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Genetic diversity of an undescribed cryptic maskray (*Neotrygon* sp.) species from Fiji

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ABSTRACT

Context. The extinction risk of sharks and rays exceeds that of most other vertebrates. Genetic analyses can help identify conservation risks. **Aims.** Identification of Fiji's maskray and testing the null hypothesis of no genetic differentiation within the species over time. **Methods.** Mitochondrial DNA cytochrome oxidase subunit 1 (*COI*) barcoding was used for species identification, and DArT-seq technology to monitor the genetic diversity. Cohort samples were collected in 2015 and 2022. A subset from each cohort was barcoded. The genetic survey was complemented by a size comparison between the two cohorts. **Key results.** Barcoding of the *COI* gene showed a maximum similarity of 97.84% to Kuhl's maskray (*Neotrygon kuhlii*) and 96.83% to the Coral Sea maskray (*Neotrygon trigonoides*), but no higher-level distinct species match to reference sequences in the Barcode of Life Datasystem. Genotyping of 56 individuals in two cohorts yielded 21,293 single nucleotide polymorphisms (SNPs), and 3871 SNPs per individual were retained. The neutral genetic diversity remained stable over time. The 2015 cohort showed positive inbreeding, with one full-sibling pair identified in each cohort. Body size comparisons indicated a significant reduction in disc length and width in the 2022 cohort. **Conclusions.** The smaller body size of the 2022 cohort may hint at increased fishing pressure, but genetic diversity has not been affected. Thus, the null hypothesis is accepted. **Implications.** These findings provide insights into the genetic diversity of Fiji's maskray and enable a genetic comparison with current *Neotrygon* species known in the region. Taxonomy confirmation is needed, but the presence of a cryptic or potentially new maskray in Fiji seems plausible.

Keywords: batoids, *COI* barcoding, Dasyatidae, elasmobranchs, morphology, Oceania, single nucleotide polymorphisms, taxonomy.

Introduction

Globally, shark and ray populations are declining due to overfishing and climate pressures (Dulvy *et al.* 2021; Osgood *et al.* 2021). Despite considerable interspecific and intra-specific life-history variation (Last *et al.* 2016*a*; Bradley *et al.* 2017), many sharks and rays exhibit late maturity, low fecundity, long gestation, and slow growth, making them particularly vulnerable to fishing pressure (Cortés 2000). Additionally, their reliance on nurseries (Heupel *et al.* 2007) and philopatric behaviour (Chapman *et al.* 2015) increase the risk of local extirpation. Small populations are prone to inbreeding and genetic drift, resulting in a loss of genetic diversity (Rus Hoelzel *et al.* 2006; Allendorf *et al.* 2012). This, in turn, reduces the species' ability to adapt to environmental changes and evolve (Frankham 2003; DiBattista 2008; Hernández *et al.* 2015). Therefore, it is crucial to preserve genetic diversity to avoid these adverse consequences.

Genetic data support conservation research and management, by facilitating the detection of genetically distinct populations, the measurement of genetic connectivity, and the identification of the risks associated with demographic change and inbreeding (Allendorf *et al.* 2012). Advances in the use of molecular markers have significantly improved the delineation of population structures in sharks and rays (Feldheim *et al.* 2001; Luikart *et al.* 2018; Ovenden *et al.* 2018). For example, extensive population structure in the white-spotted eagle ray (*Aetobatus narinari*) in the Indo-Pacific is evident (Schluessel *et al.* 2010). Also,

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there is a strong regional differentiation in the thornback ray (*Raja clavata*) between the Mediterranean basin, the Azores and the European continental shelf (Chevolot *et al.* 2006). High divergence across oceanic basins and lower differentiation along continuous coastal habitats was linked to the short-tailed stingray (*Dasyatis brevicaudata*) (Le Port and Lavery 2012). Strong ocean currents and bathymetry can limit connectivity in species with active dispersal; for example, the Indonesian through-flow current influenced genetic differences in species from the maskray complex (*Neotrygon* sp.) (Borsa *et al.* 2012; Puckridge *et al.* 2013), while the steep bathymetry of the Southern California Bight drove genetic differences in round stingrays (*Urobatis halleri*) (Plank *et al.* 2010), at small geographical scales. Generally, biogeographic barriers, ocean distances, behaviour – particularly reproductive philopatry – movement ecologies, and habitat preferences affect gene flow and genetic differentiation in elasmobranchs (Dudgeon *et al.* 2012; Hirschfeld *et al.* 2021; Devloo-Delva *et al.* 2023; Postaire *et al.* 2024).

However, the current elasmobranch literature on intraspecific genetic differentiation is skewed towards sharks (Hirschfeld *et al.* 2021), with stingrays (Myliobatoidei) (Aschliman *et al.* 2012) remaining one of the least understood groups of vertebrates in terms of molecular genetics (Beheregaray 2008). Furthermore, population genetic structures can vary spatially and temporally, affecting the accuracy of inferences based on changing spatial patterns over time (Kornfield *et al.* 1982; Lacson and Morizot 1991). Temporal genetic studies on shorter time scales, typically conducted over the course of a few years or within a single generation have primarily relied on seasonally recurring aggregations (Lieber *et al.* 2020) as well as the comparison of spatio-temporal genetic patterns within juvenile populations and between juvenile and adult cohorts (Glaus 2019; Kuguru *et al.* 2019; Liu *et al.* 2023). Collecting time-separated samples enables temporal genetic monitoring and assessing changes in genetic variation (Domingues *et al.* 2018). This can reveal significant temporal genetic differentiation, suggesting stochastic effects from a small effective population size or previously undetected genetic subpopulations or stocks (Hedgecock 1994). Furthermore, collecting samples at multiple time points reduces the likelihood of including closely related individuals within the sample. Thus, preventing genetic structure from being an artefact of related individuals sampled at the same location (Devloo-Delva *et al.* 2019). Altogether, documenting genetic diversity (and where analyses permit, the calculation of effective population sizes over time) is fundamental in marine conservation genetics and particularly critical for long-lived species such as rays (Waples *et al.* 2008; Goldman *et al.* 2012; Domingues *et al.* 2018).

Species of the genus *Neotrygon*, commonly known as 'maskrays,' are native to the Indo-West Pacific region (Last *et al.* 2016*a*). *Neotrygon* (Castelnau 1873) was previously treated as a subgenus of *Dasyatis*. Based on more recent morphological and molecular analyses, the subgenus was elevated to the generic level (Last *et al.* 2016*b*, 2016*c*), and now belongs to the family Dasyatidae. Molecular analyses have also revealed discrete genetic diversity within this group and indicated the occurrence of several cryptic species (Arlyza *et al.* 2013; Puckridge *et al.* 2013). Due to morphological ambiguity, it is often difficult to distinguish between closely-related maskrays (Arlyza *et al.* 2013; Puckridge *et al.* 2013). Thus far, at least 11 species have been described from the species complex' under the genus *Neotrygon* (Last *et al.* 2016*a*, 2016*c*; Hata and Motomura 2024). Maskrays are demersal, inhabiting intertidal sand flats, coral reefs, lagoons and slopes and as many other stingrays, they are likely susceptible to habitat degradation (Jabado *et al.* 2018). According to the Red List criteria of the International Union for the Conservation of Nature, maskrays are assessed as: Data Deficient (*Neotrygon ningalooensis* (Ferretti and White 2015), *Neotrygon kuhlii* (Kyne and Finucci 2018); Least Concern (*Neotrygon caeruleopunctata* (Sherman *et al.* 2021*a*), *Neotrygon leylandi* (Pierce and Kyne 2015), *Neotrygon orientalis* (Sherman *et al.* 2022*a*), *Neotrygon picta* (Pierce *et al.* 2015), *Neotrygon trigonoides* (Sherman *et al.* 2021*b*), *Neotrygon varidens* (Sherman *et al.* 2022*b*); and Near Threatenend (*Neotrygon annotata* (Jacobsen *et al.* 2015), *Neotrygon australiae* (Sherman *et al.* 2021*c*).

Fiji's maskray (Fig. 1*a*, *b*) has traditionally been recognised as Kuhl's maskray (*N. kuhlii*) (Fig. 1*c*), while its colour patterns rather resemble the Coral Sea maskray (*N. trigonoides*) (Fig. 1*d*). In-country species identification for the maskray in Fiji is lacking and so are data on its ecology, life-histories, and genetic-population structure. Hence, mitochondrial DNA (mtDNA) ytochrome oxidase subunit 1 (*COI*) barcoding (Ward *et al.* 2005) is required to verify Fiji's maskray species. As the maskray is the most frequently caught and traded ray in the country's small-scale fishery activities (Glaus *et al.* 2024*a*) combined with the low reproductive output of congeneric species (Pierce *et al.* 2009), it might be susceptible to even light fishing pressure.

This study used *COI* barcoding to identify Fiji's maskray. Moreover, temporal monitoring of genetic diversity (utilising genome-wide single nucleotide polymorphism (SNP) markers), spanning 7 years through the analysis of time-separated samples was undertaken. The null hypothesis of no genetic differentiation within the species over time was tested. This documentation of the species' temporal genetic diversity was complemented by comparing disc length (DL) and disc width (DW) between two temporal groups. Together, these results contribute valuable insights into the genetic diversity of Fiji's maskray and enable a genetic comparison with current *Neotrygon* species known in the region.

Fig. 1. Congeneric maskray species: (*a*, *b*) Fiji's maskray, (*c*) Kuhl's maskray, (*d*) Coral Sea maskray. Photo credit: (*a*) Alison Smith, (*b*) Tom Vierus, (*c*, *d*) both derive from iNaturalist.

Materials and methods

Sample collection

To obtain samples for molecular species identification, temporal genetic diversity analysis, and DL and DW measurements for size comparison, surveys at the Suva fish market on Fiji's main island Viti Levu (Fig. 2) were conducted in 2015 and in 2022 (Glaus *et al.* 2024*a*). Additionally, the Sigatoka fish market (Fig. 2) was visited occasionally in 2022. The Suva fish market is Fiji's prime market. A variety of reef fishes are sold alongside invertebrates such as octopus, freshwater mussels, crabs, and lobsters (Mangubhai *et al.* 2017; Tukana *et al.* 2023). The majority of rays sold there are caught within the Rewa Estuary, and Tailevu, including Bau (Fig. 2), but they also originate from nearby locations and as far as Kadavu, although precise catch locations and GPS data are unknown (Glaus *et al.* 2024*a*). In total, 49 samples were collected in 2015 and 40 in 2022. DW and DL size measurements were recorded whenever possible, while a subset of samples per cohort was used for DNA *COI* barcoding (see section below). Prior to sampling, market vendors were informed about the purpose of the surveys. DL and DW measurements and tissue sample collection were only conducted with the verbal permission of each respective vendor. In many rays, the disc includes the combined head, trunk, and pectoral fins (Last *et al.* 2016*a*). DL was measured from the tip of the head to the end of the pelvic fins, while DW was measured from the tip of the left pectoral fin to the tip of the right pectoral fin. Vendors were also asked to specify where the respective maskrays were captured. Tissue samples (fin clips, 1 cm^2) were stored in 95% ethanol until DNA extraction. Samples then underwent polymerase chain reaction (PCR) amplification, library preparation and sequencing (Sanger sequencing for barcoding and Illumina sequencing for SNP genotyping). No maskrays were bought for this study to avoid any inadvertent incentives or demand.

mtDNA *COI* barcoding

The mtDNA *COI* gene is one of the most widely used gene markers for species identification (Ward *et al.* 2008). Tissue samples from both cohorts underwent *COI* barcoding. Specifically, seven samples from the 2015 cohort were barcoded at Diversity Arrays Technology, Australia, using DArT-MP proprietary processes, while 12 tissue samples were processed at ETH Zurich and sequenced at Microsynth, Switzerland. The same primers were used by Diversity Arrays Technology and by the ETH Zurich. For the latter, DNA was extracted with the Qiagen Blood and Tissue kit, following standard protocols (Qiagen Inc., Valencia, CA, USA). A 652-bp fragment from the 5′ region of the *COI* was PCR amplified using FishF2 (5′ -35′TCGACTAATCATAAAGATATCGGCAC3′), FishF2N (5′ATCTTTGGTGCATGAGCAGGAATAGT3′), and FishR2 (5′ACTTCAGGGTGACCGAAGAAGAATCAGAA3′) primers (Ward *et al.* 2005). Resulting sequences were identified by

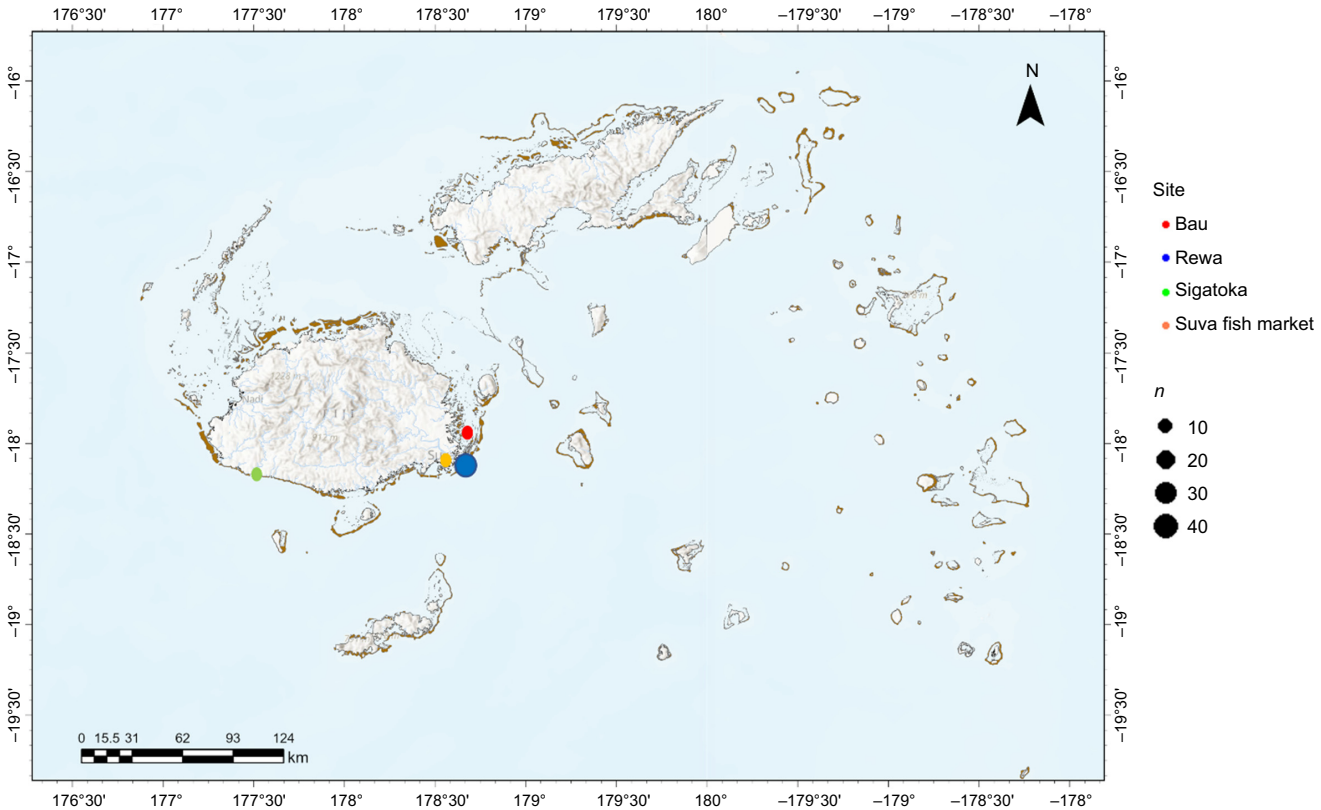

Fig. 2. Map of the Fiji Islands. Tissue samples of 89 maskrays were collected; 49 samples in 2015 and 40 samples in 2022. In total, 56 maskrays were genotyped successfully. Maskrays used for SNP genotyping were captured in the Rewa Estuary (blue, $n = 45$), Bau (red, $n = 7$) and Sigatoka (green, $n = 2$). Orange refers to the Suva fish market, where most tissue samples were acquired. Catch sites were unknown for two specimens sold at the Suva fish market.

using the Identification Engine at the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007) and by Basic Local Alignment Search Tool (BLAST) (Madden 2003). See Table 1 for Accession numbers.

The *COI* sequences from the 19 specimens (across the two cohorts) were combined with a selection of publicly available *COI* data from 24 *Neotrygon* specimens available in BOLD and NCBI GenBank (BOLD Process IDs and GenBank Accession numbers are included here for each *Neotrygon* specimen). The final alignment comprised of 43 specimens of *Neotrygon* across 695 nucleotide positions. *COI* pairwise genetic distances were calculated in MEGA-X (Kumar *et al.* 2018) with the phenetic relationship among the specimens inferred by a maximum likelihood fitting tree with 10,000 bootstrap replications used following determination of the best fit nucleotide model of Hasegawa-Kishino_Yano (H-K-Y + G (Hasegawa *et al.* 1985). Additionally, newly sequenced Fijian *Neotrygon* individuals from this study were submitted to GenBank; Accession numbers are in Table 1.

DArT-Seq: extraction and SNP sequencing

SNP genotyping was also performed at Diversity Arrays Technology using DArT-Seq, where genomic DNA was extracted using standard robotic methods. DNA was then processed for reduced representation library construction, sequenced and genotyped by DArT-Seq following previously developed and tested complexity reduction protocols for scalloped hammerhead sharks (*Sphyrna lewini*) (Marie *et al.* 2019). Briefly, genome complexity reduction was achieved with a double restriction digest using a *Pst*I and *Sph*I methylation-sensitive restriction enzyme combination. Libraries were sequenced on an Illumina HiSeq 2500 platform, and raw reads obtained following sequencing were processed using Illumina CASAVA ver. 1.8.2 software for initial assessment of read quality and sequence representation. Enzymes and libraries were the same as used for *S. lewini*, albeit a different adapter sequence (NeotrygonP_ad_sp4) with shorter fragments was used. The DArT-PL proprietary software pipeline, DArTtoolbox was implemented for further filtering and variant calling to generate the final genotypes set.

SNP quality control filtering

Quality control filtering steps were performed on the dataset; these included (excluding duplicate SNPs possessing identical Clone IDs); removing loci with a call rate (proportion of individuals scored for a locus) < 90%; and maintaining SNPs with a read depth > seven and minor allele frequencies <2%. Detection of loci under selection was tested using BayeScan

Table 1. Comparing the percentage similarity in *COI* sequences between both cohorts and reference sequences using BLAST in GenBank and BOLD (pairwise identity).

2015 cohort ID	BLAST similarity %	BLAST reference	BOLD similarity %	BOLD reference	Accession#
SV_2015_37	97.15%, *N. kuhlii*	KU498038.1	97.10%, *N. kuhlii*	GBGC10627-13	OR976143
SV_2015_4	97.10%, *N. kuhlii*	KU498038.1	97.10%, *N. kuhlii*	GBGC10627-13	OR976144
SV_2015_38	96.99%, *N. kuhlii*	KU498038.1	96.94% *N. kuhlii*	GBGC10627-13	OR976145
SV_2015_47	97.15%, *N. kuhlii*	KU498038.1	97.26%, *N. kuhlii*	GBGC10627-13	OR976146
SV_2015_40	97.15%, *N. kuhlii*	KU498038.1	97.10%, *N. kuhlii*	GBGC10627-13	OR976147
SV_2015_48	96.84%, *N. kuhlii*	KU498038.1	97.25%, *N. kuhlii*	GBGC10627-13	OR976148
SV_2015_33	97.25%, *N. kuhlii*	KU498038.1	97.04%, *N. kuhlii*	GBGC10627-13	OR976149
2022 cohort ID	**BLAST similarity %**	**BLAST reference**	**BOLD similarity %**	**BOLD reference**	**Accession#**
SV_110622_4	96.71% *N. kuhlii*	KU498038.1	96.71% *N. kuhlii*; *N. trigonoides*; *N. westpapuensis*	LIFS993-08 ANGBF13149-18 SOPNG125-18	OR839855
SV_230422_7	96.18% *N. kuhlii*; *N. trigonoides*	KU497960.1 KU498033.1	96.08% *N. kuhlii*; *N. trigonoides*	ANGBF13157-18 GBGC12837-13	OR839856
SV_090422_1	96.91% *N. kuhlii*	KU498038.1	96.89% *N. kuhlii*	ANGBF13226-18	OR840698
SV_260322_5	96.25% *N. kuhlii*	KU497960.1	96.25% *N. kuhli*	ANGBF13157-18	OR839857
SV_130522_1	96.02% *N. kuhlii*; *N. trigonoides*	KU497960.1 KU498033.1	95.93% *N. kuhlii*; *N. trigonoides*	ANGBF13157-18 GBGC12837-13	OR839858
SV_090422_3	96.77% *N. kuhlii*	AB485685.1	96.77% *N. kuhlii*	GBGC10609-13	OR839859
SV_260322_1	95.87% *N. kuhlii*; *N. trigonoides*	KU498017.1 KU498033.1	96.83% *N. kuhlii*; *N. trigonoides*	ANGBF48132-19 ANGBF48182-19	OR839860
SV_110622_1	96.68% *N. kuhlii*	KU498038.1	96.62% *N. kuhlii*; *N. trigonoides*	ANGBF13226-18 LIFS993-08	OR839861
SV_210522_1	96.26% *N. kuhlii*	KC250643.1	96% *N. kuhlii*; *N. trigonoides*	ANGBF13226-18 LIFS993-08	OR839862
SV_140422_1	95.59% *N. trigonoides*	KU498033.1	94.44% *N. kuhlii*; *N. trigonoides*	ANGBF13157-18 GBGC12837-13	OR839863
SV_140422_4	96.72% *N. kuhlii*: *N. trigonoides*	KU497960.1 KU498033.1	97.84% *N. kuhlii*	GBGC10609-13	OR839864
SV_110622_2	95.57% *N. kuhlii*; *N. trigonoides*	KU498017.1 KU498033.1	95.54% *N. kuhlii*; *N. trigonoides*	ANGBF48132-19 ANGBF48182-19	OR839865

GenBank accession numbers given for samples from the 2015 and 2022 cohorts.

ver. 2.1 (Foll and Gaggiotti 2008). The most conservative neutral model in BayeScan was used to minimise falsely detected loci under selection (Lotterhos and Whitlock 2014). Runs consisted of 100,000 iterations with a burn-in length of 50,000 iterations (Foll and Gaggiotti 2008; Foll 2012). Once probabilities had been calculated for each locus, the BayeScan function plot_R was used in the R ver. 3.2.0 statistical package (Venables *et al.* 2009) to identify putative outlier loci. A range of false discovery rate (FDR) values from 0.01 to 0.20 were evaluated based on preliminary testing and recommendations by Gondro *et al.* (2013).

Allelic diversity, population structuring and relatedness

Allelic diversity indices including average observed (H_o), expected (H_e), and unbiased expected heterozygosity corrected for population sample size ($H_{n.b.}$) were computed in Genetix v.4.05.2 (Belkhir 1999), together with the inbreeding coefficient (F_{IS}). Pairwise F_{ST} estimates (Reynolds *et al.* 1983) were calculated in Arlequin ver. 3.5.1.3 followed by correction of significance levels for pairwise testing (Excoffier and Lischer 2010). Also, an analysis of molecular variance (AMOVA) was performed in Arlequin ver. 3.5.2.2 (Excoffier 2015) using 10,000 permutations to estimate *F*-statistics to detect population genetic partitioning between cohorts. ML-Relate (Kalinowski *et al.* 2006) and COLONY (Jones and Wang 2010) were used for relatedness analysis within cohorts. For COLONY, a weak sibship prior was chosen as input parameter. The maskrays were analysed both as a single population comprising all specimens, as well as two separate populations representing the 2015 and 2022 cohorts.

The number of genetic groups in the Fiji maskray SNP dataset for the genotyped individuals was estimated using

the Bayesian model-based clustering algorithm implemented in Structure (ver. 2.3.4) (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009) run using an admixture model (without *a priori* knowledge of location) with correlated allele frequencies. Following preliminary model testing, Structure was run with K (number of clusters) set between 2, 8 and 10 independent runs per K. The Structure algorithm inferred the proportion of ancestry for each individual from each cluster; each independent run had a burn-in of 50,000 followed by 100,000 Markov chain Monte Carlo (MCMC) iterations.

The seven K Structure runs were then processed using the Greedy algorithm on the CLUMPAK (Clustering Markov Packager Across K) server (Kopelman *et al.* 2015) to find the preferred value of K (based on ΔK) (as per (Evanno *et al.* 2005) which demonstrated the uppermost level of structure in the SNP dataset. Clustering analysis on CLUMPAK was visualised with distruct (Rosenberg 2004).

K-means clustering and Discriminant Analysis of Principal Components (DAPC), a non-model based method in the R package adegenet ver. 1.4.2 (Jombart 2008; Jombart and Ahmed 2011) additionally provided description of genetic clusters in the SNP data (based on discriminant functions), whereby genetic proximity of individuals to clusters was determined based on all included neutral loci. An optimised a-score for DAPC was used to determine the number of principal components (n = 14 PCs) that were retained for the assignment analyses. The K-means clustering algorithm in adegenet (run for K = 1–K = 6) also compared clustering solutions based on Bayesian Information Criteria (BIC) after transformation of the data using a Principal Component Analysis (data not shown).

Morphology analysis

Histograms in R were used to visualise the DL and DW distribution in both cohorts (Wickham *et al.* 2019). Mean sizes-at-maturity in a congeneric maskray were 314 mm DW in females and 294 mm DW in males (Pierce *et al.* 2009). Here, only deviations from means were detected in two females and one male. The respective male belonged to the 2022 cohort and had calcified claspers, which suggest that it reached sexual maturity (Awruch 2015). Due to the uncertainties surrounding the application of size-at-maturity estimates from a congeneric maskray in Australia to the maskray species in Fiji, and acknowledging that size alone cannot reliably determine sexual maturity (as larger individuals may still be immature, while smaller ones could already have reached maturity (Pierce *et al.* 2009), all specimens in both cohorts were retained. Overall, the size-related dataset included 49 maskrays from the 2015 cohort and 40 maskrays from the 2022 cohort. To determine whether there was a significant difference in DL and DW between the two cohorts, Welch's t-tests were performed in R (Wickham *et al.* 2019), due to unequal variances (West 2021).

Compliance with ethical standards

This research was approved by The University of the South Pacific (USP), by the Provincial Councils, and by Pacific-European Union Marine Partnership PEUMP Project Management. All sampling procedures were approved by the USP Research Committee and performed in accordance with relevant guidelines and regulations. Also, in accordance with protocols of the USP, Provincial Councils were consulted, to explain the research, objectives, methodologies and expected outcomes prior to the data collection.

Results

COI barcoding

Only seven samples from the 2015 cohort (due to compromised samples and lower quality extracted DNA post sample processing), and a subset of 12 samples from the 2022 cohort underwent *COI* barcoding. For both cohort subsets, the molecular barcoding results did not yield a definitive species match. The closest match for both cohorts was with *N. kuhlii* at 97.25% for the 2015 cohort and at 97.84% for the 2022 cohort, respectively (Table 1). As these percentage matches demonstrate (Fig. 3), these 19 Fijian maskray specimens clustered together and separately to all other sequenced *Neotrygon* individuals.

SNP filtering

In total, 16 samples (also due to compromised samples and lower quality extracted DNA post sample processing) from the 2015 cohort and all 40 samples from the 2022 cohort were genotyped. Genotyping by sequencing resulted in 21,293 SNPs prior to quality control filtering (Carson *et al.* 2014), and 3871 filtered SNP loci. Among the SNPs that passed all quality control filters, no SNPs were identified as outlier loci putatively under positive selection (FDR 1%).

Genomic diversity, relatedness and DAPC

Except for H_o, which is higher in the 2022 cohort, population-level indices of genetic diversity, including $H_{n.b.}$, H_e, were similar or identical across the two temporal cohorts (Table 2). Based on the 3871 SNP loci in the filtered data set screened across both cohorts, 1225 and 3807 loci were polymorphic in the 2015 and 2022 cohort, respectively. Additionally, a large number (n = 2498) of SNP loci in the 2015 cohort were not successfully genotyped (possibly due to poorer quality sample DNA, see above). Inbreeding estimates were higher in the 2015 cohorts, with a F_{IS} value of 0.25, compared to a F_{IS} value of 0.00 in the 2022 cohort. F_{ST}-estimates (F_{ST} = 0.003, P = 0.150) and AMOVA results (F_{ST} = −0.00296, P = 0.988) indicated there was no genetic differentiation between the two temporal cohorts (Table 2).

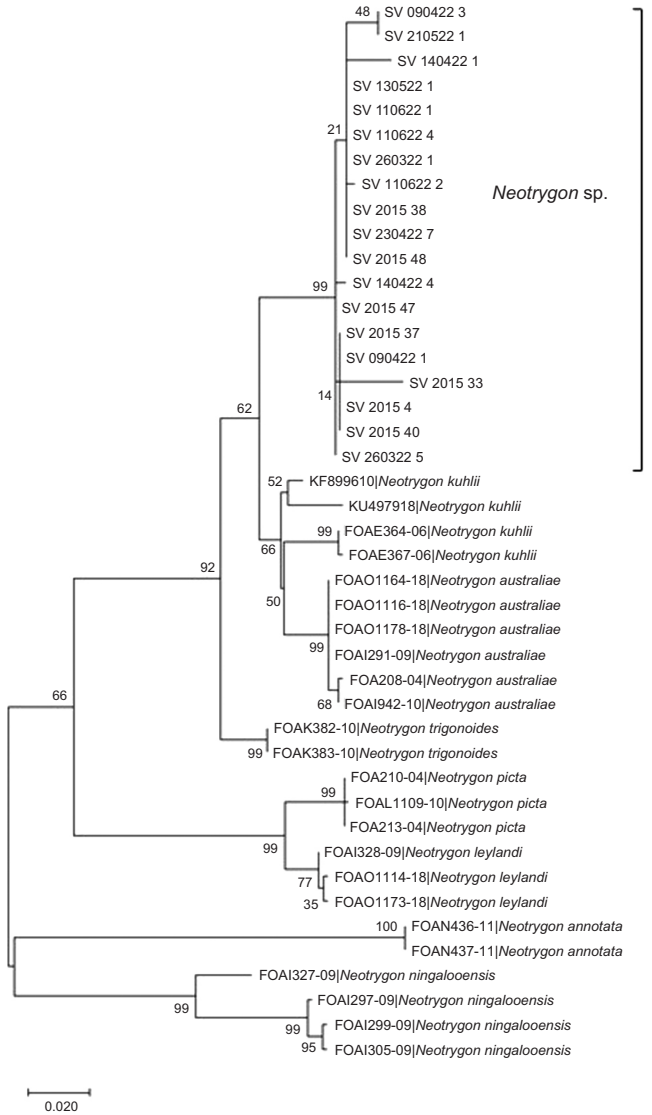

Fig. 3. Maximum-likelihood tree of eight species of *Neotrygon*, including Fiji maskrays sequenced at the *COI* mtDNA gene in 43 specimens and based on the H-K_Y model. Tree shown here has the highest log likelihood following 10,000 bootstrap replications. Each specimen is identified by either its BOLD Process ID or GenBank Accession Number. In this tree, each species forms a group. Numbers on nodes indicate bootstrap values.

Both cohorts yielded a single full-sibling pair, which were identified in COLONY and ML-Relate. COLONY results indicated a probability of 1.00, and hence a high confidence level in the inferred sibship dyads. No relatedness was detected between the two cohorts.

Based on Structure with the admixture model and correlated allele frequencies among the two cohorts, the ΔK Evanno criterion (Evanno *et al.* 2005) was identified as multi-modal peaks at $K = 3$ and 7 (Fig. 4), and the Probability K method by Pritchard *et al.* (2000) was identified as a single

Table 2. Comparison of genetic diversity and differentiation measures between the 2015 and 2022 cohort samples, and the combined cohort of the Fiji maskray, based on 3871 SNPs.

Cohort	*N*	H_o	H_e	$H_{n.b.}$	F_{IS}	Pairwise estimate	AMOVA
2015	16	0.22	0.30	0.30	0.27		
2022	40	0.30	0.29	0.30	0.00		
All samples	56	0.28	0.30	0.30	0.07	$F_{ST} = 0.003$, $P = 0.150$	$F_{ST} = -0.00296$, $P = 0.988$

Genotyping metrics: *N* (samples genotyped); H_o (observed heterozygosity); H_e (expected heterozygosity); $H_{n.b.}$ (unbiased expected heterozygosity); F_{IS} (inbreeding coefficient) per cohort; F_{ST} based on pairwise estimate and AMOVA.

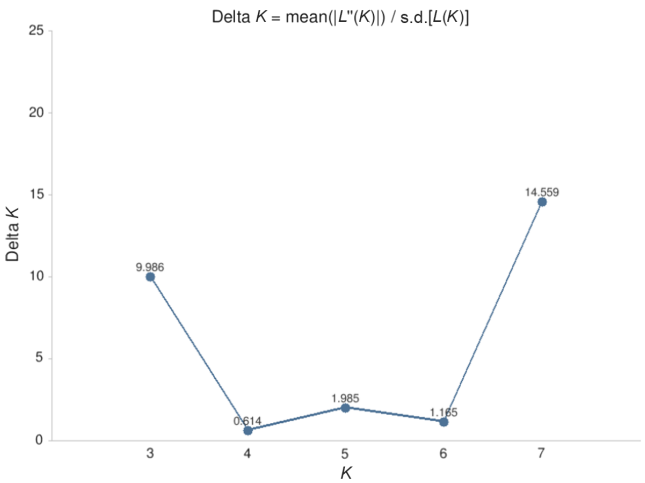

Fig. 4. Evanno output from seven sets of Structure runs, analyses based on SNPs in the Fijian maskray cohorts. A bi-model Delta *K* is shown.

peak at $K = 7$ (see Supplementary material Fig. S1, Prob(K). = 1.000). Based on these two Bayesian Structure models, the optimal number of clusters in the data was somewhat unclear. The output from the summary Clumpak pipeline is given in Fig. S2. Based on DAPC, one linear discriminant function was identified. The DAPC density plot likely represents the difference in the numbers of polymorphic and of usable loci across the individuals (Fig. S3) with the composition plot highlighting that the two groups are not entirely separate, with several individuals from the 2015 cohort displaying membership probabilities more closely with that of the 2022 cohort (Fig. 5). Based on BIC, the number of clusters detected by DAPC was $K = 2$ (Fig. S4).

Morphology analysis

Welch's *t*-test results showed that individuals from the 2022 cohort exhibited significantly smaller DL and DW compared to their conspecifics in the 2015 cohort (Table 3, Fig. 6).

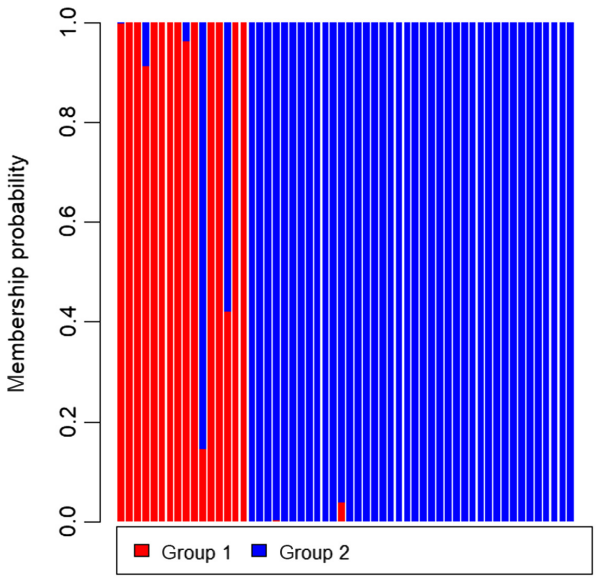

Fig. 5. Fijian maskray composition plot based on membership probabilities from DAPC analysis based on 3871 neutral loci where each individual is represented as a vertical bar and the colours correspond to probabilities of membership in the two cohorts.

Table 3. Summary of disc length (DL) and disc width (DW) in two cohorts using Welch's *t*-test.

Specimens measured	DL (cm)	DW (cm)
N 2015: 49 *N* 2022: 40		
T	4.05	3.75
Mean 2015	32.88	36.70
Mean 2022	29.42	33.95
P	<0.001	<0.001

Mean values for 2015 and 2022 are shown, indicating significant differences ($P < 0.001$) in disc length and width between the two cohort samples. T, *t*-test statistic.

Discussion

To our knowledge, this is the first study monitoring the genetic diversity in a stingray using time-separated samples. The mtDNA barcoding analysis did not yield conclusive species identification results, as it revealed a minimum genetic divergence of over 2% from the Kuhl's and of over 3% from the Coral Sea maskray. In Australasian samples, it was demonstrated that 99% of the 210 species identified based on morphological characteristics could be distinguished using *COI* barcoding (Ward *et al.* 2008). However, the *COI* gene also has limitations for elasmobranchs due to its small size (655 bp) and relatively slow evolution rate (White *et al.* 2022). This limitation was addressed by suggesting the larger and faster-evolving ND2 gene (1044 bp) as a species-discriminating marker, which generally aligned with *COI* results but offered improved resolution in some cases where *COI* failed to distinguish between groups (Naylor *et al.* 2012). Although Ward *et al.* (2008) reported that approximately 96% of within-species sequences exhibited less than 2% divergence, considering this as threshold value for separate species can be misleading (White *et al.* 2022), as *COI* sequences in sharks and rays exhibit highly variable divergence rates both between and within species (Ward *et al.* 2008; Finucci *et al.* 2018).

Fiji's maskray is widely distributed across the Fiji archipelago (Glaus *et al.* 2024*b*). The distribution of Kuhl's maskray remains uncertain. Currently documented in the Solomon Islands within the Western Central Pacific (Last *et al.* 2016*a*), its presence might extend further across Oceania. The Coral Sea maskray inhabits regions in Queensland, Australia, and New Caledonia. However, also in this species, the extent of species distribution and potential overlap remains unclear. While personal observations (KG), photographic records, and morphological differences in specimens documented for this study, along with genetic results, suggest distinctiveness of Fiji's maskray, definitive conclusions regarding distribution overlaps cannot be drawn. Overall, additional taxonomic clarification is necessary to identify Fiji's maskray, including morphological analyses and the use of NADH dehydrogenase 2 (ND2) genetic markers. Indeed, ongoing investigations, including detailed morphological and morphometric analyses aim to clarify its taxonomic status and provide a more accurate species description (K. Glaus and S. Appleyard, pers. comm.).

Genetic diversity and variation over time

Genotyping of over 3800 nuclear SNPs provided the following insights into the genetic diversity present in the Fijian maskray. The maskray in Fiji exhibited low levels of genetic diversity (as detected by SNPs), as evidenced by the low values of observed and unbiased expected heterozygosity ($H_o = 0.28$, $H_{n.b.} = 0.30$). These SNP based diversity values are nevertheless higher than in other tropical sharks such as the Galapagos shark (*Carcharhinus galapagensis*) (0.188–0.193) (Pazmiño *et al.* 2017), and the bull shark (*Carcharhinus leucas*) (0.128–0.214) (Glaus *et al.* 2020), comparable to the grey reef shark (*Carcharhinus amblyrhynchos*) (0.139–0.312) (Momigliano *et al.* 2017), and very similar to the spotted eagle ray (*A. ocellatus*) from Fiji (0.310–0.320) (Glaus and Appleyard 2024). Lower genetic diversity of shark and ray species is probably more associated with bottlenecks and the slow rate of molecular evolution. Regardless of whether the cause is historical or cotemporary, the current levels of genetic diversity should be considered for conservation policies (Martin *et al.* 1992; Rus Hoelzel *et al.* 2006; Allendorf *et al.* 2008).

The lack of genetic differentiation between the two cohorts suggested that both stochastic effects due to a small effective population size, and genetic subpopulations were absent. Positive F_{IS} values in the 2015 cohort indicated a significant

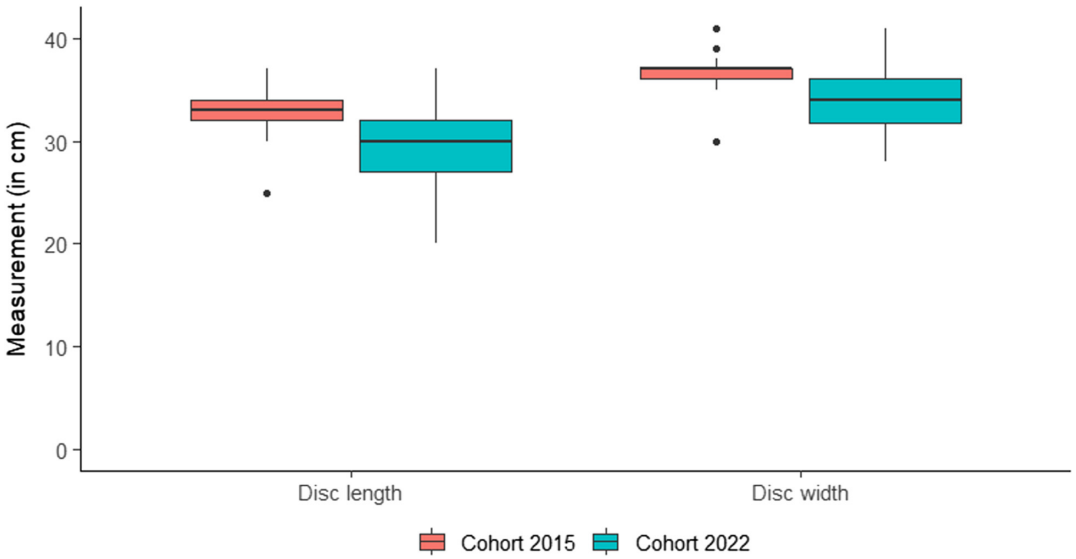

Fig. 6. Boxplot indicating decreases in disc length and disc width when comparing maskrays collected in 2015 with specimens obtained in 2022.

deficit of heterozygotes in the 16 sampled individuals. This suggested the presence of some level of relatedness and family structure within the 2015 samples. Although the sample size for the 2015 cohort was almost 2.5-times smaller compared to the 2022 cohort, one full-sib pair was identified per cohort. In congeneric maskrays, age at maturity ranges 3–6 years (Jacobsen and Bennett 2010), with maximum age estimates of 13 years for females and 10 years for males (Pierce *et al.* 2009). Therefore, identifying at least half-sibling pairs across the two cohorts would have been possible. Non-random mating is one possibility for the positive F_{IS} values in the 2015 cohort, which can result in a decrease in genetic diversity and an increase in the frequency of homozygous genotypes. However, genetic diversity remained consistent. Genetic diversity may have been preserved through the potential of gene flow between unsampled populations near our sample capture sites and the 2022 cohort. However, this cannot be confirmed as it is not known how many populations were sampled.

Furthermore, the results of the AMOVA analyses suggested despite a considerable number of SNPs in the 2015 cohort not being called (which could be attributed to the quality of the 2015 samples) there was no significant genetic differentiation between the two cohorts. The DAPC analysis indicated that differences in the number of polymorphic and usable loci between the two cohorts likely accounted for the observed variation. Additionally, while Structure was not helpful in suggesting the number of population groups based on Bayesian clustering inferred from the delta *K* statistic (likely due to the uneven sample size across the 2015 and 2022 and the difference in number of SNP loci successfully genotyped) (Puechmaille 2016), we prefer to consider individuals in the dataset demonstrated genetic proximity to two genetic clusters (Fig. 5), albeit with non-significant genetic differentiation among the 2015 and 2022 cohort. Overall, the limited quality and sample size of the 2015 cohort with only 16 individuals and the 7-year sampling regime may have impacted the significance of the results with regard to the lack of genetic variation. Therefore, longer-term sampling regimes and larger sample sizes are necessary to further investigate the genetic diversity in maskrays from Fiji and its potential implications for conservation.

Morphological comparison

The decreases in DL and DW could be reflective of population alterations, such as changes in birth rates, survival rates, or migration patterns. Sampling bias provides an additional explanation. While the sampling method remained consistent, the specimens in 2022 were measured throughout the year. In contrast, measurements in 2015 were conducted only in October and November, which may coincide with the species' reproductive season (Pierce *et al.* 2009). However, when comparing specimens collected only during October and November in both cohorts, the size decrease in DL and DW for the 2022 cohort remained evident. The same applied when only randomly selected subsets of each cohort were used for DL and DW size comparisons. Thus, a sampling bias is unlikely. The variation in size could, however, be attributed to unequal sex ratios (Goldman *et al.* 2012), but the absence of recorded sex data for the 2015 cohort hindered the possibility of conducting DL and DW size comparisons and partitioning the dataset by sex.

The 2022 cohort was sampled 2 to 13 months after international travel restrictions were lifted in December 2021. The rapid collapse of Fiji's tourism due to the COVID-19

pandemic severely undermined employment occupations, which led to urban-rural return migrations (Davila *et al.* 2021). This resulted in additional pressure on fishing resources and disputes over access and tenure (Connell 2021). Thus, probably more fishers were active in the Rewa Estuary and surrounding areas before and during the 2022 sampling period, than in 2015. Fishing efforts target larger individuals, which tend to be more fecund (Pauly *et al.* 1998). Particularly, females grow faster and reach larger sizes than males, as evident in many elasmobranchs (Klimley 1987; Francis 1996; Cortés 2000; Pierce and Bennett 2009; Pierce *et al.* 2009). The smaller size of the 2022 maskray cohort compared to 2015 may be a sign of increased fishing pressure, however the genetic diversity of the species has not decreased as found in this study. As catch monitoring time-series data for the maskray, as for other elasmobranchs, in Fiji are absent, it can neither be confirmed nor ruled out, whether the documented size decrease is indicative for increased fishing pressure.

Conclusion

Our study on Fiji's maskray (*Neotrygon* sp.) suggests a potential new maskray species in the study area, which clearly requires further taxonomic resolution. Overall, the study resulted in two main findings: (1) detection of consistent genetic diversity over time; and (2) a significant reduction in body size after 7 years. The identification of the same level of genetic diversity over time indicated that the overall genetic makeup of the maskray population remained relatively constant. Consequently, the null hypothesis is accepted. As this was the first genetic study on maskrays in Fiji, our results enhance the understanding of the maskray's genetic composition and emphasise the importance of ongoing monitoring efforts to gain deeper insights into the population dynamics of this species.

Supplementary material

Supplementary material is available online.

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Data availability. The unfiltered SNP report, data on size distributions, and R scripts will be made available on the Data Dryad Digital Repository.

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