Restocking of *Anabarilius grahami* in Lake Fuxian, Southwest China: morphological and genetic effects

DEAR EDITOR,

The restocking of the endangered Kanglang white minnow (*Anabarilius grahami*) in Lake Fuxian, China, has been conducted for 13 years. However, few studies have reported on the effectiveness of the captive breeding and release of this species. Here, we investigated variations in morphology, including body shape and skeletal deformities, and genetic features among hatchery-born and recaptured *A. grahami* from Lake Fuxian. Results showed that current hatchery-reared fish displayed a stubbier body shape than their wild conspecifics from the 1980s. Furthermore, high skeletal deformity ratios were found in two aquafarms (Luchong, 50%; Haikou, 45.2%), and the release of malformed fish elevated the skeletal deformity rate of wild stocks found near the Lake Fuxian release sites (west coast, 19.0%; east coast, 12.5%). Based on variations in the cytochrome *b* (cyt *b*) gene, existing *A. grahami* populations showed relatively high haplotype diversity and low nucleotide diversity. Hatchery populations exhibited reduced genetic variations based on microsatellite markers and reintroduction led to markedly lower genetic diversity around the west coast release sites of Lake Fuxian. Analysis of molecular variance (AMOVA) of cyt *b* and microsatellite analysis showed that the greatest genetic variations were found within populations, and genetic distance and Bayesian clustering analysis showed that the 14 populations clustered into one group. Based on morphological and genetic tests, we discuss corresponding recommendations, including release size, feed formulations, breeding strategies, and release tags, to minimize potential risks and improve hatchery practices for better restocking of this species.

Hatchery-reared fish released into the wild tend to differ from their wild conspecifics in a wide range of morphological and genetic attributes. Comparative biological studies have indicated that hatchery-reared fish show faster growth and greater morphological changes from the larval stage to maturity than that observed in wild fish (Lorenzen, 1996; Stringwell et al., 2014; Thorpe, 1991). Released hatchery-born fish with robust body shape tend to spend more time hunting than their wild conspecifics; in contrast, wild fish tend to exhibit higher foraging efficiency and lower predation risk (Ersbak & Haase, 1983; Malavasi et al., 2004; Sosiak et al., 1979). In addition to morphological adaptations to their captive environments, hatchery-reared fish also experience a high occurrence of skeletal deformities due to a variety of abiotic and dietary factors (Cobcroft & Battaglene, 2009; Darias et al., 2010; Mazurais et al., 2009; Sfakianakis et al., 2006). Despite this, little is known about the distribution and performance of skeletally deformed individuals following their release in the wild. In addition, hatchery conditions can also influence genetic features of captive populations. Comparisons between hatchery-reared and wild-type fish commonly show an increase in inbreeding and a reduction in total effective population size (N_e) (Ryman-Laikre effect) in captive systems (Waples et al., 2016). After their release into the wild, hatchery-reared fish may increase genetic risks within and among populations, such as genetic diversity loss and reduced population fitness (Ford, 2002; Fraser, 2008; Hindar et al., 1991; Waples, 1999). To achieve a successful reintroduction program, hatchery-reared fish should include wild-like individuals to improve their chances of adapting to the natural environment upon release (Svåsand et al., 1998). Thus, rearing conditions and genetic management are both important factors in the maintenance of wild characteristics during the establishment of captive populations (Lorenzen et al., 2012).

The Yun-Gui Plateau is a highland region located in the Yunnan and Guizhou provinces of Southwest China. It is the birthplace and upstream area of many major Asian rivers (e.g.,

the Salween, Mekong, and Yangtze rivers). Yunnan possesses the greatest diversity of fish in China, accounting for 40% of the nation's freshwater fish species (Chen, 2013). Unfortunately, most fish resources have declined in recent decades due to large-scale economic activities, biological invasion, and over-exploitation (Chen et al., 2012). Aquaculture-based restocking has been carried out in Yunnan Province for the last 20 years to increase populations of native fish species, such as the Kanglang white minnow (*Anabarilius grahami*) and Dianchi golden-line barbel (*Sinocyclocheilus grahami*) (authors' unpublished data).

Anabarilius grahami is a cyprinid fish with restricted distribution in Lake Fuxian (a typical Yun-Gui Plateau lake). As one of the "Four Famous Fishes in Yunnan", *A. grahami* has special economic value and popularity. Although it is a small-sized fish, it accounted for 70%–80% of annual fish production in Lake Fuxian before the 1990s (Li et al., 2003). However, wild populations of *A. grahami* decreased sharply following the unintentional introduction of the exotic icefish species (*Neosalanx taihuensis*) in 1985, with annual production of *A. grahami* declining from >400 tons before the 1990s to <1 ton (almost impossible to capture) in the early 2000s. In contrast, annual production of *N. taihuensis* increased rapidly in the 1990s, reaching more than 1 500 tons, a level it maintains today. Competitive disadvantages have been ascribed for the population decline of the endemic *A. grahami* species because its main food and ecological niche overlap significantly with that of *N. taihuensis* (Qin et al., 2007). Overfishing of *A. grahami* and collateral damage when catching *N. taihuensis* are also considered to have had an impact (Li et al., 2003). The drastic decline in the *A. grahami* population has resulted in a shift from an abundant economic species to an endangered one. In 2004, it was evaluated as vulnerable in the China Species Red List (Wang & Xie, 2004) and categorized as one of the most threatened fish in the world (Liu et al., 2009). Fortunately, artificial breeding was achieved in 2003 (Li et al., 2003) and hatchery-reared *A. grahami* individuals have been released into the wild since 2008. To date, more than 10 million individuals have been released in Lake Fuxian (about 800 000 per year) and annual production has increased to about 15 tons. However, the post-release fitness of hatchery-reared individuals and their impact on wild populations have not yet been investigated.

In this study, we analyzed the morphological and genetic differences in *A. grahami* populations (*n*=581; Table 1) collected from Lake Fuxian, from hatchery stock around Lake Fuxian (released populations), and from the Endangered Fish Conservation Center (EFCC, for conservation target) of the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS). All animal experiments were approved by the Ethics and Experimental Animal Committee of KIZ, CAS (Approval ID: SMKX-2016024; approved on 15 December 2016). To test morphological changes under hatchery conditions, we measured specimens collected from Lake Fuxian and deposited in KIZ in the 1980s using traditional measurements to acquire body shape characteristics and used radiography to check skeletal deformities. We compared

Table 1 Details on 14 fish populations used in this study

Population name	Population number (*n*)	Generation	No. checked (*n*)
Specimens in 1986	–	Wild	29
Specimens in 1988	–	Wild	26
EFCC001	~5 000	Wild	30
EFCC002	~5 300	Wild	30
EFCC003	~20 000	F1	32
EFCC004	~10 000	F2	36
EFCC005	~10 000	F2	30
F_Northwest	–	Released F1	33
F_West	–	Released F1	58
F_East	–	Released F1	40
F_South	–	Released F1	43
Jiuxi	~5 000	F1	45
Huoyanshan	~6 000	F1	41
Niumo	~2 000	F1	47
Luchong	~1 000	F1	30
Haikou	~1 000	F3	31

–: Not available.

these characteristics to current populations (hatchery-reared and recaptured individuals). Furthermore, we also examined changes in body shape and skeletal deformity based on the reintroduction program in Lake Fuxian. Finally, we analyzed genetic features of the populations (excluding KIZ specimens due to the difficulty in extracting DNA) to clarify changes in the genetic structure of populations in Lake Fuxian and hatcheries using mtDNA cyt *b* and microsatellite variations, and evaluated the effects of hatchery-reared fish on wild populations.

The morphometric data of each population are listed in Supplementary Table S1 and the body shape ratios are listed in Supplementary Table S2. Principal component analysis (PCA) indicated that the first three components, which included all seven morphometrics, explained 73.2% of the variation (Supplementary Tables S3, S4). Although total variance of the first three components did not reach 85%, scatter plots based on the data showed that wild populations in the 1980s had different morphological characters to current populations (Supplementary Figure S1). The highest CPL/CPD and SL/BD (CPL: caudal-peduncle length, CPD: caudal-peduncle depth, SL: standard length, BD: body depth) and lowest SL/CPL values were found in the previously collected specimens from Lake Fuxian. Compared with wild individuals (specimens collected in 1986 and 1988), broodstocks introduced from Lake Fuxian showed a changed body shape, with lower CPL/CPD and SL/BD values and higher SL/CPL values (Supplementary Table S2). The artificially bred offspring had much lower CPL/CPD and SL/BD values and higher SL/CPL values, as well as a stubbier body shape (deeper bodies and shorter caudal peduncles) than their wild conspecifics (Supplementary Figure S2A, C). Thus, after 10 years of release, the body shape of existing populations of *A. grahami* in Lake Fuxian differs from that of their progenitors but is concordant with hatchery-reared fish

(Supplementary Figure S2B, C).

Skeletal deformities were found in the jaw, vertebra, rib, neural arch and spine, and hemal arch and spine (Supplementary Figure S3). The skeletal deformity rates are shown in Supplementary Figure S4, with the highest deformity rates found in aquafarms around Lake Fuxian (Luchong: 50.0% and Haikou: 45.2%, Supplementary Table S5). Wild populations introduced as broodstock (EFCC001 and EFCC002) from Lake Fuxian in the early years showed no skeletal deformity. First-generation offspring produced by artificial breeding in 2003 (EFCC003), together with their offspring (EFCC004 and EFCC005), also showed no skeletal deformities. Interestingly, samples derived from Lake Fuxian demonstrated significant differences in skeletal deformities among the recapture sites. The skeletal deformity rate was higher in the western region of the lake close to the fish release site, but decreased with distance from the site (west>east>northwest and south: 19.0%>12.5%>0% and 0%) (Supplementary Figure S5). Pearson correlation analysis showed that skeletal deformity had a significantly negative relationship with distance to the release site (r=–0.991, P<0.05, n=4) (Supplementary Figure S6). The percentage of each deformity type also differed among groups (Supplementary Table S6). In the two populations (Luchong and Haikou aquafarms) with the highest skeletal deformity rates, combined deformities were dominant. Comparing specimens recaptured in the western and eastern regions of the lake, more serious deformities were found in the former.

A 1 080 bp fragment of the cyt *b* gene was obtained from the caudal fin of specimens. Haplotype diversity (*H*) ranged from 0.533 in Luchong to 0.956 in F_east, F_northwest, and EFCC004. Nucleotide diversity (*Pi*) was lowest in EFCC001 (0.002 84) and EFCC002 (0.002 83), and highest in Huoyanshan (0.007 00) (Supplementary Table S7). A total of 68 polymorphic sites were found, which defined 60 haplotypes. The H5 haplotype was the most frequent type in most populations. More than half of haplotypes were unique and distributed among samples (Supplementary Figure S7). Thus, this endangered fish exhibited relatively high haplotype diversity but low nucleotide diversity. Tajima's neutrality tests (*D*) (Tajima, 1989) and Fu's tests (F_S) (Fu, 1997) showed no significant differences among populations (Supplementary Table S7). Both within and between group cyt *b* sequence distances were small (within group distances ranged from 0.002 to 0.007 and between group distances ranged from 0.003 to 0.008, Supplementary Table S8).

The genetic features of 11 microsatellite loci are given in Supplementary Table S9. After analyzing the 11 loci in 14 populations, the highest effective alleles (*Ne*=1.778), observed heterozygosity (*Ho*=0.467), expected heterozygosity (*He*=0.411), and polymorphism information content (*PIC*=0.348) were found in EFCC001. The lowest values were shown in the EFCC004 and EFCC005 populations (F2), which were produced by EFCC003 (Supplementary Table S9). Differences in genetic diversity were found in specimens collected from the four sites at Lake Fuxian, with lowest values

(*Ne*=1.605, *Ho*=0.418, *He*=0.353, and *PIC*=0.300) in the west coast site compared to the other three sites. For aquafarms around Lake Fuxian, relatively high and low genetic diversities were found at Huoyanshan (*Ne*=1.690, *Ho*=0.449, *He*=0.390, and *PIC*=0.334) and Niumo (*Ne*=1.613, *Ho*=0.385, *He*=0.339, and *PIC*=0.298), respectively (Supplementary Table S9).

The inbreeding coefficients (*Fis*) were negative in most populations, except for EFCC004 (0.054), EFCC005 (0.029), and Haikou (0.098), but were not significantly different (Supplementary Table S9). Significant deviations in the Hardy-Weinberg equilibrium were observed in the F_northwest (northwest Lake Fuxian) and EFCC002 populations (Supplementary Table S9). Locus SSR12 exhibited significant deviation in both F_northwest and EFCC002 (Supplementary Table S10) and showed a high level of heterozygote excess in these populations.

The AMOVA (cyt *b*) and microsatellite variation results revealed that the greatest genetic variation occurred within populations (Supplementary Table S11). Based on pairwise Φ_{ST} analysis using cyt *b* and microsatellites, higher significant values were observed between populations reared at the EFCC and others (Supplementary Tables S12, S13). The highest Φ_{ST} values based on cyt *b* and microsatellite variations were found between EFCC001 and Luchong (0.367) (Supplementary Table S12) and between EFCC003 and Luchong (0.185) (Supplementary Table S13), respectively. For specimens collected from the four Lake Fuxian sites, pairwise genetic differentiation between each population was non-significant (Supplementary Table S12). However, significant differences were found in microsatellite analysis for three pairs (i.e., F_west×F_Northwest, F_west×F_East, F_west×F_South), with small *P* values of 0.012, 0,009, and 0.013, respectively (Supplementary Table S13).

For Bayesian clustering analysis (STRUCTURE) (Pritchard et al., 2000) of microsatellite data, the most probable *K* value (number of clusters) calculated from Ln P(D) and delta *K* was *K*=3. However, the value of delta *K* was very small (0.358) for *K*=3 (Figure 1A). The graph using *K*=2, 3, and 4 showed that the 14 populations clustered into one group (Figure 1B). The unweighted pair group method with arithmetic mean dendrogram showed that populations related to the Haikou aquafarm (EFCC003, EFCC004, and EFCC005) were separated from other populations. After checking the four Lake Fuxian sites, samples recaptured from the west were separated from the other three sites (Figure 1C).

In principle, restocking programs are a double-edged sword. On the one hand, they can cause negative effects, such as loss of genetic diversity, intraspecific interactions, and ecosystem destruction; on the other hand, they can help depleted populations regain a capacity for increase (Lorenzen et al., 2012). This study on the restocking program of endangered *A. grahami* should help enhance our understanding of the recovery processes. Ten years ago, *A. grahami* was still an endangered fish and the wild population was nearly extinct. However, due to aquaculture-based
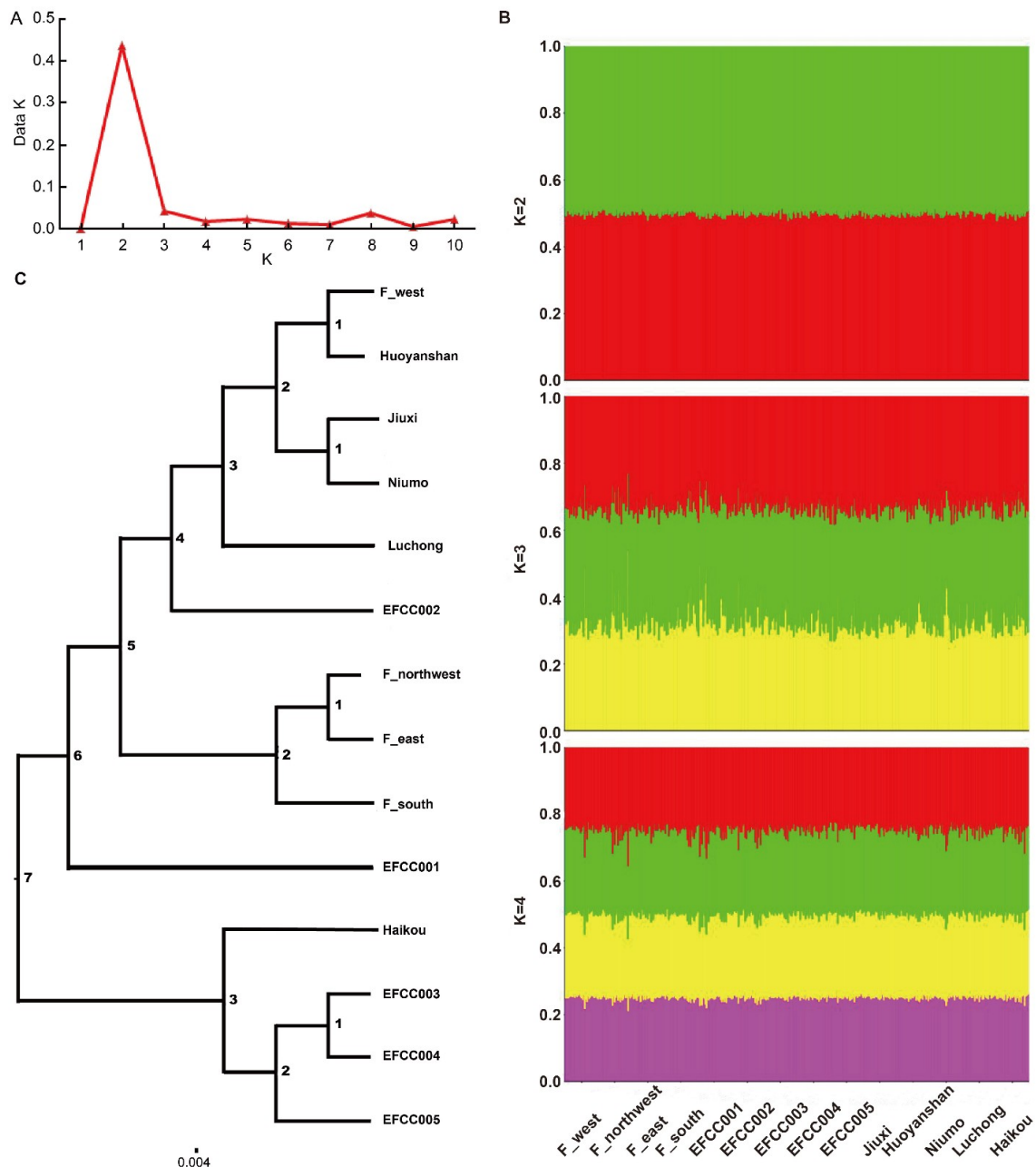
Figure 1 Bayesian analyses of *A. grahami* samples using microsatellite data

enhancement, the population in Lake Fuxian has experienced partial recovery. Before the release program, natural breeding had disappeared in the lake for almost 10 years. Now, hundreds of mature fish can be observed at spawning sites during the breeding season, even if their spawning size is far smaller than that observed historically (authors' observation). Thus, restocking appears to be an effective way to boost population recovery. For endangered species, however, the aim is not only to produce more fish to supplement wild populations but also to preserve morphological and genetic diversity and integrity.

Domesticated organisms tend to exhibit extreme morphological, behavioral, and physiological characteristics rarely seen under natural conditions (Balon, 2004; Teletchea & Fontaine, 2014). A similar situation has also occurred for *A. grahami*, with domesticated individuals displaying a stubbier body shape (deeper body depth dorsoventrally) than that of their wild conspecifics. Adequate food and a lack of predators can lead to low natural pressure selection, and thus the development of extreme phenotypes in domesticated organisms. Although they can persist under favorable conditions at aquafarms, the mortality rate in the wild can be

high (Trut et al., 2009). According to previous studies, other farmed-raised species, including common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) (Matsuzaki et al., 2009; Omori & Kon, 2019), also show stubbier body shapes and reduced swimming performance compared to wild populations (Johnsson et al., 2001; Reinbold et al., 2009). The streamlining of the body shape is thought to reduce drag and swimming costs (Enders et al., 2004), making foraging more energetically efficient in salmonids (Drinan et al., 2012; Pakkasmaa & Piironen, 2001; Vehanen & Huusko, 2011). According to the distribution and behavior of *A. grahami*, swimming ability is very important for their survival in deep lakes (Yang, 1992). Thus, individuals with a stubby body shape may exhibit reduced swimming performance and poorer competition fitness against icefish. This may be one of the reasons why the *A. grahami* survival rate (about 10%) is very low, despite the release of more than 10 million individuals in Lake Fuxian.

In addition to body shape, skeletal deformities may be another factor influencing fish survival following release. Skeletal deformities have been observed in both wild and hatchery-reared teleosts, but the prevalence of deformity differs (Georgakopoulou et al., 2010). Based on the current study, we found that the earlier caught wild specimens of *A. grahami* from Lake Fuxian had normal skeletons, but hatchery-reared fish in some aquafarms around Lake Fuxian have developed skeletal deformities. Genetic issues related to broodstock, malnutrition, temperature, and lack of natural selective pressure can elevate the prevalence of deformities under culture conditions (Georgakopoulou et al., 2010; Tave et al., 1983; Witten & Huysseune, 2009). Our examination of the F1 and F2 generations produced at the EFCC and aquafarms around Lake Fuxian found no deformities in the former, even though the genetic diversity between the two populations (EFCC004 and EFCC005) was very low. Thus, loss of genetic variation might not be a key factor causing skeletal deformity in *A. grahami*. Of note, feed formulations used by the EFCC are modified according to the different developmental stages of *A. grahami*; in contrast, many aquafarms neglect this approach, with fish from farms with higher skeletal deformities generally fed a low protein and lipid content diet. Therefore, we speculate that nutrition may be an important factor leading to deformities in some aquafarms. As the bone malformation rates were not checked before re-introduction into Lake Fuxian, the released populations with high deformity rates caused an elevation in the incidence of deformity. However, these individuals were only distributed in locations close to the release sites. This indicates that individuals with skeletal deformities may lack swimming abilities for long-distance migration. Further studies should be done to evaluate swimming performance between normal and abnormal fish.

Like phenotype discrimination in each population, aquaculture-based enhancement also influenced genetic features. Almost all *A. grahami* population sites showed relatively high haplotype but low nucleotide diversity, consistent with previous studies on *Aphyocypris kikuchii* (Lin et al., 2008), *Coilia ectenes* (Ma et al., 2012), and *Sinibrama macrops* (Zhao et al., 2016). Thus, *A. grahami* may have originated from a population with a small effective size, with the subsequent accumulation of a relatively high number haplotypes but few nucleotide sequence variations, as observed in prior research (Avise, 2000). Considering the microsatellite data, which are related to nucleotide sequences, we also found that heterozygosity and polymorphism information content in *A. grahami* were low. Low genetic diversity is a disadvantage in population recovery in Lake Fuxian as it can obstruct biodiversity conservation and negatively affect species viability (Allendorf & Luikart, 2007; Frankham et al., 2002). Thus, to mitigate negative effects, genetic features need to be checked before release and populations with low genetic diversity should be excluded. However, releasing activities over the past 10 years have been done without genetic evaluation, resulting in low genetic diversity in the Lake Fuxian population, especially in western regions close to the release sites. To elevate and maintain genetic diversity, genetic management techniques in hatcheries should be taken, e.g., minimal kinship selection, maximal founder representation, and minimal relatedness selection (Fisch et al., 2015). In addition to genetic diversity, our results also showed slight inbreeding in some generations of *A. grahami*. Inbreeding is an important factor influencing small and isolated populations and can result in loss of genetic diversity (Frankham et al., 2002; Hartl et al., 1997). Thus, some methods such as pedigree-based crosses and minimal relatedness selection should be taken to avoid inbreeding and loss of heterozygosity (Fisch et al., 2015).

Over the last decade, the body shape, health status, and genetic characteristics of *A. grahami* have changed compared to that of the early conspecifics. Hatchery-reared fish appear to perform poorly in Lake Fuxian, which may be due to phenotypic mismatch (stubby-body shape and skeletal deformities). The current release strategy has lasted for more than 10 years, however, how long the influences of morphology will last and the effects on offspring based on the current released populations require more study. The highest skeletal deformity and lowest genetic diversity rates were found in the west coast region of the lake, with fish from the western region separated from fish collected at the other three sites. We speculate that released populations with high skeletal deformities or relatively low genetic diversities may lack long-distance migration abilities. Thus, they settle in areas near their release sites and exhibit different features to individuals from other sites.

For maximum benefit of conservation programs, we should use available methods to minimize risks in population recovery (Jonsson & Jonsson, 2006). Although conservation stocking has had a positive effect on *A. grahami* population size in Lake Fuxian, there are some potential risks that can lead to negative effects in population recovery in the future. Firstly, released *A. grahami* fish with stubby bodies may have a reduced survival rate due to their lower swimming ability and poorer competitive fitness against icefish. Secondly, whether serious skeletal problems in parents impact their offspring

remains unclear. Finally, the reduced genetic diversity of existing populations would be a major disadvantage to species conservation. Based on the above analyses, we offer the following recommendations:

I. To enhance their ability to compete with icefish, *A. grahami* should be cultured with this competitor before release. Only individuals with a length greater than the standard (10 cm) should be released, because individuals at earlier stages may not have the ability to compete with icefish for food.

II. Feed formulations for *A. grahami* need to be adapted to different developmental stages to ensure normal skeletal morphology. The feed formulations in aquafarms around Lake Fuxian, especially Luchong and Haikou, should be improved (e.g., increased protein, lipid, and microelement content) based on *A. grahami* requirements. Before release, fish should be checked for skeletal deformities, and populations with a high rate of deformity should not be released.

III. Multiple guidelines involved in genetic management should be considered and adopted to maintain genetic diversity and avoid inbreeding. To elevate and maintain genetic diversity, the populations of EFCC003, EFCC004, EFCC005, Niumo and Luchong could be bred with EFCC001 or EFCC002 for hybridization. To avoid inbreeding, the EFCC004, EFCC005, and Haikou populations could be bred with distantly related populations, such as Huoyanshan and Jiuxi. Populations with low genetic diversity should not be released.

IV. Tags such as visible implant fluorescent elastomer (VIE) and otolith marker should be applied to study the growth, survival rate, migration, and dynamics of released fish.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission for field surveys in Lake Fuxian was granted by the Administration of Lake Fuxian, Yunnan Province.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.W.Z., X.F.P., and J.X.Y. conceived and designed the experiments. Y.W.Z., X.A.W., and W.F. performed all the experiments and analyzed the data. Y.W.Z. drafted the manuscript and all authors discussed and revised the manuscript. All authors read and approved the final version of the manuscript.

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