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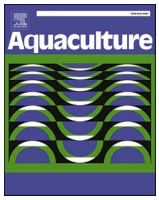

Sandfish generations: Loss of genetic diversity due to hatchery practices in the sea cucumber *Holothuria* (*Metriatyla*) *scabra*

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ABSTRACT

Hatcheries are indispensable for seed production of many commercial aquaculture species. However, for mass-spawning species in particular, they can be capricious environments where genetic diversity among progeny may be lost due to small effective broodstock population sizes, variable parental contributions and differential family survival. Understanding the genetic impacts of hatchery production is therefore important for addressing these problems and optimising propagation methods. We used 6051 genome-wide Single Nucleotide Polymorphisms (SNPs) to analyse genetic diversity, parental contributions and offspring kinship during a commercial-scale hatchery production run of sandfish (*Holothuria scabra*), a high-value sea cucumber grown in mariculture operations across the Indo-Pacific region. Broodstock contributions were highly skewed, with up to 26% of the parent pool contributing and kinship analyses determined that just two parents sired between 44.4 and 67.5% of all offspring genotyped. Effective population sizes were reduced as expected between broodstock and offspring groups (N_{eLD} = 1121.2 vs. 19.4, respectively), while losses of allelic diversity but not overall heterozygosity were apparent. Numbers of families surviving (13–16) to the juvenile stage were low, suggesting low effective population sizes among offspring cohorts is an issue for sandfish hatchery operations. To address variability in family compositions and broodstock contributions, pedigree tracking and batch spawning may be used to optimize broodstock management and hatchery protocols, to ensure production of genetically diverse offspring for routine culture and restocking operations. As many sandfish broodstock remain wild-sourced, maintenance of healthy wild populations as reservoirs of genetic diversity is important, along with selection for spawning of genetically diverse individuals which are as distantly-related as possible.

1. Introduction

Hatcheries are indispensable for closed culture of many aquatic species through seed production. Together with nurseries, they play a critical role in aquaculture production systems, but also function as important tools for wild population recovery efforts, conservation management, and sustainable fisheries enhancement initiatives. In wild populations, the accumulation of genetic diversity is a slow process taking thousands of years, which in captive populations can be lost over the span of a single generation if conservation of genetic diversity is not considered in breeding and rearing management decisions (Porta et al., 2007). Genetically diverse populations are more resilient to environmental variability and disease outbreaks, but also lend themselves to positive responses during trait selection in breeding and domestication programmes (Hughes et al., 2019).

Many aquaculture hatchery operations rely on broodstock sourced from wild populations, however, captive broodstock population sizes, together with their resultant offspring cohorts, are often much smaller

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and less genetically diverse compared to wild counterparts (Frost et al., 2006). Because many aquaculture species undergo complex early development processes with delicate larvae that have specialised feeding and other culture requirements, replication of optimal rearing conditions in artificial hatchery environments can be challenging, potentially amplifying losses of genetic diversity through variable mortality. In closed populations reared in artificial environments, the process of genetic drift through founder effects, differential family survival and selection for domestication can also contribute to reduced genetic diversity in hatchery produced progeny cohorts (Frost et al., 2006; Lind et al., 2009).

Important considerations for hatchery operations therefore include the selection of genetically diverse broodstock pools, utilisation of pedigree information and an understanding of how hatchery processes may influence spawning efficiencies and differential survival rates in larval and juvenile offspring cohorts. Risks associated with breeding practices which ignore pedigree information include an overall reduction in genetic variability among individuals and families, inbreeding depression in future generations and overall reduction in adaptive fitness (Lind et al., 2009; Nowland et al., 2017; Ravago-Gotanco and Kim, 2019). When the goal of hatchery production is to supplement depleted populations to assist stock recovery efforts, the release of genetically diverse and distantly-related individuals becomes particularly important.

In order to appropriately manage broodstock and rear offspring in a hatchery context, the availability of accurate and reliable pedigree information is necessary. Unfortunately, when dealing with mass-spawning species which produce a large number of small-sized offspring, the generation and retention of genealogical information for a large number of full-sib families is impractical or highly challenging due to limited controls over reproduction (Frost et al., 2006; Lind et al., 2010). For species which require social or chemical cues from conspecifics to trigger gamete release such as barramundi, *Lates calcarifer* (Frost et al., 2006) and silver-lip pearl oysters, *Pinctada maxima* (Lind et al., 2010), production of discrete full-sib families presents further challenges, because mass-spawning remains the most reliable method of obtaining large numbers of larvae using limited infrastructure.

Once larvae are produced, culture infrastructure constraints, such as the availability of a large number of tanks, often ensure that the rearing of pedigreed full-sib cohorts separately to track individual families through the hatchery or nursery cycle is highly limited. Under mass spawning and mass larval rearing conditions, the contributions of individual broodstock remain unknown, and small effective breeding numbers result in circumstances where contributions are made from very few broodstock. Fractional broodstock contributions can give rise to family size variability and subsequent differential survival rates, which impact the genetic variability of offspring cohorts (Brown et al., 2005; Frost et al., 2006).

The availability of molecular pedigree technologies has overcome some of the challenges associated with collecting pedigree information from mass spawning species. As these approaches utilise DNA-based genetic markers which function as biological tags, they eliminate the need for physical parent and offspring identification measures before and during the hatchery production process (Frost et al., 2006). Mass spawning and rearing practices can also be implemented, and family relationships determined via tissue sampling at desired stages during the rearing process.

The sandfish, *Holothuria* (*Metriatyla*) *scabra* is high-value tropical sea cucumber from the Indo-West Pacific region that is both harvested from the wild and maricultured to supply markets within the global *bêche-de-mer* trade (Purcell et al., 2018). Demand for sandfish has placed high fishing pressure on wild stocks, with many remaining in a depleted state (Pakoa and Bertram, 2013). Recent research has focused on developing and optimising culture methods for this species (Hair et al., 2016; Hamel et al., 2022; Juinio-Meñez et al., 2017) to cater for trade demand, assist restocking efforts and conserve wild populations (Altamirano et al., 2021; Duy, 2010; Hair et al., 2022; Militz et al., 2018). Routine hatchery production of sandfish is now established based on research to close the life cycle and development of rearing protocols (Altamirano and Rodriguez Jr, 2022; Battaglene et al., 1999; Duy, 2010; Duy et al., 2016; Ramofafia et al., 2003).

Sandfish are mass-spawners, with induction via thermal, feeding or desiccation stimulation treatments (Agudo, 2006; Duy, 2010). Broodstock are usually wild-sourced, with individual selection criteria including size, weight, activity and appearance. Usually, a single male begins to spawn following successful induction, which triggers other males, and finally females, to release gametes, with an intervening period of up to several hours. Females are highly fecund, producing between 2 and 4 million eggs per individual (Altamirano and Rodriguez Jr, 2022). An excess of sperm is often present in the spawning tank due to many more males releasing gametes than females, which reduces fertilisation rates because of polyspermy (Agudo, 2006); and males are therefore removed to separate tanks. Females are left to spawn, and sperm may be added later to the females' tank to ensure fertilisation success. Larvae develop through three distinct stages (auricularia, doliolaria and pentactula) over a 14–21 day period, prior to metamorphosis and settlement as juveniles (Altamirano and Rodriguez Jr, 2022; Battaglene et al., 1999).

While hatchery production of sandfish is now well established, broodstock remain largely wild-sourced (Hamel et al., 2022; Pitt, 2001) and their effective breeding numbers in captivity, along with the potential impacts of hatchery procedures on the standing genetic diversity of progeny cohorts remain unknown. While the genetic diversity of cultured sandfish populations has been assessed by previous research (Lal et al., 2021; Riquet et al., 2022), this study is the first to examine the cumulative effect of hatchery practices on a sandfish cohort over a single generation. Because hatchery production is critical not only for production of cultured sandfish but also for wild population restocking efforts, identifying any potential bottlenecks in the hatchery process where valuable genetic diversity is lost, is key to ensuring that genetically fit individuals are released (Lind et al., 2012). In Fiji, sandfish juveniles are routinely produced by the Fijian Government's Galoa Fisheries Station (GFS) which is operated by the Ministry of Fisheries. Juveniles are then released in the locations broodstock were sourced from, as part of a stock replenishment programme. The GFS is currently the only source of hatchery-produced sandfish in the country, and was selected as the study site.

Given that sandfish are routinely produced in hatcheries in locations where culture operations exist worldwide, it is important to understand the impacts (if any) the hatchery process may have on cohort genetic diversity, relatedness and broodstock contribution levels to progeny. This information currently remains unknown for sandfish and therefore, the specific objectives of this study were to 1) examine the genetic diversity of a sandfish parent and offspring cohort, 2) examine the number of family groups surviving through the hatchery process to the juvenile stage and 3) assess the number of parents which contributed to the offspring cohort. This information can be used to optimize broodstock selection and hatchery protocols to ensure genetically diverse offspring cohorts are produced for routine culture and restocking operations.

2. Methods and materials

2.1. Spawning run

Wild sandfish broodstock ($n = 85$, 250–350 g) were collected from Serua Island, Serua Province, Viti Levu, Fiji (18° 16′ 57.10“ S, 177°55’ 57.03” E), and transported to the Fiji Government's Ministry of Fisheries Galoa Fisheries Station (GFS) in December 2020 (Fig. 1). Broodstock were held in a 10,000 L concrete tank without substrate and conditioned for 8 days prior to spawning using standardized methods employed at the GFS as per Hair (2012) and Agudo (2006). Typically, between 20 and 45 individual broodstock are selected for mass spawning in a single

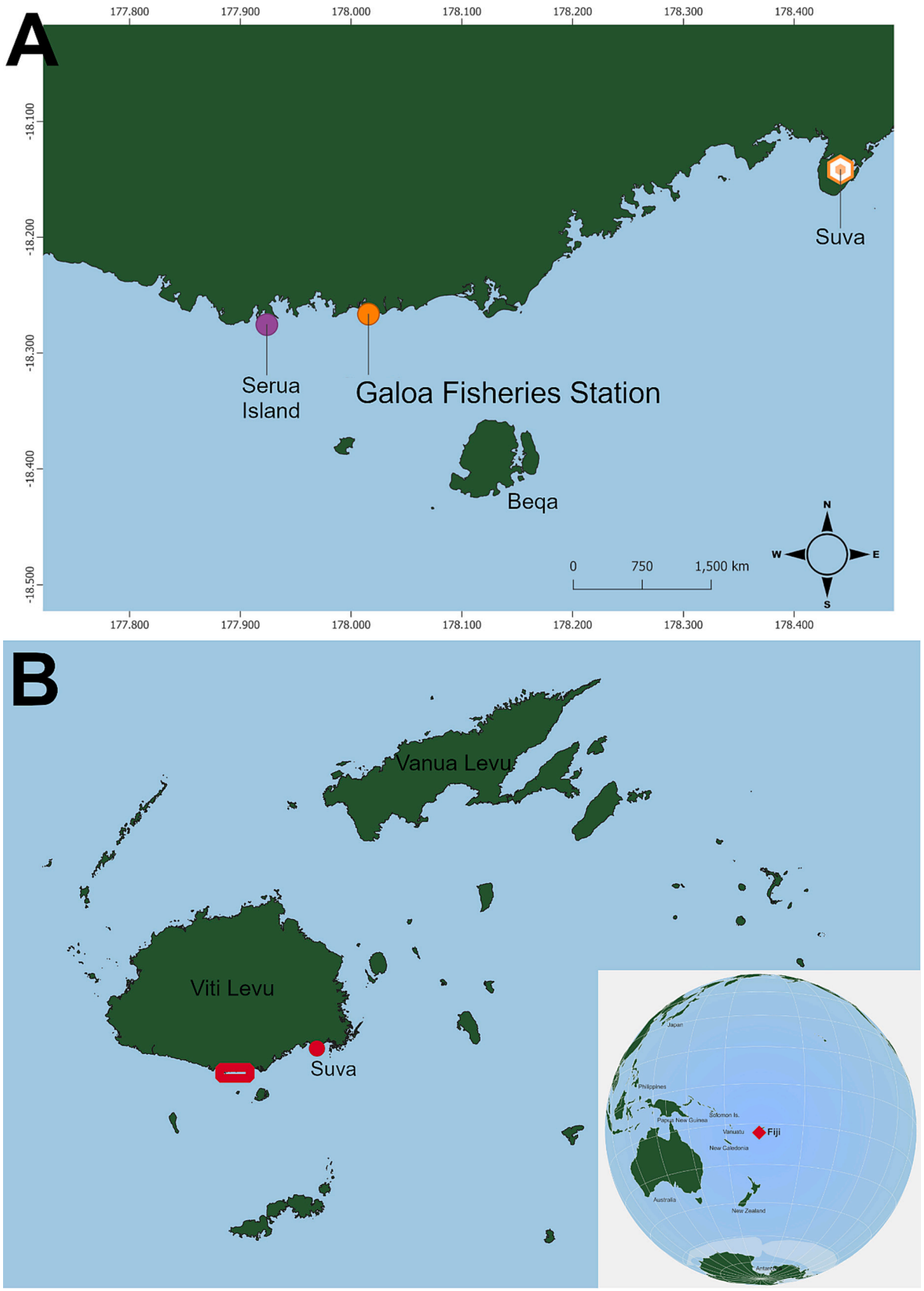

Fig. 1. A: Map of sampling location in Fiji where *H. scabra* broodstock were collected, along with the location of the Galoa Fisheries Station. B: The study area in Fiji (red rectangle); Inset: location of Fiji in region (red square). Produced using QGIS v 3.18.3-Zürich and open source geographical data obtained from The Humanitarian Data Exchange (https://data.humdata.org/dataset/cod-ab-fji) and Natural Earth (https://www.naturalearthdata.com/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tank (Agudo, 2006; Militz et al., 2018), however the GFS routinely uses 50–100, depending on the availability of wild individuals. Permission to collect sandfish and sample their tissues was obtained from the Fiji Government Ministry of Fisheries and the Fiji Ministry of iTaukei Affairs.

Broodstock were selected based on size, weight, activity and appearance (absence of body wall damage or signs of disease), and spawning induced as per Agudo (2006), Duy (2010) and Altamirano and Rodriguez Jr (2022). During conditioning for spawning, three of the 85 parent broodstock eviscerated and were thus excluded from the spawning induction process, leaving 82 individuals for tissue collection and DNA extraction. Spawning induction was timed to coincide with the period of a full moon to ensure receptivity of a high number of broodstock (Rahantoknam, 2017). Spawning was induced by immersion in a bath of *Spirulina* (1 h), desiccation for 30 min followed by thermal stimulation (raising water temperature to 5 °C above ambient for 1 h, where ambient temperature was 26 °C), in a 300 L tank. Sandfish may only reliably be sexed using external, non-invasive means upon observation of spawning activity, with males spawning first by releasing a continuous stream of sperm while standing erect off the tank floor and swaying. Females released eggs in short spurts from an enlarged gonopore (Agudo, 2006), and once observed spawning, were sequentially removed to a separate tank (150 L). As spawning progressed, 13 broodstock were able to be visually sexed. Initially four males released sperm, after which three females released eggs in succession over a 2 h period. Males continued to spawn in the 300 L tank, and after 60 min were subsequently removed to a separate tank to limit polyspermy.

Fertilisation was accomplished by collecting water containing sperm from the 300 L spawning tank and adding it to the tank containing females. Moderate aeration was supplied to the 150 L tank to permit mixing and fertilisation success monitored every 30 min over a 90 min period using microscopy to assess embryonic development. The female spawning tank was then drained, eggs rinsed and siphoned into a 50 μm washing basket before being evenly distributed among three 1000 L larval rearing tanks at a stocking density of 0.3 eggs/mL. Larvae were reared as per Agudo (2006) for a period of 35 days until settlement on *Spirulina*-coated corrugated plastic plates and fed the following commercially available microalgal concentrates: two Instant Algae® products - TW 1200® (*Thalassiosira weissflogii*) and Shellfish 1800® (mix of five marine microalgae: *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana*) (Reed Mariculture Inc.) and Algamac Protein Plus (Aquafauna Bio-Marine Inc.). Once settlement was achieved juveniles 5 mm in length were transferred to 10 pre-conditioned hapa installed in an earthen pond (5000 m^2) with approximately 500 individuals stocked per hapa net. Juveniles were reared in the hapa for a period of three months at which point they had reached an average weight of 7-10 g and length of 1–5 cm.

2.2. Tissue sample collection

As broodstock proceeded to spawn, the sex of individuals was determined and recorded and, following completion of spawning, their tissues were sampled. Despite observations that only 13 of the 85 broodstock collected were directly observed spawning at the beginning of the spawning period, the remaining 72 individuals were also tissue sampled in the event that they too had contributed gametes to the larval pool. A randomly selected pool of 200 juveniles was sampled for tissue in March 2021 following a grow out period of 3 months. Tissues were collected by excising a small piece of body wall tissue and skin from the ventrolateral anterior flanks (Supplementary Fig. 1D) which was preserved in a 20% dimethyl sulfoxide (DMSO)-salt solution (Lal et al., 2021). Tissue samples were maintained under refrigeration (4 °C) until genomic DNA (gDNA) extraction at the School of Agriculture, Geography, Environment, Ocean and Natural Sciences (SAGEONS), The University of the South Pacific in Suva, Fiji. Total genomic DNA was extracted using a modified CTAB:isoamyl alcohol protocol (Brown et al., 2022), and submitted for genotyping to Diversity Arrays Technology Ltd. (DArT PL), in Canberra, Australia.

2.3. DArTseq™ 1.0 library preparation and sequencing

Diversity Arrays Technology (DArT PL) proprietary genotyping by sequencing (DArTseq™) reduced-representation libraries were prepared as described by Kilian et al. (2012) and Sansaloni et al. (2011), with optimisation for the sandfish genome as described by Lal et al. (2021). In summary, genome complexity reduction was achieved with a double restriction digest using *Pst*I and *Sph*I methylation-sensitive restriction enzymes (REs). Custom DArT PL barcoded adapters (6–9 bp) were ligated to RE cut-site overhangs and target "mixed" fragments selectively amplified using custom designed primers for each sample. Samples were subsequently cleaned using a GenElute PCR Clean-up Kit (Sigma-Aldrich, cat.# NA1020-1KT) and then normalised and pooled using an automated liquid handler (TECAN, Freedom EVO150). Single-end 77 bp sequencing was carried out on the Illumina HiSeq 2500 platform at the DArT PL facility in Canberra, Australian Capital Territory, Australia.

2.4. Marker filtering, genotype calling and filtering

Illumina CASAVA v.1.8.2 software (Illumina Inc., San Diego, California, USA), was used for checks of read quality, sequence representation and generation of FASTQ files. Cleaned FASTQ files were then used for further filtering, variant calling and calling of final genotypes using the DArT PL proprietary software pipeline DArTtoolbox (Cruz et al., 2013). Individual samples were de-multiplexed according to adapter barcodes, and subsequently aligned and matched to catalogued sequences in both NCBI GenBank and DArTdb custom databases to check for viral and bacterial contamination. Any matches for viral or bacterial sequences were removed from downstream datasets.

SNP and reference allele loci were identified in reduced-representation loci (RRL) clusters and assigned scores of: "0" = reference allele homozygote, "1" = SNP allele homozygote and "2" = heterozygote, based on their frequency of occurrence. For robust variant calling, all monomorphic clusters were removed, SNP loci had to be present in both allelic states (homozygous and heterozygous), and a genetic similarity matrix produced using the first 10,000 SNPs called to assess technical replication error (Robasky et al., 2014), and remove clusters containing tri-allelic, aberrant SNPs and overrepresented sequences.

Once SNP markers were identified, their homozygote and heterozygote call rates, frequency, polymorphic information content (PIC), average SNP count, read depth and repeatability were calculated using DArT PL's KD Compute package. Once the raw genotype dataset was supplied by DArT PL, it was further filtered to retain a single, highly informative SNP at each genomic locus. A nested criteria of call rate and average polymorphic information content (PIC, highest to lowest rankings for both criteria) removed duplicated loci with identical Clone IDs. The dataset was further filtered for global call rate (95%), read depth (>7), average PIC (1%) Minor Allele Frequency, MAF (2% per sample group) and average repeatability (95%).

All loci were then assessed for departure from Hardy-Weinberg Equilibrium (HWE) using Arlequin v.3.5.1.3 (Excoffier et al., 2005), using an exact test with 10,000 steps in the Markov Chain and 100,000 dememorisations. Identification of loci under potential selection was achieved independently using two software packages, BayeScan v.2.1 (Foll and Gaggiotti, 2008) and HacDivSel (Carvajal-Rodriguez, 2017). Bayescan computations were carried out at 0.01, 0.05, 0.1, 0.2 and 0.5 False Discovery Rate (FDR) thresholds as per Brown et al. (2022). Putative F_{st} outlier loci identified during both runs were removed and segregated into a separate outlier marker dataset, and all remaining SNPs tested for departure from Hardy-Weinberg Equilibrium (HWE) in Arlequin v.3.5.1.3 (Excoffier et al., 2005), using an exact test with

10,000 steps in the Markov Chain and 100,000 dememorisations. Loci under selection were identified and pruned to generate a final dataset containing only selectively neutral loci.

2.5. Genomic diversity

To assess levels of genomic diversity within and between parent and offspring sample groups, allelic diversity indices including the average observed (H_o) and average expected heterozygosities corrected for population sample size ($H_{n.b.}$) were computed using Genetix v.4.05.2 (Belkhir et al., 1996). Genetix was also used to calculate Wright's inbreeding coefficients (F_{is}) per sample group and mean numbers of alleles per locus (A, MAF ≥5%). The number of private alleles (A_p, at MAF ≥5% under the rarefaction method) was computed using HP-RARE v.1. (Kalinowski, 2004), while the average multi-locus heterozygosity (MLH) per population was determined after Slate et al. (2004). The GenAlEx package (Peakall and Smouse, 2006) was used to determine the percentage of polymorphic loci, number locally common alleles (frequency ≤ 5%), and the effective number of alleles (N_{eff}). Effective population size based on the linkage disequilibrium method (N_{eLD}) was also estimated for each population using NeEstimator v.2.01 (Do et al., 2014).

2.6. Parentage and relatedness

Parentage assignment and kinship analyses for identification of parent-offspring, full-sib or half-sib relationships present among samples were completed using COLONY2 v2.0.5.8 (Jones and Wang, 2010) and CERVUS (parentage assignment only) (Kalinowski et al., 2007; Marshall et al., 1998) software packages. Prior to these computations the dataset was pruned, as using a large number of SNPs for pedigree reconstruction is unnecessary and may impede parentage assignments by their non-independence (Huisman, 2021). Therefore, a smaller subset of loci with a high genotyping rate (>90%), in relatively low linkage disequilibrium and high MAF (>30% and thus potentially the most informative SNPs in the absence of rare alleles), was selected as per Huisman (2021) and Huisman (2017). Filtering for MAF was accomplished manually, while LD pruning was carried out in the PLINK v.1.07 toolset (Purcell et al., 2007).

COLONY2 employs a full-pedigree maximum likelihood method to assign parentage and calculate sibship and accounts for the presence of null alleles, genotyping errors and mutations (Harrison et al., 2013; Jones and Wang, 2010). Computations were run without updating allele frequencies with run progression, specifying the presence of inbreeding, specifying monogamy for both males and females, not inferring clones and scaling full sib-ship relationships. Strong sib-ship priors for all relationship determinations were requested, population allele frequency was specified as unknown (i.e., calculated during the run), and the full-likelihood (FL) method with medium length runs at high precision selected.

A total number of three runs were carried out using both datasets, each using a different random number seed, all assuming a genotyping error rate of 0.05. All other options remained at their default settings. COLONY2 permits designation of individual samples as belonging to either offspring, or parents as candidate males and females. For all runs, parent individuals whose sexes during spawning were directly observed, were assigned their respective sexes (i.e., candidate male or candidate female), whereas parents that were not observed spawning were all specified as candidate females. This is because sandfish sexes are not distinguishable until observed spawning (see tissue sample collection section of methods for further details). In terms of parentage assignment computations, COLONY2 putative mother and father identities are arbitrary designations and only represent opposite sexes, and therefore if a candidate male parent was designated as female, that individual would still be detected as a parent.

Upon run completion, family assignments computed from each run were inspected in the ".*BestFSFamily" and ".BestConfig_Ordered" output files, and tabulated following assessment of family inclusion and exclusion probabilities. All full-sibling and half-sibling dyads detected during each run were also ordered by probability, and then tallied for each sample group using an inclusion threshold of $p \leq 0.05$. The filtered dataset generated for COLONY analyses (875 SNPs) was also used for CERVUS v.3.0.7 (Kalinowski et al., 2007; Marshall et al., 1998) computations. Parentage analyses were carried out using the parent pair analysis option, with one set of three runs specifying parent sexes as unknown and another set of three runs specifying known sexes (i.e., those male and female broodstock which were directly observed to spawn). For the runs specifying known sexes, all broodstock which were not directly observed to spawn were randomly assigned as male or female. For all runs as per the COLONY manual, first an allele frequency analysis was completed, after which a simulation of parentage analysis was carried out. For the simulation analysis, parameters were set as follows; 10,000 offspring simulated with 79 candidate parents, confidence estimated using delta and relaxed and strict confidence level limits of 80% and 90%, respectively. The final parentage analysis runs used outputs from both the allele frequency and simulation of parentage analyses, and were set to include the two most-likely parents based on joint LOD scores. Relaxed and strict confidence limits were set at 80% and 95%, respectively.

2.7. Resolution of family genetic structure

To investigate putative family relationships visually among sandfish offspring, network analyses were carried out using the Netview R package (Steinig et al., 2016). Netview R population networks are generated based on a shared allele 1-identity-by-state (IBS) distance matrix created in the PLINK v.1.07 toolset (Purcell et al., 2007). Each network computes the maximum number of nearest neighbours per individual (Neuditschko et al., 2012; Tsafrir et al., 2005). Individual networks are then visualised and edited in the Cytoscape v.2.8.3 network construction package (Smoot et al., 2011). The IBS matrices and corresponding networks were constructed at various thresholds of the maximum number of nearest neighbour (mk-NN) values between 1 and 50, after which the optimal network for representation was selected based on cluster stability (Steinig et al., 2016).

3. Results

3.1. Hatchery run, larval rearing and tissue sampling

A total of four females (sample IDs: A1-A4) and 9 males (sample IDs: A5, A6, A63-A69) had been directly observed to release gametes. The sexes of all 13 broodstock directly observed spawning were recorded for subsequent parentage assignment analyses. It is highly likely other broodstock had also spawned during this period, however as the tank water became opaque due to the presence of gametes and brooder discharge (Supplementary Fig. 1C), observations of individual broodstock genders were difficult.

At the end of the hatchery run, approximately 5000 juveniles had survived to the settlement stage. While 200 juvenile sandfish were sampled for tissues and DNA extraction from the offspring cohort, 3 individuals experienced sample failure during library preparation and were excluded. Similarly, of the 82 broodstock sampled for tissues, a further 3 experienced sample failure and were removed from the sample pool. Consequently, a total of 79 broodstock and 197 offspring (collectively 276 individuals) were genotyped by DArT PL.

3.2. DArTseq genotyping and SNP filtering

A total of 37,343 polymorphic genome-wide SNPs were genotyped across 276 individuals (79 parents and 197 offspring) in the raw dataset supplied by DArT PL, at call rates ranging from 20 to 100%. Following

filtering for global call rate (95% threshold), which removed 19,654 SNPs (47.4% loss) and read depth (>8, median dataset value = 9.08), 6935 SNPs were retained (overall 81.4% loss). Further pruning of the dataset (MAF; 2% per sample group, i.e., parents and offspring) for average SNP repeatability (95%), average PIC (1%), screening for F_{st} outlier loci (13 SNPs at FDR = 0.1%) and loci departing from HWE (9 SNPs: $p < 0.001$) resulted in 6051 selectively-neutral SNPs being retained for downstream analyses.

The filtered genotype dataset containing 6051 SNPs required further refinement to minimise missing data and optimisation for parentage and relatedness analyses. A call rate threshold of 98.6% was selected to initially retain 1415 SNPs (76.6% loss), after which a final dataset of 875 SNPs was generated following higher MAF (65%) and Linkage Disequilibrium, LD ($r^2 > 0.5$) pruning. This reduced dataset was used for both COLONY and CERVUS computations.

3.3. Genomic diversity

The effective population size estimate for the offspring sample group was substantially smaller (N_{eLD} = 19.4 [19.4–19.4]) compared to the broodstock sample group (N_{eLD} = 1121.2 [1079.4-1166.4], Table 1).

Allelic diversity estimates were largely similar between the two sample groups demonstrating the retention of genetic diversity within one generation, evident in the mean number of alleles per locus (A = 1.87 ± 0.004 vs. 1.85 ± 0.004), percentages of polymorphic loci and effective number of alleles (N_{eff} = 1.48 ± 0.004 vs. 1.476 ± 0.004) estimates for broodstock and offspring, respectively (Table 1). There were no locally common alleles (MAF ≥5%) reported in either sample group, however private alleles were detected in 8 loci across 35 individuals in the offspring cohort.

Heterozygosity metrics in Table 1 suggest a mild increase in the offspring cohort relative to their parents, with both observed heterozygosity estimates (0.247 ± 0.002 vs. 0.285 ± 0.002) and average multilocus heterozygosity (0.234 ± 0.069 vs. 0.285 ± 0.015) being higher for broodstock and offspring, respectively. This may be the result of heterozygote excess through pooling of diverse genotypes. Similarly, inbreeding coefficient estimates were higher among broodstock individuals (F_{is} = 0.144 ± 0.002) compared to the offspring cohort (F_{is} = 0.018 ± 0.002).

3.4. Kinship

Offspring assignments made to family groups by COLONY2 for all 197 sandfish offspring genotyped are reported in Table 2. COLONY2 resolved 13 full sibship groups (each comprising 2 or more individuals), which collectively contained 184 individuals or 93.4% of the total offspring pool sampled. A total of 13 singleton offspring could not be assigned to any of these 13 groups. Family compositions were heavily skewed towards 5 of the 13 family groups generated, which contained 82.7% of all offspring sampled. These 5 groups included two large (dyad sizes of n = 81 and 52) and three small families (dyad sizes of n = 12, 9 and 9, respectively). The remaining 8 families contained between 2 and

Table 2
Full-sibling family assignments determined by COLONY2 software across 197 hatchery produced sandfish juveniles.

Full-sib family index	Prob (Inc.)	Prob (Exc.)	Family membership assignments & number of individuals in each family
1	1.0	1.0	J1, J101, J103, J108, J109, J112, J115, J117, J118, J120, J128, J130, J144, J146, J148, J149, J156, J158, J159, J16, J161, J162, J165, J20, J25, J28, J3, J30, J31, J35, J40, J44, J47, J51, J55, J56, J6, J61, J63, J66, J69, J71, J76, J78, J79, J8, J82, J83, J86, J88, J90, J92 **(52)**
2	1.0	1.0	J10, J100, J104, J105, J106, J11, J110, J111, J114, J119, J121, J122, J123, J124, J125, J127, J13, J134, J136, J139, J14, J140, J143, J145, J147, J15, J151, J153, J154, J155, J160, J164, J166, J167, J17, J171, J173, J177, J18, J181, J182, J183, J185, J187, J188, J189, J19, J191, J195, J204, J21, J27, J36, J37, J39, J4, J42, J43, J45, J46, J48, J5, J52, J53, J57, J58, J62, J64, J68, J7, J70, J72, J77, J80, J84, J85, J87, J9, J95, J97, J99 **(81)**
3	1.0	1.0	J107, J163, J26, J32, J34, J65, J67, J73, J81 **(9)**
4	1.0	1.0	J116, J33, J75 **(3)**
5	1.0	1.0	J12, J129, J49, J94 **(4)**
6	1.0	1.0	J131, J138, J150, J175, J190, J192, J196, J197, J203, J205, J91, J98 **(12)**
7	1.0	0.9998	J133, J22 **(2)**
8	1.0	1.0	J135, J184, J23 **(3)**
9	1.0	1.0	J137, J168, J170, J172, J174, J179, J180, J186, J96 **(9)**
10	1.0	0.2865	J142, J41 **(2)**
11	1.0	1.0	J169, J193, J199 **(3)**
12	1.0	1.0	J176, J200 **(2)**
13	1.0	1.0	J113, J50 **(2)**
14–25 (singletons)	1.0	0.2836–1.0	J102, J126, J132, J152, J157, J178, J194, J202, J24, J29, J74, J89, J93 **(1 for each of 13)**

Table 1
Genetic diversity indices and relatedness computed for the *H. scabra* broodstock and offspring groups sampled. Parameters calculated include the effective population size by the linkage disequilibrium method (N_{eLD}; 95% confidence intervals indicated within brackets), mean number of alleles per locus (A), standardized private allelic richness (Ap, MAF ≥5%: the total number of loci with private alleles detected per population is shown in bold along with the numbers of individuals containing private alleles, effective number of alleles (N_{eff}), number of locally common alleles (MAF ≥5%) found in <50% of both sample groups tested, percentage of polymorphic loci, observed heterozygosity (H_o), average expected heterozygosity corrected for population sample size ($H_{n.b.}$), inbreeding coefficients (F_{is}) and average individual multilocus heterozygosity (MLH). All computations were generated using a dataset containing 6051 genome-wide SNPs.

Population	n	N_{eLD} [95% C.I.]	A (≥5%)	Ap (≥5%)	N_{eff}	Locally common alleles (≤5%)	% polymorphic loci	H_o (± SD)	$H_{n.b.}$ (± SD)	F_{is} ($p <$ 0.01)	Av. MLH (± SD)
Broodstock	79	1121.2[1079.4 - 1166.4]	1.870 ±0.004	0 0.001±0.000	1.480 ±0.004	0	99.87%	0.247 ±0.002	0.294 ±0.002	0.144 ±0.002	0.234 ±0.069
Offspring	197	19.4 [19.4–19.4]	1.850 ±0.004	**(8, n = 35)**	1.476 ±0.004	0	100.0%	0.285 ±0.002	0.291 ±0.002	0.018 ±0.002	0.285 ±0.015

4 members.

3.5. Family genetic structure

To further independently assess the putative number of family groups present among offspring through visualization of genetic structure, Netview R was utilised. A total of 13 broad genetic clusters were resolved (Fig. 2), corresponding to the number of family groups identified by COLONY2 analyses.

3.6. Parentage assignments

3.6.1. COLONY

Parentage assignments made by COLONY2 (Supplementary Material S2) indicate the contribution of 52 parent genotypes to the 197 sandfish offspring. Of these, 14 individuals were "known" candidate parents i.e., broodstock whose genotypes were supplied for analyses, whereas the remaining 38 individuals were "unknown" hypothetical parent assignments by COLONY2. Within the total broodstock pool genotyped (79 individuals), these known candidates represent a 17.7% contribution.

Within the pool of 14 known candidate parents, all four female broodstock observed spawning (A1-A4) were present, along with a single male (A66). Of the 38 unknown candidate parents, 22 and 16 were designated as candidate mothers and fathers, respectively. The relatively large number of unknown broodstock contributions may be a result of genotyping error, marker resolution or the possibility that broodstock (n = 3) individuals which had failed during genomic library preparation and thus weren't genotyped had made contributions.

The majority of offspring (172 out of 197; 87.3% assignment success) were assigned to at least one known candidate parent, with the remaining 25 assigned to unknown hypothetical parents. Of the assignments to known candidate parents, a large proportion of offspring (133 in total; 67.5% of all genotyped) were sired by just two broodstock individuals, distributed over two families. Neither of these broodstock were directly observed to spawn during the hatchery run. Within the remaining 39 offspring assignments made to known candidate parents, 25 offspring were assigned to female broodstock observed spawning (A1: 3, A2: 12, A3: 9 and A4: 1 offspring, respectively), with just 1 assignment to a male broodstock observed spawning (A66). These skewed proportions suggest that broodstock not directly observed spawning during the run likely contributed to the larval pool produced. There were no individual offspring assignments to two known parents, whereas all 25 offspring were assigned to two unknown parents.

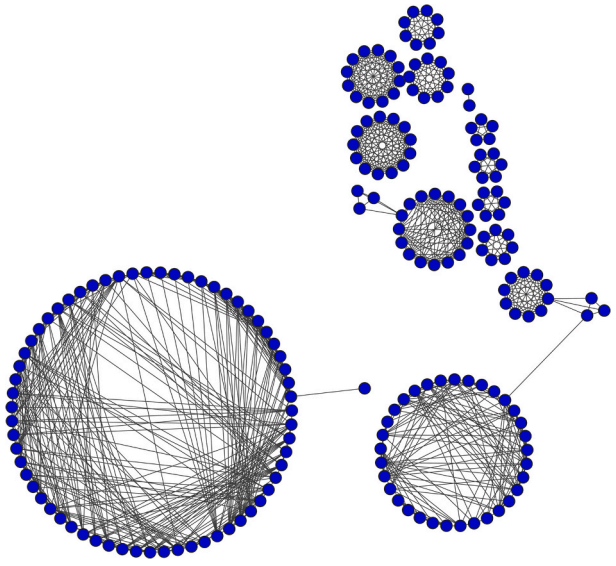

Fig. 2. High resolution network of 197 sandfish offspring generated using Netview R. The network has been constructed at a maximum number of nearest neighbour (mk-NN) threshold of 13, using 6051 SNPs. Each circle represents a single individual sandfish and the network mapped using the circular topology framework option in Cytoscape v 3.9.1.

3.6.2. CERVUS

Parentage assignments made by CERVUS are reported in Supplementary Material S3, with 180 of the 197 offspring genotyped (91.4%) assigned with high confidence (Logarithm of the Odds, LOD, score values >0). All offspring were assigned to the broodstock genotypes supplied (i.e., "known" parents), with a 26.6% contribution (21 individuals) from the total broodstock pool (n = 79) sampled. These proportions are higher than those reported by COLONY2 (17.7%). CERVUS is capable of assigning two candidate parents to each offspring, however only the first candidate parent assignments are reported here as a higher proportion of positive pair LOD scores were obtained (78.2% success) c.f. second candidate parent assignments (54% success).

Of the 21 candidate parents assigned, 11 individuals (52.4%) were broodstock observed spawning during the hatchery run, supporting the COLONY2 findings that a substantial proportion of broodstock not directly observed spawning made contributions to the larval pool. These included 4 females (A1-A4) and 7 males (A5, A6 and A64-A69). Similar to the COLONY2 assignments, a large proportion of offspring (44.4%) were sired by the same two broodstock individuals (A37: 50 offspring and A70: 30 offspring). Together with 5 other broodstock individuals (A28, A2, A38, A1 and A3) which were assigned 20, 12, 11, 9 and 9 offspring respectively; these 7 individuals collectively accounted for 78.3% of all broodstock contributions.

Parentage assignments made between COLONY2 and CERVUS overlapped with a moderate degree of consensus. Of the 21 known broodstock identified by CERVUS, 12 were also identified by COLONY (A1–4, A27, A28, A37, A38, A51, A61, A66, A70). Both methods also identified two broodstock (A37 and A70), which had the highest contributions relative to all broodstock genotyped. Additional comparison metrics are provided in Table 3. Similar numbers of families were identified (13 vs. 16) between both methods, as was the assignment success proportions of 87.3% (COLONY2) vs. 91.4% (CERVUS). Results were also concordant on the contributions of the 4 female broodstock directly observed spawning, and the number of broodstock which were not observed spawning (10 vs. 9).

In summary, a minimum of 13 families (range of 13–16) survived to the juvenile stage among the offspring cohort. Paternal and maternal contributions arising from between 14 and 21 of 79 broodstock generated these families, however the proportions of offspring between families were heavily skewed (44.4–67.5% of all offspring genotyped) towards just two parent individuals (A37 and A70). Both these parents were not among the 13 broodstock (4 female and 9 male) directly observed spawning during the hatchery run, highlighting that later spawners may potentially be important contributors in sandfish

Table 3
Comparison of outputs between COLONY2 and CERVUS assignments.

	COLONY2	CERVUS
Total numbers of offspring assigned	172/197* (87.3%)	180/197# (91.4%)
Total # families assigned (half & full-sib)	13	16
Total # singletons assigned	13	5
# known female broodstock assigned to all offspring	4 (A1-A4)	4 (A1-A4)
# known male broodstock assigned to all offspring	1 (A66)	7 (A5, A6, A64–67, A69)
# unknown broodstock assigned to all offspring	10	9

* To one known broodstock candidate parent.
# Only offspring which were assigned positive LOD scores for the most likely candidate parent.

hatchery runs.

4. Discussion

For aquaculture species, the long-term sustainability of hatchery production to support commercial grow-out operations, selective breeding efforts or augmentation of wild populations is reliant on the conservation and retention of genetic diversity in offspring cohorts. In hatchery-produced sandfish, our results demonstrate for the first time that while the loss of genetic diversity over the span of a single generation is mild, there is potential for the generation of genetic bottlenecks over successive generations through fractional broodstock contributions and accumulation of private alleles. The survival of a low number of families (13–16) through to the juvenile stage is a substantial concern, suggesting low effective population sizes among offspring cohorts is an issue for sandfish hatchery operations. To address variability in family compositions within the biological constraints associated with seed production of this species, broodstock selection and management will be key.

4.1. DArTseq genotyping, assessment of marker quality and yield

While a range of different DNA-based marker systems have been used to assess the genetic diversity of sea cucumber species, this study is the first to utilise genome-wide SNPs to examine the direct effects of hatchery practices over a single generation. Previous studies have employed allozymes (Uthicke and Benzie, 2001), microsatellites (An et al., 2013; Nowland et al., 2017; Riquet et al., 2022), mitochondrial COI barcodes (Skillings et al., 2011; Skillings et al., 2014; Uthicke et al., 2010), rRNA and nuclear histones (Soliman et al., 2016a; Soliman et al., 2016b). The advantage of genome-wide SNPs over more traditional markers is their flexibility in offering a large number of loci for high-resolution investigations of genomic diversity (Lal et al., 2021), which can be pruned down to the hundreds of independent, informative markers required for kinship analyses and pedigree generation (Huisman, 2017, 2021; Prakash et al., 2022). The genotyping approach selected here will be useful in creating and monitoring genetic pedigrees, as sandfish breeding operations advance and selection for commercially or ecologically important traits in cultured stocks become commonplace for this species.

4.2. Genetic diversity loss between parents and offspring

Loss of genetic diversity within the confines of a hatchery environment appears to be exacerbated in several broadcast spawning species, which produce a great number of offspring distributed across large family numbers. Previous research has documented losses in captive populations of white leg shrimp (Knibb et al., 2020), yellow croaker (Wang et al., 2012), barramundi (Frost et al., 2006), silver-lip pearl oysters (Lind et al., 2009), sea trout (Bernaś et al., 2020) as well as sandfish (Lal et al., 2021; Riquet et al., 2022). It is theorised that apart from differential broodstock contributions and resultant small effective breeding numbers, differential survival rates and size-based grading or culling practices can influence retention or loss of genetic diversity (Frost et al., 2006).

Species' biological attributes are also contributing factors, such as responses to an artificial (and potentially suboptimal) culture environment, settlement cues, family genotypes and social interactions (Frost et al., 2006; Lind et al., 2010; Mills et al., 2012). The latter is particularly important in barramundi culture, as this finfish displays social aggression and cannibalism, resulting in smaller individuals being preyed upon, thereby skewing family growth and survival rates (Frost et al., 2006). Larval settlement is a critical step in the development of many taxa and represents a milestone that is not reached by the vast majority of larvae which hatch. Variability in settlement success within a hatchery setting can contribute to loss of genetic diversity, when individuals and families are lost from the offspring cohort.

Sandfish undergo a biphasic settlement history in the wild, recruiting first to seagrass leaves and thereafter to sandy substrates (Mercier et al., 2000). The first settlement of doliolaria larvae in a hatchery environment is facilitated by the provision of *Spirulina*-coated plates, after which larvae metamorphose into epibenthic pentactula larvae. A further metamorphosis into early juveniles occurs after approximately 20 days (Altamirano and Rodriguez Jr, 2022), with a distinct shift to deposit feeding. Not all larvae undergo successful metamorphosis and settlement, and because sandfish effectively undergo two of these events during development, it is possible these are critical points where genetic diversity may be lost in the hatchery cycle. Further research to track families through settlement may shed some light on the dynamics associated with these final phases of the hatchery culture process.

The genetic diversity of wild and cultured sandfish populations has been examined in previous studies, including Ravago-Gotanco and Kim (2019), Uthicke and Purcell (2004), Lal et al. (2021), Nowland et al. (2017), Brown et al. (2022) and Riquet et al. (2022). Where cultured and wild populations have been compared, all studies have reported reductions in the effective population size of hatchery-produced populations with increased relatedness among offspring dyads. Nowland et al. (2017) reported the presence of moderate levels of genetic diversity despite sustained long-term fishing pressure in 10 and 2 wild sandfish populations sampled from Papua New Guinea and Northern Australia, respectively. Similarly, the current study detected retention of genetic diversity among the wild-sourced broodstock individuals. Riquet et al. (2022) examined five batches of hatchery-produced sandfish in New Caledonia and, comparing them to wild individuals, reported reductions in effective population size sufficient to detect evidence of genetic drift over a single generation, mirroring results of the current study where rare alleles were detected in 17.7% of all offspring genotyped. These authors also found a loss in genetic diversity of ~5–35% compared to wild populations, which they concluded was too small to prevent loss of evolutionary potential and subsequent population extirpation if these individuals were used for restocking or stock enhancement.

4.3. Parental contributions and kinship among offspring

Low effective population size is a crucial issue for sandfish hatchery operations. Parentage assignment analyses reported between 17 and 26% of the broodstock genotyped contributed to the 13–16 families generated during the hatchery run, with the largest proportion of offspring skewed towards just two parent individuals (A37 and A70). Differential broodstock contributions skewing genetic compositions of offspring have been observed in other mass spawning species. In Japanese flounder (*Paralichthys olivaceus*), for example, a single male out of six was found to sire 99% of larvae in an offspring cohort (Sekino et al., 2003), while in barramundi one male sired ~50% of all offspring in one mass spawning event while one sire and dam contributed 77% and 80%, respectively, to all progeny in another event (Frost et al., 2006). Similarly, Lind et al. (2009) reported large skews in full-sib family representation of up to 40% from a single family in the silver-lip pearl oyster. These authors also attempted to reduce the unpredictable variances in family sizes resulting from mass spawning by collecting eggs separately from individual females and fertilising them with mixed sperm from several males. It was found that generally higher N_e values were produced for progeny cohorts using this method, although partitioning individuals into full-sib family groups still resulted in large skews in family contributions due to differential survival (Lind et al., 2009). For the silver-lip pearl oyster, the use of additional broodstock in mass spawning operations did not solve the problem of decreased genetic variability in progeny cohorts. Relatively few broodstock were seen to dominate offspring cohorts and cause large skews in full-sib family representations, even when extra broodstock were included (Lind et al., 2009).

It is suggested that in natural marine populations, high fecundity and

stochastic larval viability ensures that the variability of individual reproductive success is a "sweepstakes" event (Hedgecock, 1994; Hedgecock and Pudovkin, 2011). In a hatchery setting where broodstock are mass spawned and fertilisation success is randomised, the same phenomenon may occur (Lind et al., 2009), or perhaps be magnified. As sandfish share similar reproductive characteristics to silver-lip pearl oysters (both species are mass spawners, conspecific presence is required to induce spawning and both possess high fecundity), similar levels of family representation in hatchery produced cohorts are evident as shown in our study.

4.4. Recommendations for broodstock, hatchery and nursery management

While hatchery-based propagation of juvenile sandfish is invaluable for seed production to supply grow-out operations and aid recovery of depleted and/or extirpated populations, caution must be exercised when selecting broodstock from source populations and the release locations of hatchery-produced juveniles. Uthicke and Purcell (2004) suggested that population genetics needs to be considered when releasing hatchery-produced sandfish into areas where non-local broodstock are used. This practice is followed in Fiji, Indonesia and the Philippines, where routine releases for wild population augmentation and sea ranching operations are carried out (Altamirano et al., 2021; Brown et al., 2022; Dumalan et al., 2019; Sembiring et al., 2022).

It is important that hatchery-based restocking programmes ensure that adequate levels of genetic diversity are maintained in juveniles and that restocking sites overlap with the natural dispersal limits of the source population (Ravago-Gotanco and Kim, 2019). These measures will ensure avoidance of 'genetic pollution' effects, arising from the admixture of potentially less diverse individuals into a natural population, translocation of individuals into sites where natural dispersal regimes may not usually operate (Brown et al., 2022), or swamping of locally-adapted genotypes (Evans et al., 2004).

Interventions to minimise the loss of genetic diversity in hatchery systems will not be a "one size fits all" approach, as among other influences, pressure points where genetic variability is lost will vary among species due to life history differences and adaptations to artificial culture environments. For sandfish, while losses in allelic diversity but not overall heterozygosity were apparent over a single generation in this study, skewed broodstock contributions and the survival of a reduced number of families and consequently higher relatedness among sibling dyads is problematic. Potential solutions to this problem include broodstock selection and management interventions, as well as evaluation of mating schemes which incorporate pedigree information.

When selecting broodstock, genetically diverse individuals which are as distantly-related as possible should be used. For the majority of sandfish hatchery operations, broodstock are currently wild-sourced (Hamel et al., 2022; Militz et al., 2019; Pitt, 2001), highlighting the importance of maintaining healthy wild populations as reservoirs of genetic diversity. The use of a large number of broodstock during mass spawning is also a potential solution, however there are practical limitations to the number of broodstock which can be maintained and spawned within the confines of hatchery infrastructure.

Established protocols for sandfish broodstock selection advise that between 20 and 60 individuals should be selected for a typical hatchery run (Agudo, 2006; Altamirano and Rodriguez Jr, 2022), however not all individuals spawn following induction and female contributions are usually lower (Brown et al., 2022; Pitt, 2001). The present study used 85 broodstock, however kinship analyses suggest only up to 21 of these contributed to the larval pool generated. These observations concur with results obtained by Lind et al. (2009) who conducted controlled spawning experiments in the silver-lip pearl oyster, and found that the use of additional broodstock did not necessarily improve contributions and family composition skews in offspring cohorts.

Given that sandfish can only reliably be mass spawned during routine hatchery operations following conspecific cues, a possible solution requiring investigation may be to batch spawn several groups of broodstock, and then combine larval pools for communal rearing. If combined with pedigree information on individual broodstock, it is possible that fractional broodstock contributions may be mitigated to an extent. In commercial operations which may choose to maintain captive broodstock pools instead of sourcing from wild populations, potentially deleterious founder effects could be minimised (Militz et al., 2019). As sandfish culture ultimately advances into selection of commercially important traits through selective breeding, captive broodstock with documented traits and heritability metrics will become established.

5. Conclusions

This study is the first to examine the cumulative effect of hatchery practices on a sandfish cohort over a single generation using high-resolution genomic data. It highlights directions for future research into optimising sandfish broodstock selection, management, spawning and larval rearing procedures, to ensure retention of genetic diversity over consecutive generations.

Practical considerations for hatchery production of sandfish should include selection of as many, genetically diverse, distantly-related broodstock as practically possible, and the generation and retention of pedigree information where applicable. For culture operations which rely on wild source populations for broodstock, fishery management measures informed by population genetic data to ensure their genetic diversity are indispensable. Genetic monitoring of translocation and restocking management efforts is also imperative, to ensure minimisation of potential genetic pollution effects.

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CRediT authorship contribution statement

Kelly T. Brown: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Paul C. Southgate:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Epeli M. Loganimoce:** Formal analysis, Writing – review & editing. **Teari Kaure:** Investigation, Writing – review & editing. **Brian Stockwell:** Writing – review & editing, Supervision. **Monal M. Lal:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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