CSIRO PUBLISHING

Marine and Freshwater Research, 2015, **66**, 375–380
http://dx.doi.org/10.1071/MF13313

Short Communication

Parental contribution to progeny during experimental spawning of jungle perch, *Kuhlia rupestris*

M. L. Hoskin[A,D], *M. J. Hutchison*[C], *A. C. Barnes*[A], *J. R. Ovenden*[B] *and L. C. Pope*[A]

[A]The University of Queensland, School of Biological Sciences, Brisbane, Qld 4072, Australia.
[B]The University of Queensland, School of Biomedical Sciences, Brisbane, Qld 4072, Australia.
[C]Queensland Department of Agriculture, Fisheries and Forestry, Bribie Island Research Centre, 144 North Street, Woorim, Qld 4507, Australia.
[D]Corresponding author. Email: matthew.hoskin@uqconnect.edu.au

Abstract. When releasing captive-bred animals into wild populations, it is essential to maintain the capacity for adaptation and resilience by minimising the effect on population genetic diversity. Populations of the jungle perch (*Kuhlia rupestris*) have become reduced or locally extinct along the Queensland coast; thus, captive breeding of *K. rupestris* for restocking is presently underway. Currently, multiple individuals are placed in a tank to produce larvae, yet the number of adults contributing to larval production is unknown. We performed a power analysis on pre-existing microsatellite loci to determine the minimum number of loci and larvae required to achieve accurate assignment of parentage. These loci were then used to determine the number of contributing participants during a series of four spawning events through the summer breeding season in 2012–2013. Not all fish contributed to larval production and no relationship was found between male body size and parentage success. In most cases, there was a high skew of offspring to one mating pair (62% was the average contribution of the most successful pair per tank). This has significant implications for the aquaculture, restocking and conservation of *K. rupestris*.

Additional keywords: conservation, fish, genetics, restoration.

Received 27 November 2013, accepted 20 August 2014, published online 9 December 2014

Introduction

Overfishing and the destruction of habitat has led to the reduction of population sizes, and even the extirpation of populations, in many freshwater fish species (Chapin *et al.* 2000). To combat this, reintroduction programs release captive-reared individuals to either supplement existing populations or to replace extinct populations (Keller *et al.* 2000; Purcell and Kirby 2006; Roche *et al.* 2008). Often, captive-bred individuals join the local breeding population, thus contributing offspring to populations in the future (Champagnon *et al.* 2012). This means that released individuals need to have diverse genotypes that mirror the genetic composition of the wild breeding stock. This differs fundamentally from restocking for commercial purposes, where the primary aim is to enhance the experience of recreational fisheries, rather than regenerate or maintain pre-existing genetic diversity and structure. Historically, mismanagement of genetic diversity in restocked populations has led to changes in behaviour, morphology and demography of a species (Laikre *et al.* 2010).

To maintain the genetic integrity of wild populations when augmenting them with hatchery-bred fish, it is important to monitor parental contribution to offspring. For example, in barramundi (*Lates calcarifer*), parental contribution to offspring during spawning in captivity was found to be highly variable, such that over half of one cohort was sired by a single male (Frost *et al.* 2006). If not properly managed, related individuals could be selected as future broodstock, resulting in high levels of inbreeding over future generations (Frost *et al.* 2006). Changes in the genetic make-up of wild populations as a result of incorrect management have also been shown in Murray cod, *Maccullochella peelii*, where microsatellite analysis determined that the effective breeding number of *M. peelii* within some fish farms was only half of the adult population, leading to reduced allelic richness in restocked populations (Rourke *et al.* 2009). Consequently, a best practice for restocking with hatchery-reared populations was established by the New South Wales government, which identifies criteria for site selection, design and operation, and the management of broodstock, breeding programs, water quality and fish health (Rowland and Tully 2004).

Kuhliidae is a family with a single genus, *Kuhlia*, consisting of 12 species with an Indo-Pacific distribution, distributed from Africa to American Samoa (Pusey *et al.* 2004; Feutry *et al.* 2012*a*). *K. rupestris* (Araki *et al.* 2009) is a prised sport fish and a highly desirable food fish (Herbert *et al.* 1995). Captive experiments (Hogan and Nicholson 1987) and otolith analyses

(Feutry *et al.* 2012*a*, 2012*b*) have indicated that *K. rupestris* reproduces in marine to brackish water, with a comparatively short pelagic larval duration of ~41 days. However, the species has not been observed breeding in the wild, so their behavioural patterns, and even exact environmental requirements, remain unknown.

Exploitation and human-induced barriers to migration (e.g. weirs and dams) have led to a significant reduction in south-eastern Queensland populations (Hutchison *et al.* 2002). Natural recovery by *K. rupestris* has not occurred in south-eastern Queensland, despite the removal of some fish passage barriers more than 10 years ago (Hutchison *et al.* 2002). Genetic connectivity over an evolutionary timeframe has been demonstrated among jungle perch populations throughout the Coral Sea (Feutry *et al.* 2013). However, modelling of larval dispersal indicated that southern Queensland populations are likely to receive limited recruits from other locations, potentially contributing to the slow recovery of populations in this region (Feutry *et al.* 2013). Therefore, a carefully managed reintroduction program is considered the best approach for restoring *K. rupestris* to its former southern distribution. A breeding program for *K. rupestris* has been established that addresses population declines in south-eastern Queensland by reintroducing *K. rupestris* to areas where they have become locally extinct, and where factors that initially led to the extinction have been removed.

In order for the reintroduction program of *K. rupestris* to be successful, released individuals should be the offspring of as many combinations of broodstock parents as feasible, in roughly equal proportions (>10 pairs sourced from the most suitable location; Rowland and Tully 2004). Currently, *K. rupestris* is spawned in groups as it is thought that one individual releasing gametes triggers group spawning (e.g. Samoilys 1997). This was based on earlier trials, where spawning of single pairs resulted in low fertilisation rates, and there were difficulties finding 'running' males (males producing sperm). As a result, parentage is likely to be mixed and it is unknown which individuals may have contributed. To shed light on parentage contribution, we used genetic analysis with microsatellite loci to determine which adults within captive spawning groups reproduced successfully over the 2012–2013 breeding season.

Materials and methods

To determine the minimum number of microsatellite loci required to confidently assign parentage, a simulation study was undertaken using existing microsatellite genotype data for *K. rupestris* from the Queensland coast (Peters *et al.* 2009; Feutry *et al.* 2013). Fifteen microsatellite loci were ranked on the basis of polymorphic information content (PIC) (Hearne *et al.* 1992).

The software package CERVUS v.3.0.3 (Kalinowski *et al.* 2007) was used to sample random groups of three females and nine males from the Queensland-coast dataset to act as parents. We chose this number of parents because this represented the largest possible group per tank, and was therefore the most difficult situation that could be encountered in our study. The genotypes of 10 000 larvae from randomly selected parents were simulated, and parentage of each was assigned at the 90% level of confidence. Each simulation was performed using the following parameters: default genotyping error rate (0.01), a missing data rate calculated from the existing genotype data, and the assumption that all the parents were genotyped. Simulations were performed for increasing numbers of loci, with loci added according to PIC rank. Ten replicates were performed for each number of loci. The percentage of simulated offspring assigned with high confidence to the correct parent was recorded as an estimate of the power of that particular set of loci to assign parentage.

Brood fish and larval sampling

Fin clips were obtained from PIT-tagged adults participating in spawning events at the Bribie Island Research Centre. Tissue was stored in 20% dimethyl sulfoxide (DMSO) 5 M NaCl at 4°C. Fish were also measured, weighed and sexed as part of the general health assessment.

Spawning tanks were kept between 26 and 28°C, salinity was between 34 and 36 parts per thousand (ppt), light was controlled on a 14-h daylight schedule, and tanks were aerated with air stones. Spawning took place as close to the new moon as possible (within 4 days). Adults used in the trial ranged in bodyweight from 102 to 2706 g; fork length ranged from 187 to 480 mm.

Broodstock tanks were on a recirculating biofiltration system with UV treatment of water. Salinities were maintained between 3 and 5 ppt to reduce osmotic stress and to prevent outbreaks of freshwater parasites. Temperatures ranged from 21°C in winter to 27°C in summer. Fish were fed 5 days per week to satiation on a mixed diet including commercial pellet feeds, prawns, fish, mussels and terrestrial insects. Fish had been held in captivity for at least 6 months before spawning and the majority of fish had been in captivity for more than 2 years. Broodstock were obtained by electrofishing from rivers and streams in Queensland, including Wyuna Creek on Fraser Island for Tanks A and B, the Daintree River north of Cairns for Tank C, and St Helens Creek near Mackay for Tank D.

Larvae from four spawning tanks were sampled for the present study (Table 1). The first two tanks (A and B) were both sampled in November 2012. Tanks C and D were sampled in December 2012 and January 2013, respectively. Within both November tanks (A and B), it was possible to perform temporal sampling, as distinct batches of larvae were produced over more than 1 day. For Tank C, egg batches were released within a 7-h period, and mixing is likely to have occurred between batches. Eggs were pooled in subsequent analyses. In Tank D, only one spawn was fertilised. Following egg collection and subsequent hatching, larvae were collected within 6 days through a 200-μm screen, washed and individual larvae were transferred to a well of a 96-well plate and stored at −20°C. DNA extraction took place within 1 month of collection. The total number of eggs spawned per tank was high (average = 829 000) and the hatching rate as a percentage of the total number of fertilised eggs was also high (average = 63%). Under these circumstances, it seems unlikely that selective egg mortality per parent was sufficient to introduce bias in our estimate of the number of females that successfully reproduced.

DNA extraction and genotyping

DNA was extracted from adult fin clips using a Qiagen DNEasy DNA extraction kit (Qiagen). DNA from larvae was extracted

Table 1. The number of breeding adults from spawning trials of jungle perch (*Kuhlia rupestris*) determined by genotyping larvae
Data were collected from four groups of potential parents (tank). Tank A and B larval samples also were collected from separate batches of larvae (A1, A2; B1, B2). 'M' and 'F' refer to the number of adult males and females in each spawn tank, and larvae (L) refers to the number of larvae genotyped. 'Detect with 90%' is the minimum parental contribution detectable with 90% confidence given the number of larvae genotyped. 'Breeding females', 'Breeding males' and 'Breeding pairs' refer to the numbers in the tank that produced the larvae that were genotyped. '>%' represents the greatest percentage of assigned parentage achieved by a single female, male or pair, and '<%' represents the smallest amount

Tank	M	F	L	Detect with 90% (%)	Breeding females			Breeding males			Breeding pairs		
					Number	>%	<%	Number	>%	<%	Number	>%	<%
A total	6	3	37	6	2	81	19	4	89	3	5	78	3
A1			32	7	2	93.75	6.25	3	93	3	3	91	3
A2			5	37	1	100	n.a.	2	60	40	2	60	40
B total	7	3	39	6	1	100	n.a.	4	59	5	4	59	5
B1			28	8	1	100	n.a.	2	82	17	2	82	17
B2			11	19	1	100	n.a.	2	81	18	2	81	18
C	7	3	14	15	3	50	7	5	43	7	7	36	7
D	6	3	27	9	1	100	n.a.	3	74	4	3	74	4

using Chelex (Bio-Rad; Estoup *et al.* 1996). All individuals were genotyped for four loci (Peters *et al.* 2009). Microsatellite amplification, size analysis and scoring were undertaken according to the methods of Peters *et al.* (2009). One pair of loci was PCR multiplexed, allowing three PCRs to be performed to amplify the four loci. PCR products were separated by capillary sequencing using an Applied Biosystems (ABI) 3130 sequencer. GeneMapper 3.7 (Applied Biosystems, Waltham, MA, USA) was used to determine allele sizes relative to GeneScan 500 LIZ size standard (Applied Biosystems).

Statistical analyses

Parentage of offspring was estimated using CERVUS 3.0.3 (Kalinowski *et al.* 2007). Parentage was assigned using the likelihood method. First, a likelihood ratio was calculated, by dividing likelihood that the candidate parent is the true parent by the likelihood that the candidate is not the true parent. From this, the LOD score is obtained, by taking the natural log of the likelihood ratio. The difference between the LOD of the most likely parent and the second most likely parent is called delta LOD. Simulations were then performed to determine the critical value of delta LOD scores required to assign parentage with >90% confidence. For each tank, allele frequencies and simulation analyses were performed for the specific set of candidate parents, with 10 000 offspring simulated to assign both parents with sexes of adults known. The simulation settings used were as described earlier, using four loci. A parentage analysis was then performed to assign parents to larvae, with the simulation results used to determine confidence levels. Parents were assigned using a minimum of three loci and 90% confidence. Once parentage was assigned, the numbers of males and females breeding per tank, and the proportion of parentage each individual and pair had achieved, was estimated.

To test for an effect of body size on the proportion of parentage success, we performed a Spearman's rank correlation, comparing ranked weight with ranked proportion of spawn sired ('parentage'). Tests were performed separately for each sex and each tank. One-tailed *P*-values were combined across tanks (separately for each sex) using Fisher's combined *P*-method with a Bonferroni correction for multiple tests. Tests were performed in R version 3.1.0 (R Core Team 2014) using the package MADAM (Kugler *et al.* 2010) to perform the combined Fisher's *P*-test.

Results

Power analyses

Power analyses indicated that three loci (KRU03, KRU11, KRU17) would be sufficient to assign high rates of parentage with high confidence (97.57% of offspring assigned at 90% confidence). Only small improvements were made using more than three loci (4 loci 99.45%, 5 loci 98.78%, 6 loci 99.95% offspring assigned). However, to account for the possibility of some missing data, larvae were genotyped at the four most polymorphic loci (KRU02). Adding a fourth locus did not greatly increase costs because all four loci could be run in a single lane, and the additional locus could be amplified in a multiplex PCR, meaning the total number of PCRs remained at three.

Using the general binomial probability formula, we determined that 22 samples would be required to detect a 10% contribution by an individual with 90% confidence, and therefore represented our ideal minimum sample size. For Tank C, our sample size was lower than this. Using this same formula, we determined the minimum contribution we could confidently detect with 90% confidence given our sample size for each tank, and have included this estimate in Table 1.

Parentage analysis

Parentage was assigned with at least 90% confidence to all larvae genotyped for a minimum of three loci. In Tank A, there was one mismatch between a larva and an assigned parent, but this was within the error rate assumed for our data. Parentage analyses revealed that not all the adults were breeding. If all adults had contributed evenly, up to 33% of larvae per tank could be half-siblings (the same mother), and as few as 4.8% and 5.5% would be full siblings (from the same pair). For Tanks A, B and D, only three to five pairs of a possible 18–21 pairings per tank spawned successfully, and among these pairs the contribution

was highly skewed (Table 1). This resulted in low genetic diversity among the larvae produced in Tanks A, B and D, as between 81 and 100% of them came from the same mother and 59–89% came from the same male, with a single pair contributing to between 59 and 78% of the spawn (Table 1). Tank A had the lowest diversity, with 78% of offspring being full siblings. Tank C had the highest genetic diversity, with seven unique 'pairs' contributing to the spawn. In all, 8 of the 10 parents in Tank C contributed to parentage. There was a more even spread of larval parentage than in Tanks A, B and D, with a maximum female contribution of 50% and the maximum male contribution of 43%. These large numbers of families were picked up, even though the sample size was small ($n = 14$). However, even in this example, 50% of the larvae were half siblings (Table 1, Fig. 1).

For Tanks A and B, parentage could be assigned to two separate egg batches. In Tank A, two females and three males contributed larvae to the first egg batch. The same females produced eggs with two different males in the second batch of eggs. In the first egg batch from Tank B, one female produced an egg mass fertilised by two males. In the second egg batch, the same female produced a second egg mass with two different males (Table 1).

In three of four tanks for females, there was a perfect correlation between weight rank and 'parentage' rank (Spearman's correlation Rho = 1, 1, −1, 1). The combined *P*-value was not significant ($P = 0.2$), but there were insufficient data to detect a significant relationship. There was little correlation between weight rank and 'parentage' rank for males across our four tanks (Spearman's correlation Rho = 0.03, 0.14, 0.68, 0.31), and combined *P*-values were again non-significant ($P = 0.17$).

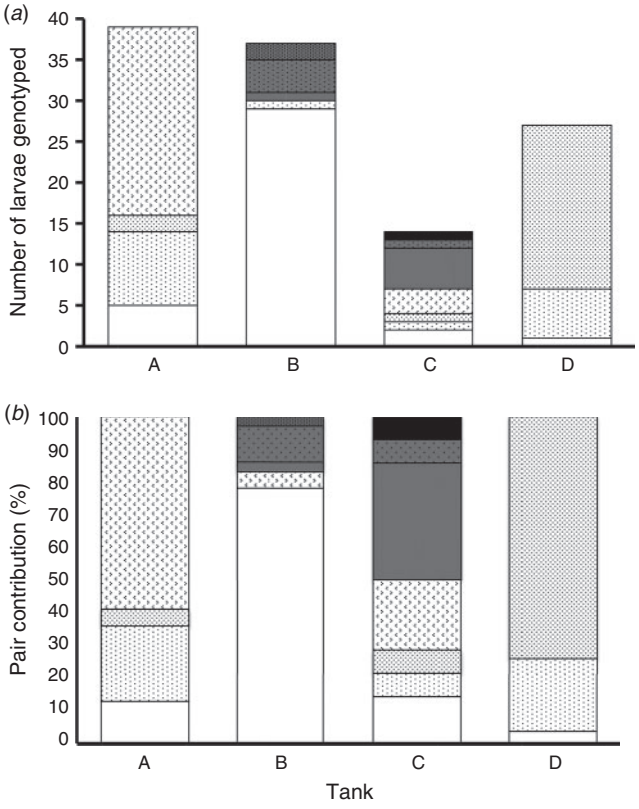

Fig. 1. Parental contribution of *Kuhlia rupestris* adults across different spawning groups, shown by (*a*) number of larvae and (*b*) percentage of larvae. All larvae from Female 1 are white, with larvae fathered by different males with Female 1 indicated by different degrees of stippling. In the majority of spawns, only a single female contributed to the larvae. All larvae from a second female are dark grey, with different degrees of stippling representing different males mating with Female 2. Where a third female contributed that is indicated in black. Note: different individuals were used for each spawn.

Discussion

The reproductive behaviour of *K. rupestris* in the wild is largely unknown, but aspects can be deduced from the present study of captive adults. Using genetic markers to assign parentage, the results from four separate parental groups (three females and six or seven males per group) determined that (1) not all adults in a tank contributed to larvae, (2) a single pair generally produced at least half of the larvae in a spawn, (3) females are capable of releasing more than one batch of eggs, (4) males are capable of siring larvae from successive egg batches from multiple females, (5) body size was not correlated with parentage contribution.

The numbers of adults that mated and produced larvae across the separate tanks was low; on average 4.75 of a possible 19.5 pairs bred per tank. A median of two females and four males bred per tank. The maximum contribution was three females and seven males and the minimum contribution was one female and three males per tank. Similar results have been reported during conservation breeding in other hatcheries of other freshwater species (e.g. (Frost *et al.* 2006; Borrell *et al.* 2011; Christie *et al.* 2012; Hold *et al.* 2013). One strategy to maximise the genetic diversity of *K. rupestris* larvae produced in a system like this would be to increase the number of spawning tanks and to use novel combinations of potential parents. The use of genetics to confirm parentage would allow the production of genetically diverse larvae with the minimum resources (number of brood stock, number of tanks).

In captive-bred *K. rupestris*, a single pair dominated the production of larvae in the majority of tanks, and it was evident that not all adults bred. Across tanks, single pairs produced between 36 and 78% of larvae, with the average contribution being 61.8%. Thus, more than half of the larvae were produced by a single pair of adults (i.e. full-siblings), and remaining larvae were largely half-siblings. Under these circumstances, the genetic diversity of the larvae was not representative of the genetic diversity of the parents. Limited genetic diversity in cultured fish for wild release can lead to the depletion of effective breeding numbers of a wild population, as well as reducing fitness and population over time (Reisenbichler and Rubin 1999; Christie *et al.* 2012; de Mestral *et al.* 2013).

The parentage of larvae from Tank A and B provided insight into the reproductive behaviour in *K. rupestris*. In these tanks, eggs were able to be collected from two distinct spawning events. Parentage analysis indicated that a female from each of these tanks produced larvae in the first spawn, then produced larvae with different males in the second spawn. The multiple releases of eggs suggest that *K. rupestris* is a serial batch

spawner. Multiple spawning events are common in other species of fish, including *Plectropomus leopardus* (coral trout; Samoilys 1997). Among other benefits, this may increase the genetic diversity of the population by increasing the chance of different males fertilising the different batches from a single female. Little evidence was found for a relationship between body size and parentage success, particularly for males (which had larger sample sizes). Although an absence of a relationship is not evidence, our results caution against assuming that only larger males will produce larvae when selecting broodstock. Further research through observation in the wild and experiments is required, to determine whether the reproductive behaviours observed in captivity reflect natural behaviour.

Compared with the resources required to increase the number of spawning tanks, monitoring genetic diversity of larvae is cost effective. The method employed here (microsatellite loci) is particularly cost effective. Because of the existence of a large population genetic database for the species (i.e. 487 individuals genotyped at 15 loci; Feutry *et al.* 2012*b*), we were able to perform accurate simulations to establish the minimum number of microsatellite loci required to confidently determine parentage at the aquaculture facility. In addition, multiplexing and resolving genotypes in one lane further reduces costs. Reducing the number of loci can also reduce the error associated with parentage assignment, potentially allowing more accurate parentage assignment where loci are sufficiently variable (Jones and Ardren 2003; Jones *et al.* 2010). As predicted by our simulations, four loci were sufficient to assign parentage to all larvae genotyped, with a minimum of three being necessary.

The results of the present study have direct implications for the restocking of *K. rupestris* in Queensland, Australia. If genetic testing were not available for the assessment of future spawnings, it would be conservative to treat the larvae from each tank as full siblings because of the high skew towards one breeding pair found in these results. On the basis of New South Wales quality assurance guidelines for threatened species (as there are no guidelines for restocking wild populations in Queensland), there should be equal proportions of larvae from a minimum of 10 different pairs of unrelated adults over 5 years for restocking an endangered fish species in the wild (Rowland and Tully 2004). For *K. rupestris* under the current larval production system, this would involve 10 spawning events because the present study has shown that larvae from one group of brood stock are largely from one pair. Currently, 10 spawning events would require ~30 female and 60–70 male *K. rupestris* individuals from habitats adjacent to the location where larvae are planned for release. This may be problematic because of small population sizes in some locations in south-eastern Queensland; however, New South Wales guidelines recommend that brood stock should be regularly rotated with adults from the wild. If spawning success of individuals were genetically monitored, or if experimental trials facilitated spawning by single pairs, a smaller number of wild broodstock would be required.

By genotyping captive-bred larvae, we showed that the majority was produced by a single breeding pair, despite the large numbers of parents that were potentially available. Future restoration of wild populations needs to take advantage of genetic monitoring to maximise the chance of success. The release of two batches of eggs from a single female has demonstrated that *K. rupestris* is capable of producing multiple batches of eggs at least under captive conditions.

Acknowledgements

We sincerely thank Raewyn, Jess Morgan, Jeff Bilby and Michael Macbeth. The Jungle perch team at the Department of Agriculture Fisheries and Forestry (DAFF) for sample collection. Jeff Bilby for his help with data management. The Jungle perch project is funded by the Australian Commonwealth Government Fisheries Research and Development Corporation. Management of jungle perch broodstock was conducted under a General Fisheries Permit and Animal Ethics Permit CA 2011/05/509.

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