provided an outstanding opportunity to study patterns in the distribution of organisms and the processes that produce those patterns (Deiner et al. 2011; Georges et al. 2014; Heads 2002; Mayr 1944; Nyári and Joseph 2013; Polhemus and Polhemus 1998; Rawlings and Donnellan 2003; Wallace 1860; Wüster et al. 2005). Studying the contemporary distributions of species, the spatial patterns of genetic diversity within species and the processes that gave rise to those patterns is important for understanding the factors that shape contemporary biodiversity, for both taxonomy and conservation management (Coates et al. 2018; Eizaguirre and Baltazar-Soares 2014; Ewart et al. 2020; Huey et al. 2014).

Biodiversity of the Australian continent bears a strong signature of progressive aridification. After its separation from Antarctica at the end of the Palaeocene (Veevers and McElhinny 1976), Australia experienced a long period of relatively stable climate favouring mesic environments. Gondwanan temperate and tropical rainforests and associated fauna were widespread until drier conditions prevailed from Early to Middle Miocene when more diverse forest types emerged (Hill 2004; Martin 2006). Substantial aridification began during the Middle Miocene *ca* 15 Mya (Fujioka and Chappell 2010; Martin 2006). It intensified during the Pliocene–Pleistocene, leading to the formation of xeric stony deserts *ca* 2–4 Mya (Fujioka et al. 2005) and extensive dune fields *ca* 1 Mya across central Australia (Fujioka et al. 2009) causing the contraction of mesic habitats to the eastern and south-western parts of Australia (Byrne et al. 2011; Martin 2006). Today, about two-thirds of the Australian continental land mass comprises arid and semi-arid lands, and the whole continent experiences major irregular and periodic seasonal droughts (Byrne et al. 2008; Chiew et al. 1998). The fossil record indicates that mesic-adapted fauna underwent mass extinction across Australia in response to aridification (Hocknull et al. 2007), replaced by diversification of those lineages that could adapt (Oliver and Hugall 2017), with the remainder surviving in isolated mesic refugia (Byrne et al. 2011).

Concurrent with the onset of aridification in Australia during the Middle Miocene, the collision between the northward-moving Australian continental plate and the westward-moving Pacific plate led to the formation of New Guinea (Cloos et al. 2005). Initially, with little emergent land available for colonisation by fauna (Gold et al. 2017; Park et al. 2020), the island rapidly increased in size via the accretion of oceanic and continental terrains in the north, a relatively stable Australian continental block underlying the lowlands in the south, and uplift of the central range principally in the Late Miocene 8–12 Mya (Pigram and Davies 1987; Stanaway 2008; van Ufford and Cloos 2005). Phylogenetic patterns of Australo-Papuan biodiversity indicate that New Guinea has provided the opportunity for the interchange of faunal elements of Asian (Esquerré et al. 2020; Hugall et al. 2008; Rowe et al. 2019) and Australian origin (Gauffre-Autelin et al. 2021; Letsch et al. 2023; Moyle et al. 2016; Sparks and Smith 2004; Stelbrink et al. 2014; Todd et al. 2014), and an evolutionary refuge for Australian lineages extirpated from their ancestral range by widespread aridification (Aplin and Ford 2014; Hocknull et al. 2007; Moyle et al. 2016).

The processes of vicariance and dispersal, and the resultant contemporary patterns of Australo-Papuan biodiversity have been shaped by the dramatic climatic and eustatic oscillations associated with Pleistocene glacial cycles (Byrne et al. 2011; Reeves et al. 2008; Williams et al. 2009). Compared to the present, sea-levels over the past *ca* 2.6 million years have ranged between −130 m during cool and hyper-arid glacial maxima, to +35 m during comparatively warm and less arid interglacial periods (Miller et al. 2020). Sea-level amplitude of each cycle varied substantially. Drops in sea-level during glacial maxima had a critical influence on terrestrial, freshwater and marine connectivity by exposing Torres Strait, the Arafura Sill and the vast Sahul Shelf. These land bridges intermittently connected Australia and New Guinea and enabled currently isolated freshwater habitats that persisted through hyper-aridity (Playà et al. 2007), to coalesce before reaching the edge of the continental shelf or before reaching the large paleolakes of Lake Carpentaria and Lake Bonaparte (Voris 2000; Yokoyama et al. 2001). Phylogenetic evidence suggests that the freshwater and estuarine habitats of Lake Carpentaria, although highly dynamic and fluctuating between freshwater, brackish and marine conditions (Reeves et al. 2008) provided dispersal avenues for a range of freshwater fauna between Australia and New Guinea (Baker et al. 2008; Huey et al. 2014; Todd et al. 2014). Conversely, rising sea-levels during interglacial periods flooded continental shelves and isolated formerly connected aquatic habitats, disrupting gene flow, promoting genetic drift, species and population-level diversification (Tschá et al. 2017; Shelley et al. 2020).

The pig-nosed turtle (*Carettochelys insculpta*, Ramsay 1886) currently occupies this very dynamic region (Rhodin et al. 2021). It belongs within the family Carettochelyidae, a once diverse and widely distributed family of turtles that originated in Asia during the early Cretaceous *ca* 145–100 Mya (Joyce 2014) that later came to occupy North America, Europe, Asia, Africa and Australasia (Glaessner 1942; Joseph-Ouni et al. 2023; Joyce 2014; Rule et al. 2022). Currently represented by a single extant species, the distribution of *C. insculpta* includes the rivers draining south from the central ranges in New Guinea and three major rivers in northern Australia (Rhodin et al. 2021). The species is of considerable conservation concern (Eisemberg et al. 2011; Georges, Doody, et al. 2008; Petrov et al. 2023), having been recently classified as Endangered by the IUCN (Eisemberg et al. 2018). Harvesting for bush meat and wildlife trafficking are considered key threatening processes (Burgess and Lilley 2014; Eisemberg et al. 2011; Samedi and Iskandar 2000; Shepherd et al. 2020). In southern New Guinea, the species occupies diverse permanent and semipermanent freshwater and saline habitats within the tropical lowlands, including rivers, lakes, lagoons, swamps, estuaries, coastal mangroves and the ocean surrounding coastal islands (Georges, Doody, et al. 2008). Migrations occur both upriver and downstream to coastal islands to nest (Eisemberg, Rose, Yaru, Amepou, et al. 2015a; Eisemberg et al. 2015b; Georges, Alacs, et al. 2008). In Australia, they are found in rivers, thermal springs, billabongs and plunge pools of the Daly, South Alligator and East

Alligator rivers of the Australian monsoonal tropics (Georges, Doody, et al. 2008) but do not extend their ranges or nesting into coastal mangrove regions or estuaries (Doody et al. 2002; Georges and Kennett 1989) as they do in New Guinea.

The distribution of *C. insculpta* is well suited to study how aquatic lineages diversified in response to climatic and eustatic changes during the Cenozoic, as they are found in both Australia and New Guinea. Fossil material attributable to carettochelyids suggests a relatively recent but widespread biogeographical history of this family throughout the Australo-Papuan region after dispersal from southeast Asia to the north. Carettochelyid fossil material from Sarawak (age unknown) has been attributed to a new species *Carettochelys niahensis* that is readily distinguished from *C. insculpta* (White et al. 2023). Further fossil material from the Miocene of Papua New Guinea (Glaessner 1942) has led to the suggestion that dispersal had occurred across Wallace's line and the islands of the Indo-Australian Archipelago by the Late Miocene (Joyce 2014), reaching Australia relatively recently (Cogger and Heatwole 1981). However, fossil material dating to the Late Miocene/Early Pliocene (*ca* 6.2–4.9 Mya) from Victoria in southeastern Australia (Rule et al. 2022) and the Pliocene from Queensland (Joseph-Ouni et al. 2023), in addition to the fossil from the Miocene of southern New Guinea (Glaessner 1942), indicates that *C. insculpta* was likely once distributed widely across eastern Australia in the Miocene and Early Pliocene (Rule et al. 2022). Their current distribution likely reflects a contracted range caused by increased aridification during the Quaternary (Rule et al. 2022) as well as more recent interchange between Australia and New Guinea via the Sahul land bridge during Pleistocene glacial maxima (Cogger and Heatwole 1981).

In this paper, we explore the biogeographic history of *C. insculpta* across its range in northern Australia and southern New Guinea. We use mtDNA sequences and genome-wide SNPs to explore phylogenetic relationships, population differentiation and estimate divergence times, interpreting these patterns in the context of paleodrainage connectivity that occurred between Australia and New Guinea during Pleistocene eustatic and climatic fluctuations. Finally, we identify conservation units and fully evaluate their genetic status, identifying populations with depauperate genetic diversity that require genetic rescue.

2 | Materials and Methods

2.1 | Field Sampling and DNA Extraction

A total of 251 *C. insculpta* were used in this study (Figure 1, Table S1), with 216 wild turtles sampled from across their range in northern Australia ($n = 111$), Papua New Guinea ($n = 99$), two locations in Indonesian Papua Province ($n = 6$) and 35 trafficked turtles determined to originate from unknown locations in Indonesian Papua Province based on forensic population assignment by Young et al. (2025). In Australia, collection sites included the three rivers in which *C. insculpta* is known to occur—Daly River ($n = 75$ individuals), South Alligator River ($n = 23$) and East Alligator River ($n = 13$). In Papua New Guinea, turtles were captured from 10 rivers and one coastal island, including the Vailala River ($n = 4$), Purari River ($n = 7$), Kikori River ($n = 30$), Turuvio Island at the mouth of the Kikori River ($n = 25$), Turama River ($n = 12$), Bamu River ($n = 7$), Aramia River ($n = 4$), Fly River ($n = 9$) and Oriomo River ($n = 1$). The Kikori River, Kikori River coast, Turama River and Bamu River samples were all collected from rookeries. Samples were also obtained from turtles held in the private collection of William P. McCord (USA) reportedly from two locations in Indonesian Papua Province, namely the Merauke River ($n = 4$) and Pulau (Eilanden) River ($n = 2$).

Tissue for DNA extraction was typically a skin biopsy (2×5 mm) excised from toe-webbing and preserved in absolute ethanol in the field, later stored at −20°C or from blood that had been frozen or dried on FTA Elute micro cards (Whatman). Genomic DNA was extracted from a small portion of the sampled tissue or card using a proteinase K and salting-out procedure (Jensen et al. 2013). Extracted DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific Australia, Melbourne), and standardised by dilution to 25 ng/μL for PCR or between 50 and 100 ng/μL for reduced representation sequencing by Diversity Arrays Technology (DArT Pty Ltd., Canberra, www.diversityarrays.com).

2.2 | Mitochondrial Sequencing

An 883 bp fragment of the mitochondrial *NADH dehydrogenase subunit 4*, 69 bp of tRNA-*His* and 26 bp of tRNA-*Ser* (referred to as the ND4 fragment) and a 741 bp fragment of mitochondrial control region and 29 bp fragment of tRNA-Pro (referred to as control region fragment) were PCR-amplified using modified primers CiND4-F, CiND4-R (H-Leu; Stuart and Parham 2004) and CiCR-F and CiCR-R (LCM15382 and H950g; Abreu-Grobois et al. 2006). Sequencing reactions and purification were performed following (Campbell et al. 2018). Products were sanger sequenced with an ABI 3730xl DNA Analyser at the Biomolecular Resource Facility (BRF) within the John Curtin School of Medical Research, Australian National University and edited using Geneious Prime 2020.2.2 (Biomatters, Auckland, New Zealand). For further details see Text S1.

2.3 | Nuclear SNP Sequencing, Genotyping and Quality Control

Skin tissues and extracted DNA were provided to DArT for processing, sequencing and informative SNP marker identification using DArTseq (Kilian et al. 2012). DArT performed a genome complexity reduction technique using double digestion of genomic DNA with two restriction endonucleases *Pst*I (5′-CTGCA|G-3′) and *Sph*I (5′-GCATG|C-3′), fragment-size selection and next-generation sequencing on an Illumina HiSeq2500 (CA, USA). Sequences were processed using proprietary DArT analytical pipelines; for full details refer to Georges et al. (2018). Initial filtering was based primarily on average and variance of sequencing depth, average allele counts and call rate across samples. Approximately one third of samples were sequenced twice

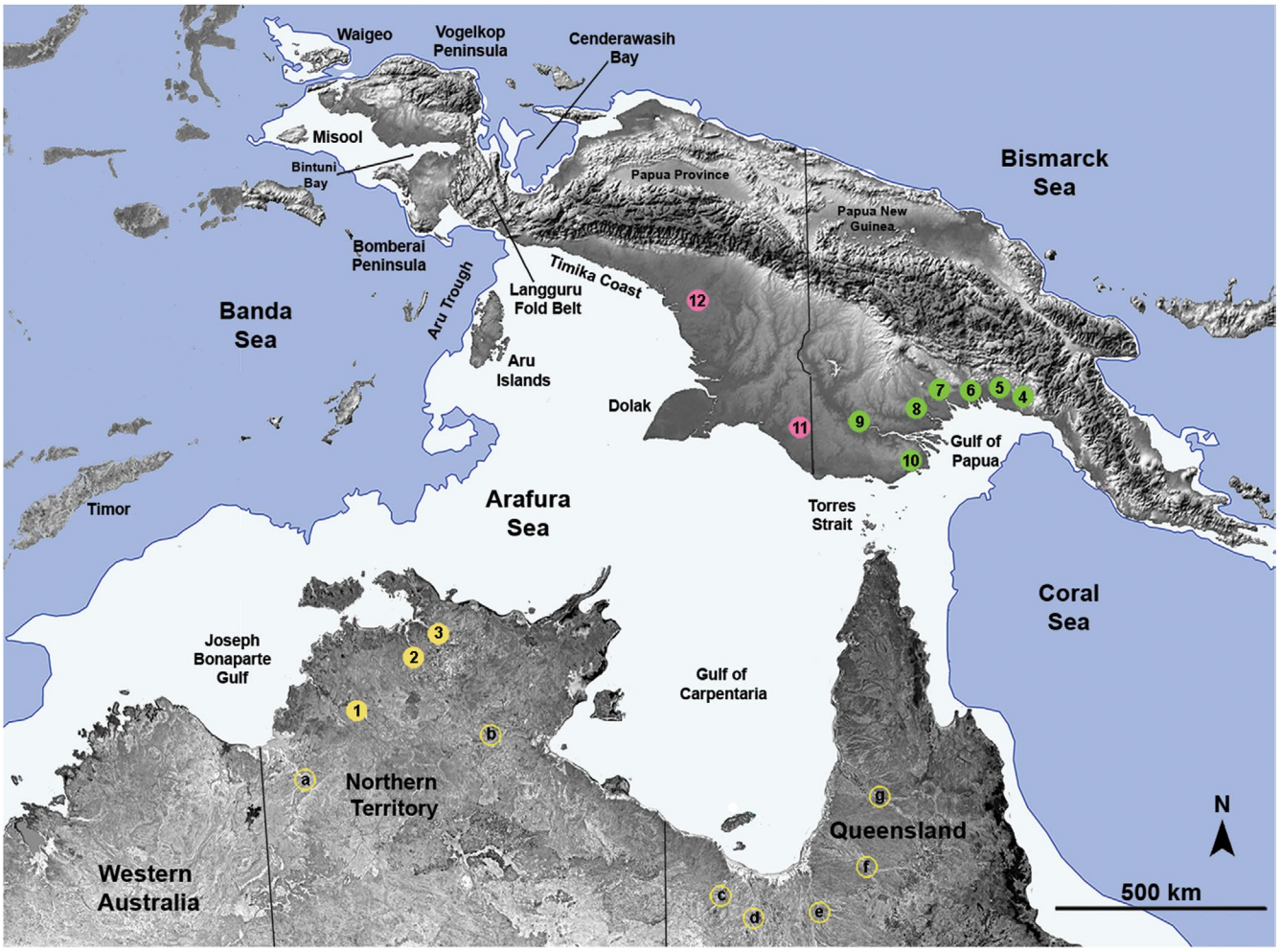

FIGURE 1 | A map of northern Australia and New Guinea showing drainages from which samples were taken. Solid circles: [1], Daly River ($n = 75$); [2], South Alligator River ($n = 23$); [3], East Alligator River ($n = 13$); [4], Vailala River ($n = 4$); [5], Purari River ($n = 7$); [6], Kikori River ($n = 30$) and Turuvio Island at the mouth of the Kikori River ($n = 25$); [7], Turama River ($n = 12$); [8], Bamu River ($n = 7$) and Aramia River tributary ($n = 4$); [9], Fly River ($n = 9$); [10], Oriomo River ($n = 1$); [11], Merauke River ($n = 4$); [12], Pulau (Eilanden) River ($n = 2$). Hollow circles show major drainages with substantial lowland reaches for which *Carettochelys insculpta* is absent: (a) Victoria River; (b) Roper River; (c) Nicholson River; (d) Leichhardt River; (e) Norman River; (f) Gilbert River; (g) Mitchell River (Qld). The light blue shaded region shows the approximate extent of the lands exposed during the height of the last interglacial (sea-level − 130 m). Full details of sample locations are in Table S1. Samples from captive turtles originated from unknown locations within Indonesian New Guinea ($n = 35$) are not included.

as technical replicates, with scoring consistency used to identify high quality SNP markers with low error rates. We applied further quality control filtering (Table S2) using the R package DARTR (Gruber et al. 2018; Mijangos et al. 2022). These filters were for reproducibility across technical replicates (< 99%), call rate removing both loci and individuals with > 5% missing data, read depth (< 8× and above > 50×) to remove low coverage SNPs and potential paralogs, and removing multiple SNPs per locus. Specimens were removed from non-bottlenecked populations with close kinship probabilities (≥ 0.23) assessed using the R package POPKIN (Ochoa and Storey 2021). We then filtered on linkage disequilibrium to account for the presence of monomorphic heterozygous loci from a possible gene duplication, resulting in a stringently filtered dataset of 16,002 SNPs used for phylogenetic and population genetic analyses. The dataset was further filtered on call rate removing any loci with missing data resulting in 8854 SNPs for estimating effective population size with NEESTIMATOR v2.1 (Do et al. 2014).

2.4 | Mitochondrial Phylogeny and Divergence Times

To estimate geographic distributions and divergence dates of *C. insculpta* lineages, we used two phylogenetic approaches with the mtDNA dataset. First, we used concatenated and partitioned mtDNA sequences to infer a phylogenetic tree of mitochondrial haplotypes and estimate relative divergence dates with BEAST2 v2.6.6 (Bouckaert et al. 2019), using the constant-size coalescent tree prior and a strict molecular clock specifying homogeneity of substitution rate across branches. A substitution rate of 7.0×10^{-3} substitutions/site/Ma was specified for the third codon position of *ND4*, based on the mean evolutionary rate estimated for turtles by Lourenço et al. (2013). The HKY nucleotide substitution model was specified for *ND4* and *tRNA* partitions in MEGA v10.2.4 (Kumar et al. 2016); for the control region, the HKY + G model was chosen with rate variation among sites modelled using a

gamma distribution with four rate categories. Samples from the posterior distribution of the Markov chain Monte Carlo (MCMC) analyses were drawn every 1×10^3 steps over a total of 2×10^7 steps. All input files and parameters were prepared using BEAUTI v2.6.3 (Bouckaert et al. 2019), and the posterior results evaluated for convergence in multiple runs by checking trace files and parameter effective sample size (ESS) were > 200 in TRACER v1.7.2 (Rambaut et al. 2018). The first 10% of MCMC steps were discarded as burn-in. A maximum clade credibility tree was reconstructed using TREEANNOTATOR v2.6.3 and edited using FIGTREE v1.4.4 (Rambaut 2012).

Second, we inferred phylogenetic relationships using a Maximum Likelihood (ML) approach in IQ-TREE v2.1.3 (Minh et al. 2020) with 10,000 ultrafast bootstraps and gene (gCFs) and site (sCFs) concordance factors. Partition schemes and models of molecular evolution were estimated using the corrected Akaike information criterion (AIC_c) in MODELFINDER (Kalyaanamoorthy et al. 2017), which determined the HKY + F substitution model for codons one and two, and TIM2 + F for codon 3 of *ND4* and HKY + F + I for the control region. All trees were rooted using the mid-point method without an outgroup, as *C. insculpta* is the sole extant species within Carettochelyidae. To assess genetic similarity within and among mitochondrial clades identified from the phylogenetic analyses, we calculated the uncorrected proportion of genetic differences (*p*-distance) in MEGA from the 883 bp of ND4 and the 741 bp of the control region respectively.

2.5 | Phylogenetic Analysis of SNP Data

We used two phylogenetic approaches on the 16,002 SNP dataset. First, relationships among all sampled turtles were inferred using maximum likelihood in IQ-TREE. We used ModelFinder, with the Bayesian information criterion (BIC) to estimate the optimal substitution model (TVM + F + I + I + R4), with 10,000 ultrafast bootstraps and the Shimodaira-Hasegawa-like aLRT test (Guindon et al. 2010) to summarise phylogenetic uncertainty. Second, we modelled a species tree based on the multispecies coalescent with SVDQUARTETS (Singular Value Decomposition Quartets; Chifman and Kubatko 2014), implemented in PAUP v4.0 (Swofford 2003). Heterozygous SNPs were replaced with standard ambiguity codes using DARTR; branch support was estimated with 10,000 bootstrap replicates.

2.6 | Population Genetic Structure

We used multiple complementary analyses to quantify the genetic structuring of wild *C. insculpta* for the purpose of identifying evolutionarily significant units (ESUs) and management units (MUs) (*sensu* Moritz 1994) across the species' range with the addition of captive individuals assigned to the Indonesian Papua Province population by Young et al. (2025). To visualise the genetic structuring of *C. insculpta* across its range, we first performed a principal coordinate analysis (PCoA) in DARTR. To complement this analysis of nuclear markers, we examined the relationships of mtDNA haplotypes within and among river drainages in a TCS network using POPART v1.7 (Leigh and Bryant 2015).

We identified diagnosable operational taxonomic units (OTUs), that is, aggregations of sampling localities based on fixed allelic differences between pairs of sampling locations (including the traded individuals) using the *gl.fixed.diff* function in DARTR. Briefly, sampling locations within the same catchment or rookery were amalgamated manually, then remaining sampling sites and any prior amalgamations were compared pairwise for fixed allelic differences. Those with no fixed differences (parameter *tloc* set to 0) were amalgamated and the process was repeated until entities were diagnosable by two or more fixed allelic differences (i.e., corroborated fixed differences). The resultant pairwise fixed differences were tested against the expected number of false positives given the observed sample sizes (Georges et al. 2018).

We calculated pairwise F_{ST}, Φ_{ST} and Fisher's exact tests among all sampling locations (including the traded individuals) using the mtDNA dataset in ARLEQUIN v3.5 (Excoffier and Lischer 2010). Statistical significance was determined for each statistic by Markov chain permutation ($\alpha = 0.05$, $n = 100{,}000$, dememorisation = 10,000). Pairwise F_{ST} (Weir and Cockerham 1984) was also calculated among sampling locations using the SNP dataset in the R package STAMPP (Pembleton et al. 2013), implemented in DARTR. We generated 95% confidence intervals and *p*-values by bootstrapping across loci ($n = 1000$), *p*-values were corrected for multiple comparisons using the FDR correction (Benjamini and Hochberg 1995) applied in the *p.adjust* function in the R package STATS.

2.7 | Genetic Diversity and Historical Demographics

We assessed patterns of genetic diversity in *C. insculpta* using both the mtDNA and SNP datasets for each OTU determined through the fixed difference analysis. From the mtDNA sequence dataset, we calculated the number of haplotypes (*H*) and haplotype diversity (*Hd*) using the R package PEGAS (Paradis 2010), accounting for unequal sample sizes by standardising to $n = 10$. Nucleotide diversity (π) was calculated using DNASP v6 (Rozas et al. 2017).

For nuclear estimates of diversity, we calculated observed heterozygosity (H_o) and unbiased expected heterozygosity (uH_e) (standardised to $n = 13$), with 5000 bootstrap replicates following the recommendations of Schmidt et al. (2021) and an estimate of invariant loci to allow comparability across species using DARTR. We tested whether H_e was significantly different between groups using the re-randomisation approach of the *gl.test.heterozygosity* function in DARTR with 1000 bootstrap replicates. Rarefied allelic richness (*Ar*) was calculated using the R package HIERFSTAT (Goudet 2005) standardised to $n = 13$. We estimated the number of private alleles (*Pa*) in reciprocal pairwise comparisons between OTUs, and between single OTUs and all other OTUs combined using DARTR. To estimate levels of inbreeding within OTUs, we calculated the inbreeding coefficient (F_{IS}) across loci with 10,000 bootstrap replicates, using the *gl.Ho* function and uH_e calculation within DARTR. We calculated the intrapopulation proportion of shared alleles using DARTR, and kinship probabilities using POPKIN v1.3 (Ochoa and Storey 2021).

Contemporary effective population size (N_e) was calculated for each OTU with the linkage disequilibrium method (LDN_e) within NeEstimator v2.1 (Do et al. 2014) using the 8854 SNP dataset, specifying random mating and an allele frequency critical value of 0.05.

2.8 | Paleochannel Reconstruction

The paleochannels of the Sahul Shelf were created from the GEBCO global elevation and bathymetry data set (GEBCO Compilation Group (2023) GEBCO 2023 Grid (https://doi.org/10.5285/f98b053b-0cbc-6c23-e053-6c86abc0af7b, last accessed 19 July 2023)) and manipulated using ArcGIS v10.1 (Environmental Systems Research Institute, California) to create the streamlines and obtain estimates of sea depth.

2.9 | Comparison of Biogeographic Scenarios

We constructed and compared four hypothetical biogeographic models using DIYABC Random Forest (DIYABC-RF) v.1.2.1 (Collin et al. 2021) for the Aus, PNG and PP groups. The first three models were dispersal models: (PP(Aus,PNG), (PNG(Aus,PP) and (Aus(PNG,PP)) (Figure S2). The fourth model was a vicariance model (Aus,PNG,PP). In the dispersal models, the initial divergence was set at $t_2 = 10{,}680–44{,}080$ generations and the subsequent divergence at t_1 (9120–38,560 generations). These values correspond to the range in estimates obtained from the phylogenetic dating (Figure 2). In all four models, a potential bottleneck was introduced at time t_1 – db to represent a dramatic drop in population size of the Australian population between t_1 and present. In the case of the first dispersal model (Aus(PNG,PP)) and the vicariance model (AUS,PNG,PP), this bottleneck represents the reduction in population size (to N1b = 50–1000) arising from aridification in Australia and extends from time t_1 to the present. In the other two dispersal models, the bottleneck arises from founder effect. The Random Forest Analysis was based on 80,080 simulated datasets in the training set and 5000 trees in the evaluation run.

The SNP data set was filtered before the ABC model testing. First, we removed all SNPs that coexisted on single sequence tags, retaining only one at random, then we filtered all loci for which there were missing data, then we filtered on read depth (> 10× in accordance with the recommendations of Collin et al. 2021). Software package dartR (function gl2diyabc-rf) was used to convert the genlight format into DIYABC-RF format. This yielded 7972 SNP loci (all autosomal), reduced to 3849 loci by setting MAF < 0.05 in the parameters passed to DIYABC-RF. We simulated 60,000 datasets in the training run, with initial effective populations sizes (N_e) set to 10,000 for the three populations, dropping to 50–1000 for the Australian population post bottleneck. Remaining DIYABC-RF parameters were left at the defaults.

The Bayes Factor (K) between two scenarios was computed as the ratio of their posterior probabilities:

$$K_{1/2} = p_1 / p_2$$

where p_1 is the posterior probability of Scenario 1, p_2 is the posterior probability of Scenario 2, and the two scenarios have equal priors. The interpretation of Bayes Factor support for Scenario 1 over Scenario 2 follows the guidelines of Kass and Raftery (1995): $K_{1/2} < 1$—evidence against; $1 < K_{1/2} < 3.2$—weak evidence; $3.2 < K_{1/2} < 10$—moderate evidence; $10 < K_{1/2} < 100$—strong evidence; $K_{1/2} > 100$—decisive evidence.

3 | Results

Mitochondrial loci *ND4* and control region were sequenced for 97 *C. insculpta*, revealing 30 distinct mtDNA haplotypes that consisted of 127 variable sites, 94 of which were parsimony informative (Table S3). *ND4* contained 30 variable sites and control region contained 97 variable sites. Within the control region, two of the mutations occurred at a single site and there were two single nucleotide indels (Table S4). The first indel was unique to Indonesian Papua Province; the second indel was unique to Papua New Guinea. For our nuclear DNA dataset, 233 *C. insculpta* were genotyped for 16,002 polymorphic SNP loci after filtering (Table S2). Both datasets contained samples spanning the entire range of *C. insculpta* (Figure 1).

3.1 | Phylogenetic Relationships and Divergence Times

The mtDNA and SNP phylogenetic analyses recovered concordant topologies with varying support for nodes (Figures 2 and 3; Figure S1). Divergence dating with the mtDNA phylogeny recovered three major lineages that emerged from an evolutionary split *ca* 660 Kya (HPD 0.267–1.102 Ma) separating the turtles from Australia and New Guinea, and another split *ca* 564 Kya (HPD 228–964 Kya) separating the turtles from Papua New Guinea and Indonesian Papua Province (Figure 2). The pairwise *p*-distance of mean sequence divergence in mtDNA haplotypes between the Australian and New Guinea lineages was 1.39% for *ND4* and 6.08% for the control region. The Australian lineage is further divided into two clades that split *ca* 147 Kya (HPD 50–265 Kya), separated by 0.45% mean sequence divergence for *ND4* and 1.52% for the control region, the first corresponding to turtles exclusively from the Daly River, and the other clade formed by the South Alligator and East Alligator rivers that split 50 Kya (HPD 12–95 Kya) separated by 0.26% for *ND4* and 0.36% for the control region, which are sufficiently distinct to be subclades. The New Guinea lineage comprises two large and diverse clades separated by the Torres Strait (Figure 2), with a pairwise mean sequence divergence of 1.1% for *ND4* and 5.57% for the control region, having split *ca* 564 Kya (HPD 228–964 Kya). The Papua New Guinea clade encompasses all catchments flowing into the Gulf of Papua. The Indonesian Papua Province clade, due to limited sampling within only two rivers, putatively encompasses all the catchments flowing south of the central ranges where the turtles occur in Indonesian Papua.

All three lineages and corresponding clades displayed strong phylogeographic structure, at the scale of single river catchments within Australia, and at larger scales inclusive of

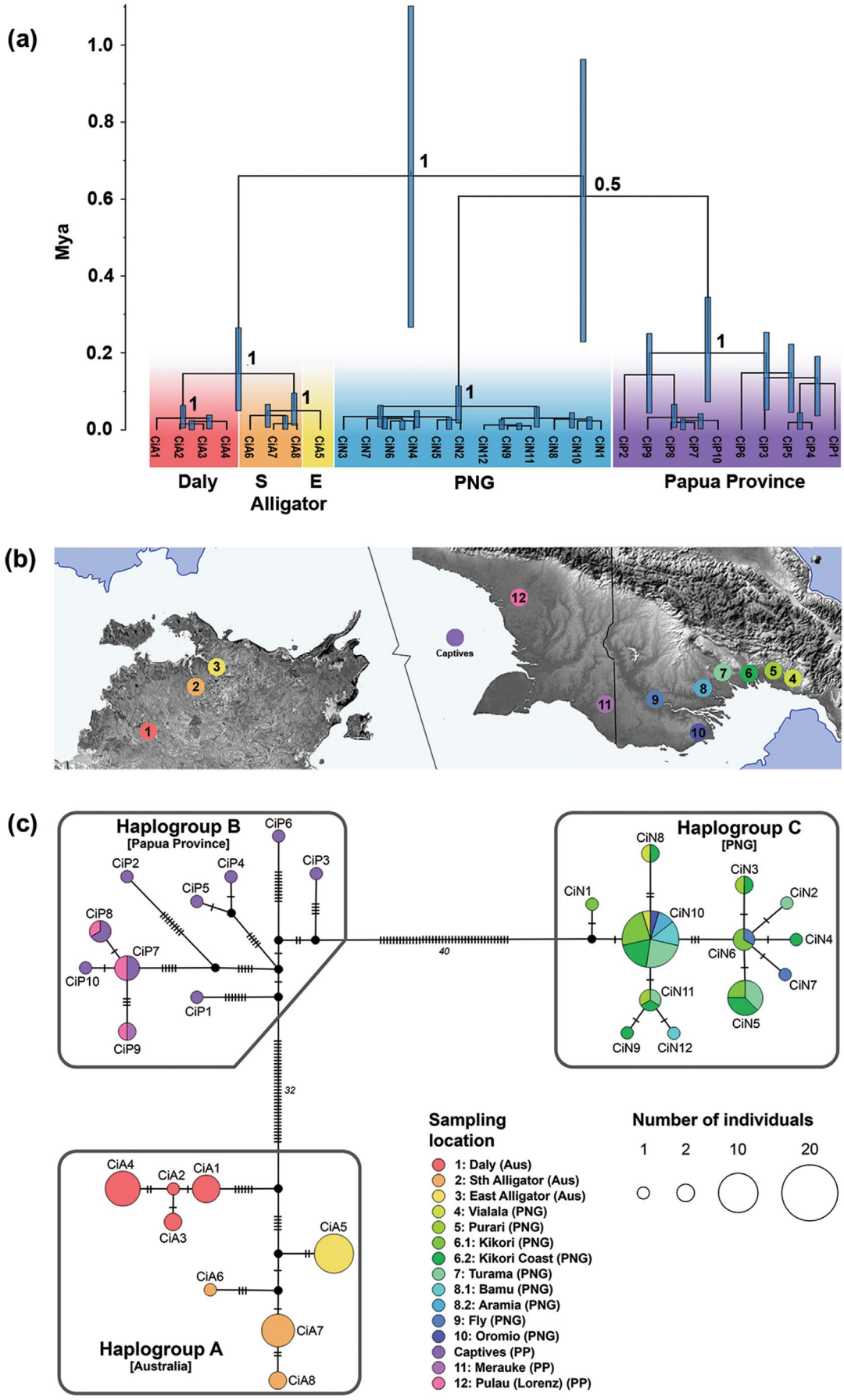

FIGURE 2 | Legend on next page.

FIGURE 2 | Dated mitochondrial phylogeny (a) and TCS haplotype network (c) for the pig-nosed turtle *Carettochelys insculpta* supporting three main lineages: (1) Australia (Haplogroup A), (2) Indonesian Papua Province (Haplogroup B) and (3) Papua New Guinea (Haplogroup C); locations shown in (b). The Australian lineage can be further subdivided into two clades and subclades representing the three catchments: Daly River, South Alligator River and East Alligator River.

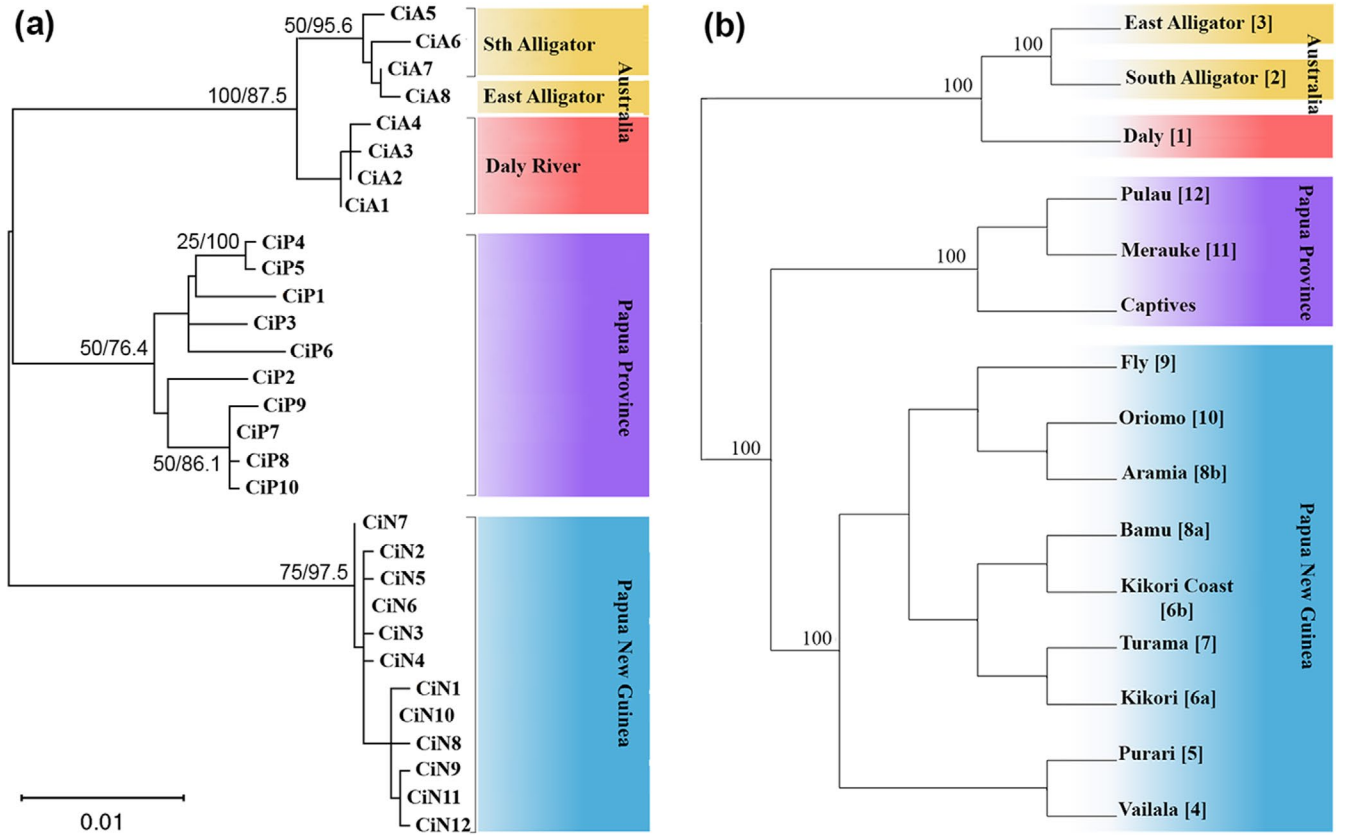

FIGURE 3 | Phylogenies for *Carettochelys insculpta* based (a) on maximum likelihood of concatenated partial mtDNA sequences (*ND4* and Control Region) using IQ-TREE and (b) a nuclear DNA species tree inferred using SVDQUARTETS applied to the SNP data. Standard ambiguity codes were used for heterozygous sites. Bootstrap support values are given on the nodes.

multiple catchments across the Gulf of Papua within New Guinea (Figure 2). Mean sequence divergence in mtDNA haplotypes among clades ranged between 0.45%–1.51% for *ND4* and 1.52%–6.52% for control region, and within clades ranged between 0.06%–0.24% for *ND4* and 0.2%–1.8% for control region sequences (Table S5).

3.2 | Population Genetic Structure

The PCA on 16,002 SNPs revealed clustering of multilocus genotypes into five distinct groups across the first four principal components, which explained 39.1%, 21.1%, 5.8% and 0.8% of the genetic variation, respectively (Figure 4). Australian turtles are represented by three distinct and isolated genetic clusters corresponding to the Daly River, South Alligator River and East Alligator River. The low percentage variation explained by the fourth component that splits the South Alligator River and East Alligator River clusters indicates that the divergence between the two is relatively minor. The captive turtles form a single large cluster with the Merauke River and Pulau River turtles from Indonesian Papua Province, consistent with the assignment of these individuals by Young et al. (2025).

The fixed difference analysis revealed five OTUs diagnosable by corroborated fixed allelic differences between final pairwise comparisons among catchments (Table 1). The number of loci showing fixed allelic differences significantly exceeded the expected false positive rate ($p < 0.0001$) in all cases. Turtles within Papua New Guinea, spanning across multiple river drainages from the Vailala River to the Oriomo River (locations 4–10, Figure 1) form the largest diagnosable unit. The individuals within Indonesian Papua Province (11 & 12) also make up a single diagnosable unit. These two New Guinea aggregations were marginally diagnosable by only two fixed allelic differences (i.e., one corroborated fixed difference). In Australia, the turtles of the three geographically isolated river catchments were each diagnosable, with even the adjacent river drainages of the South Alligator River and East Alligator River forming two units diagnosable by 34 fixed allelic differences. The Daly River formed the third diagnosable unit in Australia (Table 1). There were 115 fixed allelic differences between the Australian clade and the Papua New Guinea clade, and 50 fixed allelic

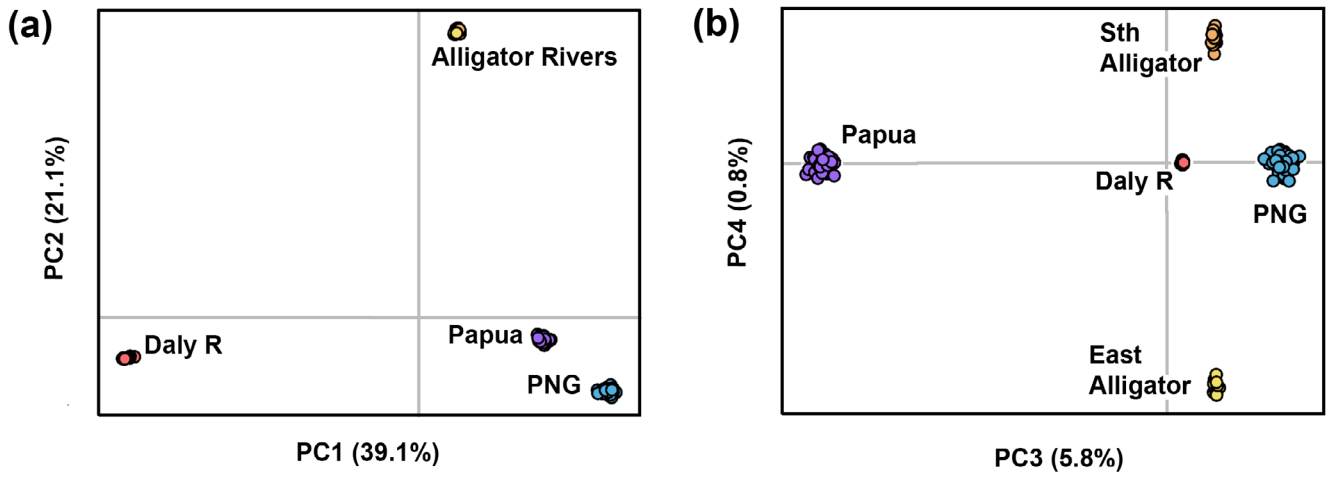

FIGURE 4 | Genetic structure for the pig-nosed turtle *Carettochelys insculpta*, based on principal components analysis applied to 16,002 loci from 233 individuals. Clusters within Australia correspond to the Daly River, South Alligator River and the East Alligator River; the Papua New Guinea (PNG) cluster contains turtles from all catchments and rookeries sampled within PNG; the Indonesian Papua Province cluster contains turtles from the Merauke River, Pulau River and the animals obtained from captive collections.

differences between the Australian clade and the Indonesian Papua Province clade. However, only 20 fixed allelic differences occur between Australia and New Guinea after their respective sampling localities were amalgamated.

Finally, F_{ST} values in final pairwise comparisons among sampling locations (including the traded individuals) showed moderate to extremely high values between clades and subclades, suggesting population structuring from large variance in allele frequencies (0.197–0.970; Table 2). Pairwise comparisons between the Daly River (location [1], Figure 1) and all other OTUs revealed the highest F_{ST} values, ranging from 0.649 to 0.970. Comparisons among Australian OTUs ranged from 0.459 to 0.970, between Australian and New Guinea OTUs 0.459 to 0.666 and were lowest between the two New Guinea OTUs at 0.197 (Table 2). The SNP F_{ST} was significantly differentiated between the Vailala River (location [4], Figure 1) and all other sampling locations in Papua New Guinea, and elsewhere (0.017–0.949).

At a finer scale, differentiation among final pairwise comparisons of rookeries and sampling locations (including traded turtles) measured by mtDNA F_{ST} and Φ_{ST} showed moderate to high population structuring (F_{ST} = 0.176–0.744; Φ_{ST} = 0.836 to 0.969; Tables S6 and S7). No significant genetic differentiation was found among Papua New Guinea coastal or upstream rookeries or catchments with mtDNA F_{ST} and Φ_{ST}. No significant differentiation was found among Indonesian Papua Province sampling locations and captive colonies. The populations of the Daly, South Alligator and East Alligator rivers were each differentiated from the other (Table S7).

3.3 | Genetic Diversity

The Australian OTUs showed the lowest mtDNA diversity levels; the number of haplotypes (H) and haplotype diversity (Hd) (standardised to n = 10) were lowest in the East Alligator River OTU and lower across all Australian OTUs (H = 1–3; Hd = 0–631; Table S8) compared to Papua New Guinea OTUs (H = 5; Hd = 0.732) and Indonesian Papua Province OTUs (H = 6; Hd = 0.861). Nucleotide diversity (π) was low across all Australian OTUs (0–0.00101; Table S8) and Papua New Guinea (0.00142) and highest in Indonesian Papua Province (0.00667).

TABLE 1 | Diagnosable units for the pig-nosed turtle *Carettochelys insculpta* arising from the fixed allelic difference analysis (sensu Georges et al. 2018).

OTU	Daly	Sth alligator	East alligator	PNG	Papua
Daly	—	75.7	51.3	5.0	31.1
Sth alligator	1721	—	15.5	35.8	54.8
East alligator	1867	34	—	28.8	48.5
PNG	506	507	573	—	0.2
Papua	318	271	321	2	—

Note: The pairwise comparisons were made using the dataset with 16,002 SNPs from 233 individuals. Counts of loci with fixed allelic differences are shown in the lower triangle and the corresponding expected number of false positives given the sample sizes are given in the upper triangle. All fixed differences in final comparisons were significant, p-values < 0.0001. The diagnosable units correspond to the Daly River; South Alligator River; East Alligator River; drainages that empty into the Gulf of Papua, Papua New Guinea (locations 1–10, Figure 1); and rivers in Indonesian Papua Province (including captive turtles, refer Young et al. (2025) (locations 11–12, Figure 1). These diagnosable units can be considered operational taxonomic units (OTUs).

SNP diversity was also lowest in the Australian OTUs, with observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), allelic richness (Ar) (standardised to n = 13) and the number of private alleles (Pa) all lower (H_o = 0.00026–0.00106, uH_e = 0.00025–0.001, Ar = 1.04–1.08, Pa = 25–249; Table 3) than Papua New Guinea (H_o = 0.00805, uH_e = 0.00815, Ar = 1.45, Pa = 1731) and Indonesian Papua Province (H_o = 0.01023, uH_e = 0.0105, Ar = 1.65, Pa = 4215), which showed the highest diversity levels. *He* was significantly different between

TABLE 2 | Pairwise F_{ST} values between final comparisons of sampling locations (lower triangle) and corresponding 95% confidence limits (upper triangle) based on the 16,002 SNPs scored for 233 individuals.

OTU	Daly	Sth alligator	East alligator	Vialala	PNG	Papua
Daly	—	0.956–0.960	0.968–0.972	0.946–0.951	0.644–0.661	0.659–0.673
Sth Alligator	0.958	—	0.469–0.516	0.829–0.842	0.532–0.550	0.485–0.503
East Alligator	0.970	0.493	—	0.82–0.833	0.514–0.533	0.450–0.468
Vialala	0.949	0.835	0.827	—	0.013–0.022	0.159–0.173
PNG	0.653	0.541	0.524	0.017	—	0.191–0.202
Papua	0.666	0.494	0.459	0.166	0.197	—

Note: All F_{ST} values were significantly different from zero.

all OTUs (p-value <0.05). Overall, there were 11,946 private alleles present in New Guinea turtles collectively in comparison with Australian turtles collectively, and 872 private alleles present in Australian turtles in comparison with New Guinea turtles.

Inbreeding coefficient (F_{IS}) estimates were low across all OTUs and slightly lower in Australian turtles ($F_{IS}=0.00186–0.02469$; Table 3) compared to those in Papua New Guinea (0.06255) and Indonesian Papua Province (0.08186). Intrapopulation genetic distance measured by the proportion of shared alleles showed that turtles within the Daly River OTU are less differentiated ($\mu=0.98$, SD = 0.006) than the South Alligator River ($\mu=0.86$, SD = 0.01) and East Alligator River ($\mu=0.84$, SD = 0.01), Papua New Guinea ($\mu=0.82$, SD = 0.004) and Indonesian Papua Province ($\mu=0.82$, SD = 0.004) OTUs (Figure 5). Kinship coefficients also showed that turtles within the Daly River OTU are more closely related than turtles within other OTUs, with a mean kinship coefficient in the Daly of ($\mu=0.50$, SD = 0.15) compared to the South Alligator ($\mu=0.19$, SD = 0.10), East Alligator ($\mu=0.14$, SD = 0.14), Papua New Guinea ($\mu=0.15$, SD = 0.05) and Indonesian Papua Province OTUs ($\mu=0.06$, SD = 0.09) (Figure 5).

Contemporary effective population sizes (N_e) were lower in Australian OTUs compared to New Guinea OTUs, with the lowest N_e of 48 in the East Alligator River, followed by the South Alligator River with a N_e of 86 and the Daly River of 88 (Table 3). The mean N_e of the New Guinea OTUs was more than 120 times larger than the Australian populations. However, most jackknife 95% confidence intervals, except for the South Alligator OTU, returned infinite as the upper interval. The Indonesian Papua Province OTU, which had the highest nucleotide diversity, expected heterozygosity and allelic richness, also had the highest N_e (10,588; Table 3).

The diyABC analysis favoured the vicariance model over the dispersal models with 4508 votes (posterior probability of $p=0.78$) compared to a sister relationship between Indonesian Papua Province and Australia (PNG(Aus,PP)) with 127 votes ($p=0.02$), a sister relationship between Papua New Guinea and Australia (PP(Aus,PNG)) with 34 votes ($p=0.01$) and a sister relationship between Australia and New Guinea (Aus(PP,PNG)) with 331 votes ($p=0.06$) (Figure S2, Table S9). The Bayes Factor in support of the vicariance model over the dispersal models ranged from strong (13.6) to decisive (135.6) according to the criteria of Kass and Raftery (1995).

4 | Discussion

This study presents comprehensive analyses of mitochondrial and nuclear genetic patterns within the endangered pig-nosed turtle *C. insculpta*, providing insight into their phylogenetic relationships, biogeographical history and current genetic status. Based on the existence of carettochelyid fossil material from southern New Guinea (Glaessner 1942), southeastern Australia (Rule et al. 2022), and possibly northeastern Australia (Joseph-Ouni et al. 2023) during the Late Miocene/Early Pliocene, it is clear that *C. insculpta* has been a long-term resident of Australia and New Guinea. They presumably expanded their range across the expanded emergent Australian continental shelf and colonised Australia during the period climatic conditions favoured the persistence of mesic environments (Byrne et al. 2011; Martin 2006). However, their patterns of persistence remain unclear following the heightened aridity experienced during the Pleistocene.

The islands of Australia and New Guinea have been periodically connected via two land bridges during the low sea-levels of Pleistocene glacial phases, as recently as *ca* 7 Kya between Australia's Cape York and New Guinea (sea-level 12 m below present); and *ca* 12 Kya between Australia's Top End and New Guinea (sea-level 53 m below present) (Chivas et al. 2013; Lambeck et al. 2014; Reeves et al. 2008). Our mitochondrial and nuclear molecular phylogenies each recover the Australian, Papua New Guinea and Indonesian Papua Province turtles as three distinct lineages or Evolutionarily Significant Units (*sensu* Moritz 1994) that diverged during the Chibanian (Middle Pleistocene) (Figure 2). The Australian lineage sister to the New Guinea lineages diverged *ca* 660 Kya (HPD: *ca* 1.102–0.267 Ma), and the New Guinea sister lineages of Papua New Guinea and Indonesian Papua Province, separated by the Torres Strait, diverged *ca* 564 Kya (HPD: *ca* 964–228 Kya). Thus, our data indicate that *C. insculpta* remained isolated without contemporary exchange (i.e., in the PCA and fixed allelic difference analyses) not only throughout the most recent land bridge connections between Australia and New Guinea during the Late Pleistocene/Early Holocene, but also the previous six glacial maxima that have occurred every *ca* 100,000 year, suggesting limited

TABLE 3 | Indicators of genetic diversity and effective population sizes for *Carettochelys insculpta*.

OTU	n	$H_o \pm$ SE	$H_e \pm$ SE	$Pl \pm$ SD	$F_{IS} \pm$ SD	$Ar \pm$ SD	Pa	N_e (95% CI)
Daly	75	0.00026 ± 0.00000043	0.00025 ± 0.0000004	647 ± 87.7	0.0038 ± 0.009	1.04 ± 0.10	249	88.1 (23.6–∞)
Sth Alligator	23	0.00106 ± 0.00000042	0.001 ± 0.00000036	984 ± 67.7	−0.00186 ± 0.008	1.08 ± 0.24	67	86 (46.4–361.8)
East Alligator	13	0.00065 ± 0.00000026	0.00061 ± 0.00000024	636 ± 49.5	0.02469 ± 0.013	1.06 ± 0.23	25	47.7 (18.5–∞)
PNG	85	0.00805 ± 0.00000302	0.00815 ± 0.00000139	7020 ± 120.5	0.06255 ± 0.002	1.45 ± 0.45	1731	7218.3 (3067.7–∞)
Papua	37	0.01023 ± 0.00000225	0.0105 ± 0.00000173	9866 ± 295.7	0.08186 ± 0.002	1.65 ± 0.39	4215	10,588 (4253.2–∞)

Abbreviations: *Ar*, allelic richness; CI, confidence limits; F_{IS}, Fisher's inbreeding coefficient; H_o, observed heterozygosity; n, sample size; N_e, effective population sizes; OTU, operational taxonomic unit; *Pa*, number of private alleles in comparison with all other OTUs; *Pl*, number of polymorphic loci; uH_e, expected heterozygosity corrected for invariant sequence tags.

dispersal opportunity and perhaps a single early dispersal event in the Middle Pleistocene.

Two possible scenarios explain the phylogenetic patterns of divergence. The first is a vicariance model (Aus,PNG,PP) that postulates the long-term persistence of interbreeding populations across Australia and southern New Guinea since the Late Miocene/Early Pliocene. By chance, only the current lineages that had diverged *ca* 660 Kya and *ca* 564 Kya survived throughout subsequent multiple Pleistocene glacial cycles as dwindling populations, with other lineages extirpated. Extant Australian populations are thus postulated to be relicts of a former broader distribution, their range likely gradually reduced by the progressive aridification of the Australian continent, eventually contracting to remaining mesic regions in northern Australia.

Since the Late Miocene/Early Pliocene, the species would likely have occurred in the larger rivers flowing into the Gulf of Carpentaria, including the Roper, Nicholson, Mitchell (Qld) rivers (Figure 1), each of which has well-developed lowland reaches with suitable habitat. Indeed, the presence of the Indonesian Papua Province clade in the Merauke River, which was a tributary of Lake Carpentaria under low sea-levels, suggests that *C. insculpta* was likely able to extend throughout Lake Carpentaria and the rivers of a large portion of the exposed continental shelf. However, Geological evidence suggests that rivers south of the Wenlock River on Cape York received less rainfall during Pleistocene heightened aridity and likely evaporated before reaching Lake Carpentaria (Playà et al. 2007). This suggests that glacial phases would have caused localised extirpations of *C. insculpta* and prevented persistence in the southern Australian Gulf catchments.

A second scenario is a dispersal model (PP(Aus,PNG) that fits well with the current distribution of *C. insculpta*. Under this scenario, *C. insculpta* was a long-term resident of Australia and southern New Guinea but was extirpated from Australia by progressive aridification, with Indonesian Papua Province serving as a refugium. Under this scenario, the populations in northern Australia and Papua New Guinea were re-established via secondary invasion from Indonesian Papua Province *ca* 660 Kya and *ca* 564 Kya respectively, and have since remained isolated. The dispersal event to Australia could have occurred via the now submerged paleochannels draining the Akimeugah Basin, the Arafura Basin and the Northern Territory. Other aquatic species such as fishes (Huey et al. 2014), crayfish (Baker et al. 2008) and freshwater prawns (de Bruyn et al. 2004), with distributions across northern Australia and southern New Guinea, also provide examples of divergences that predate the more recent glacial maxima (reviewed in Huey et al. 2014), indicating there was limited opportunity for dispersal across the land bridges of the exposed continental shelf for aquatic taxa and that heightened aridity during glacial phases was a likely barrier to freshwater biota (Unmack 2001). Support for this scenario comes from the localised co-distributions of freshwater species shared between Australia and New Guinea; the three Australian catchments *C. insculpta* currently inhabits fall within a region that shares the majority of species with New Guinea, the biogeographical Northern Province (Shelley et al. 2019). It is likely that dispersal from Indonesian Papua Province and recolonisation of Australia would have occurred into these Northern Province catchments.

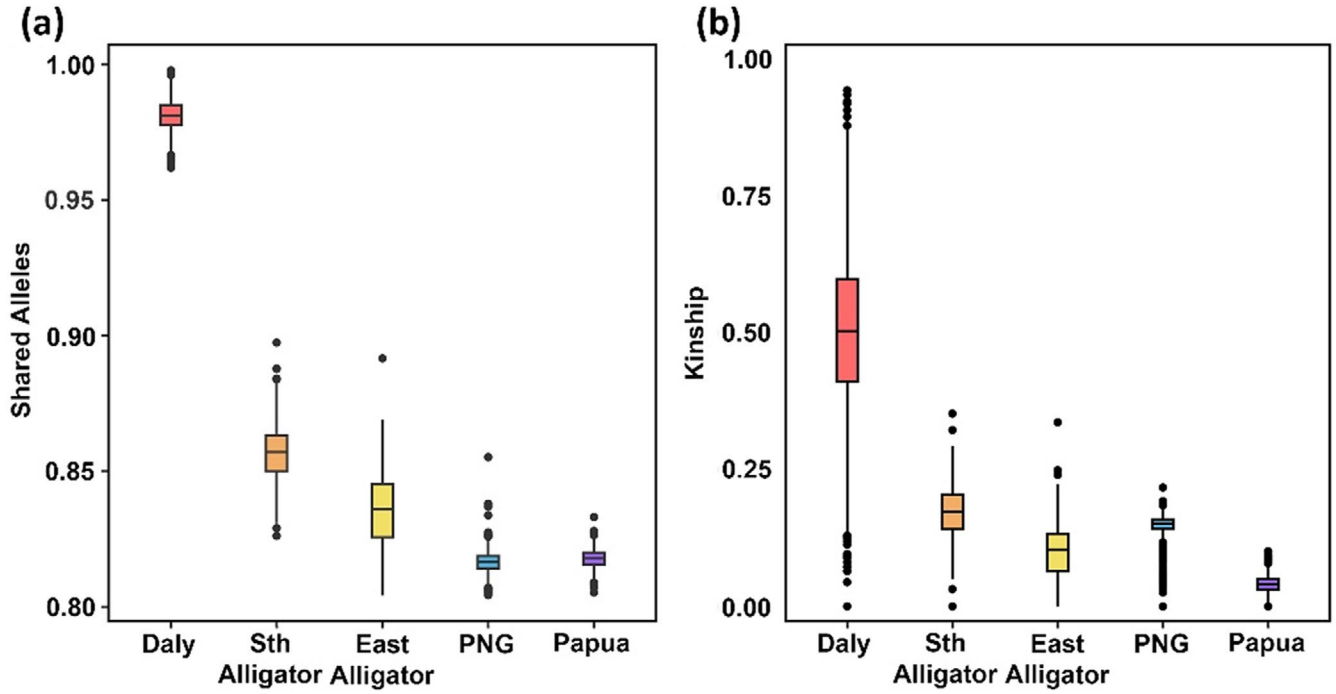

FIGURE 5 | Indicators of genetic diversity of populations of the pig-nosed turtle *Carettochelys insculpta*. (a) Proportion of shared alleles and (b) kinship probabilities based on 16,002 SNPs and 233 individuals from across the species range. The Daly River population has the lowest level of genetic diversity, as indicated by the high proportion of shared alleles and high kinship coefficient.

The ABC random forest analyses compared the above two hypotheses (together with two additional dispersal scenarios) and yielded results that are most consistent with the first scenario of vicariance. Thus, the best supported hypothesis is that the Australian populations of *C. insculpta* are relictual, reduced in extent by progressive aridification and more recently, by low river flows and local extirpation from all but the Daly and Alligator Rivers drainages. The alternate hypothesis of dispersal from the Indonesian Papua refugia is poorly supported, and the paleochannel reconstructions and associated rises (Figure 6) do not identify a clear path for dispersal from Indonesian Papua to the current distribution of *C. insculpta* in Australia. Thus the vicariance model is adopted as the one with the strongest support both in terms of the diyABC analysis and our interpretation of the patterns of paleochannel connectivity.

Historically, carettochelyids were presumed to have been tolerant of marine conditions and to have colonised new continents through trans-oceanic dispersal (Joyce 2014; Rule et al. 2022; Smith et al. 2016). However, although considered highly mobile, reasonably tolerant of saline conditions, and with nesting activities that extend to coastal estuaries and near-offshore islands (Eisemberg et al. 2011; Eisemberg, Rose, Yaru, Amepou, et al. 2015a; Eisemberg et al. 2015b), *C. insculpta* exhibits considerable genetic structure across its range in Australia, suggesting that oceanic dispersal has been limited. High levels of genetic differentiation evident in F_{ST} and numbers of fixed allelic differences observed between the adjacent catchments of the South Alligator River and East Alligator River in Australia suggest that gene flow has been highly restricted, likely not having occurred since the two rivers last coalesced in the historical paleodrainage to the north that emptied over the continental shelf (Figure 6). Behavioural adaptations to aridity in the Australian *C. insculpta* could further explain this structure, as unlike in New Guinea, *C. insculpta* in Australia restricts its activity preferentially to spring-fed reaches of the lowland segments of these rivers during the dry season of the Australian wet-dry tropics (Georges and Kennett 1989) and expands its activities to the adjacent floodplains during the wet season (Doody et al. 2002), rather than moving down to coastal estuaries and mangroves as it does in New Guinea (Eisemberg, Rose, Yaru, Amepou, et al. 2015a; Eisemberg et al. 2015b). In addition, the low levels of genetic differentiation observed between the Vailala River of Papua New Guinea and the interconnected drainages of the remainder of the Gulf of Papua evident in F_{ST} analyses also suggest restricted gene flow. These rivers are separated by only 25 km of ocean beach, indicating that *C. insculpta* in New Guinea is limited in its ability for oceanic dispersal.

The lack of genetic structuring across most drainages in Papua New Guinea and Indonesian Papua Province suggests these regions represent large populations, gene flow occurring among rivers across extensive interconnected floodplains. However, in the absence of these broad lowland river deltas, significant genetic differentiation exists between Indonesian Papua Province and Papua New Guinea turtles, evident in F_{ST} and a fixed allelic difference. The Torres Strait functions as a biogeographic barrier separating the two populations into distinct management units, coinciding with the national boundary between Papua New Guinea and Indonesian Papua (Figure 1). During glacial phases, the Torres Strait land bridge would have separated Indonesian Papua and Papua New Guinea populations. The genetic differentiation appears to have been maintained during interglacial periods when the Torres Strait is submerged, by the stretch of coastline between the Oriomo River in the east and the

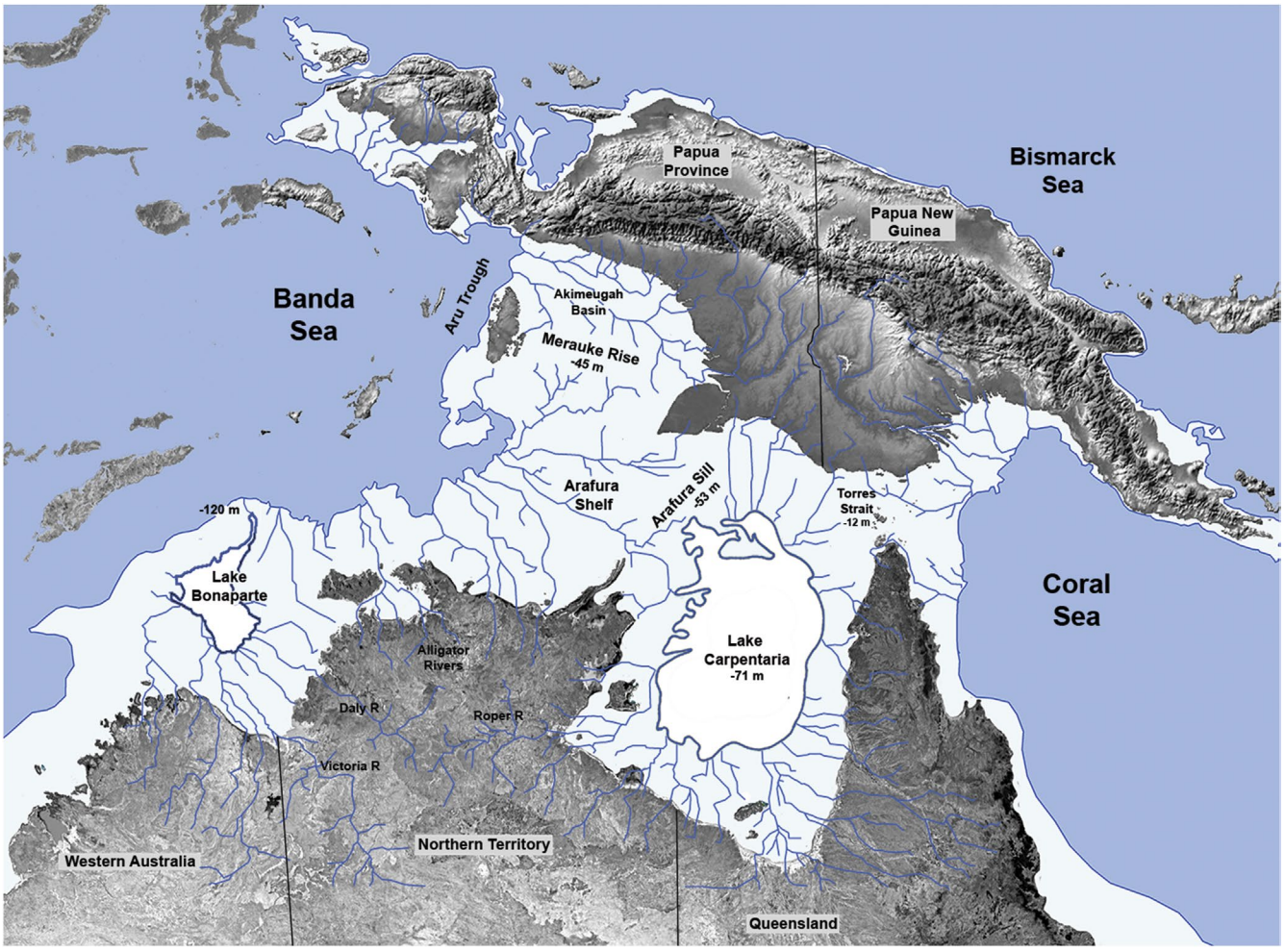

FIGURE 6 | A map of northern Australia and New Guinea showing the extent of the continental shelf and associated land bridges that would have been exposed during a sea-level of -130 m. Paleochannel reconstruction shows the Daly and Victoria rivers flow into Lake Bonaparte, whereas the South and East Alligator rivers join and discharge directly over the continental shelf. Tributaries of Lake Carpentaria flow across the Arafura Sill to discharge directly over the continental shelf. The rivers of Indonesian Papua Province are constrained to the Akimeugah Basin by the Merauke Rise and collectively discharge into the Aru Trough, and the rivers of Papua New Guinea flow east into the Coral Sea. The entrance to Lake Bonaparte would have been closed to the sea only at the lowest of Pleistocene sea-levels. The value of −45 m for the Merauke Rise is the depth at which the feature would have been fully aerial. Bathymetry data were sourced from GEBCO Compilation Group (2023 Grid).

Merauke River in the west, where the intervening rivers (e.g., Bensbach and Morehead rivers) are relatively small, lack extensive coastal mangroves and lack substantial populations of *C. insculpta* (Georges et al. 2006). The paleochannels of Indonesian Papua Province flowed west and north of the Aru Islands in the Akimeugah Basin, bounded to the south by the Merauke Rise and eventually flowing off the continental shelf into the Aru trough (Figure 6), while those immediately west of Torres Strait (Merauke, Bensbach, Morehead) flowed into Lake Carpentaria. Paleochannels draining rivers from Papua New Guinea flowed east of Torres Strait into the Coral Sea (Figure 6), maintaining isolation historically and also during contemporary times by limited dispersal between the two (albeit slight in terms of fixed allelic differences).

At a finer scale, our study detected no significant genetic differentiation in mitochondrial F_{ST} or Φ_{ST} between haplotypes between the coastal and river rookeries within the Papua New Guinea population, or between rookeries among different catchments. This indicates that *C. insculpta* does not exhibit natal philopatry to particular nesting beaches or rivers in Papua New Guinea.

The long-term isolation of *C. insculpta* populations in the monsoonal tropics of Australia from those in New Guinea has resulted in local erosion of genetic diversity in each of the three Australian populations. Local impacts of human activity on each of these three populations (Daly River, South Alligator River and East Alligator River) need to be managed separately as distinct management units. It is likely that these three populations have been in a bottleneck caused by reduced habitat quality and extent from climatic shifts to aridity and sea-level rise causing extirpations, isolation and small population sizes, which has led to the progressive erosion of genetic diversity through genetic drift. Although our estimates of inbreeding coefficients indicate a lack of inbreeding depression, the current effective population sizes are alarmingly low, two orders of magnitude lower than in New Guinea and well below the required levels to prevent inbreeding depression, loss of fitness and to retain a populations evolutionary potential (Frankham et al. 2014). Genetic diversity of turtles

in the Daly River is exceptionally low, as indicated by all indices of diversity, the minimal genetic distance between individuals, high relatedness and the exceptionally low levels of divergence in the SNP maximum likelihood phylogeny (Figure S1). The low heterozygosity observed in Australian populations is comparable to other endangered turtles, the Daly River population having a lower heterozygosity than any other presently studied turtle species (Gallego-García et al. 2021). These low diversity results highlight the need for conservation actions and management of the Australian *C. insculpta* populations.

4.1 | Conservation Implications

In light of the bottleneck and high levels of genetic drift having reduced the genetic diversity of Australian populations, the extremely high levels of genetic structuring evident in F_{ST} and fixed allelic differences among Australian populations and between Australian populations and those in New Guinea should be conservatively interpreted in a taxonomic sense (Coates et al. 2018). Our coding mitochondrial *ND4 p*-distances confirm population-level differentiation, rather than what might have otherwise been assigned as species-level differences solely based on the large differentiation of the nuclear datasets due to genetic drift. Without a companion morphological analysis, we are unwilling to recommend changes to the current taxonomy. We regard the distinct lineages that we have identified as lineages within a single widespread species subject to a complex history of isolation and interconnection in the Australo-Papuan region. Others may draw upon our data to complement further analyses based on multiple lines of evidence to resolve the taxonomy and the need to place names on the mtDNA and nuclear lineages we have identified.

The low levels of genetic diversity evident in Australian populations compared with those of New Guinea are of considerable conservation concern, implying an uncertain future with a high risk of extinction due to reduced capacity to adapt to rapid environmental change (Pauls et al. 2013; Sgrò et al. 2011) and other fitness impacts, such as an increased susceptibility to exotic disease outbreaks (Hoffman et al. 2014; Pearman and Garner 2005), which have recently decimated turtle species elsewhere in Australia (Zhang et al. 2018).

Although genetic signatures of local adaptation were not assessed in this study, the long isolation between Australian and New Guinea populations provides sufficient time for possible accumulation of genetic incompatibilities and adaptations to different environments, which if mixed, may lead to possible outbreeding depression (Frankham et al. 2011). However, local adaptations are unlikely to persist through genetic bottlenecks and high genetic drift (Ralls et al. 2018), as experienced by the Australian populations. Therefore, maximising genetic diversity should be a greater priority over minimising the introduction of putatively harmful genetic variation (Ralls et al. 2020).

As a first step, translocating male turtles between Australian populations would provide a small increase in nuclear diversity while maintaining unique mitochondrial distinctiveness of each catchment. This would be beneficial to maintain mitochondrial genetic structuring for wildlife forensics purposes with this heavily trafficked species. Optimal strategies for genetic rescue through assisted gene flow would need to be carefully considered, possibly involving captive breeding programmes to first assess the viability of outbred individuals between New Guinea and Australia.

In conclusion, we have demonstrated that long-term biogeographical processes such as aridification and sea-level fluctuations of Pleistocene glacial cycles can impact on freshwater taxa, with long-standing impacts on their genetic diversity. The patterns observed in genomic and mitochondrial divergence have allowed for the delineation of conservation units and the identification of populations requiring genetic rescue. Driving influences on the genetic structure of *C. insculpta* include a combination of historical paleodrainage connections and isolation caused by high sea-levels during the current interglacial period. Our study also highlights the crucial role fossils can play in understanding extinction and current biogeographic patterns. Many groups lack a useful fossil record, and investigators are constrained to assume that a group's current distribution is similar to its past distribution, interpreting biogeographic patterns within those constraints. *Carettochelys insculpta* provides an outstanding example of how a species' range can change over a few million years, essentially losing range from the Victoria, Australia fossil to the current Australian populations over a 3000 km straight line distance or *ca* 14° of latitude. This underscores the critical role extinction plays, albeit rarely witnessed, in understanding a species' biogeographic history.

Author Contributions

M.J.Y. designed research, performed research, contributed reagents/analytical tools, analysed data and prepared an early draft of the paper. P.J.U. provided guidance on the phylogenetic analyses. B.G. provided guidance for all analyses involving R programming and population genetics. A.G. and P.J.U. contributed substantially to the interpretation of the results and to preparing the final draft. All authors contributed to writing the final draft.

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Ethics Statement

All samples were collected with approvals from the Papua New Guinea Conservation and Environment Protection Authority (CEPA) and Australian Northern Territory government, with approvals from the University of Canberra animal ethics committee, and transported internationally under CITES permits issued by CEPA and in accordance with IATA shipping conditions.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

SNP data and R Scripts to access the SNP data are available on Dryad (https://doi.org/10.5061/dryad.qrfj6q5pb). Mitochondrial DNA sequences are available from GenBank (accession numbers PP213549–PP213645 for ND4 + tRNAs and PP213646–PP213742 for control region + tRNA-Pro).

Peer Review

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.