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Genetic structure and low-genetic diversity suggesting the necessity for conservation of the Chinese longsnout catfish, *Leiocassis longirostris* (Pisces: Bagriidae)

Zhongwei Wang[a,b], Jianfeng Zhou[a,b], Yuzhen Ye[a], Qiwei Wei[c,d], & Qingjiang Wu[a]
[a]*State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, 430072 Wuhan, P.R. China, (e-mail: qjwu@ihb.ac.cn)*
[b]*Graduate School of Chinese Academy of Sciences, Beijing, China*
[c]*The Key Lab of Freshwater Fish Germplasm and Biotechnology, Ministry of Agriculture, P.R. China*
[d]*Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Sciences, 434000 Jingzhou, Hubei, P.R. China*

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Synopsis

Genetic variation and phylogenetic relationship of *Leiocassis longirostris* populations from the Yangtze River were investigated at mitochondrial DNA level. The samples were collected from the upstream and mid-downstream of the Yangtze River. Three mitochondrial DNA fragments, ND5/6, cytochrome *b* (Cyt *b*) and control region (D-loop), were amplified and then digested by 10 restriction endonucleases. Twenty-three D-loop fragments randomly selected were sequenced. Digestion patterns of ND5/6 by *Alu*I and *Hae*III, D-loop by *Hin*fI and *Rsa*I, and Cyt *b* by *Hae*III were polymorphic. Ten and eighteen haplotypes were obtained from RFLP data and sequence data, respectively. The individuals from upstream and mid-downstream of the Yangtze River were apparently divided into two groups. The average genetic distance was 0.008 and 0.010 according to the two data. Low diversities and decreasing abundance indicated that *Leiocassis longirostris* may be in severe danger and reasonable measures of fishery management should be taken.

Introduction

Mitochondrial DNA (mtDNA) has become one of the greatest studied portions of animal genome in stock structure analysis (Poteaux et al. 2001; Szalanski et al. 2000; Takeyama et al. 2001), systematic and phylogeny studies (Simons et al. 2001; Triantafyllidis et al. 1999). The mtDNA is haploid, inherited maternally and lacks recombination, which reduces the effective population size for mtDNA to one-forth of that for nuclear genes (Lindak & Paul 1994). These properties make mtDNA a valuable marker for detecting population structure at the species level. Among all the mitochondrial genome the control region, which is noncoding, has been used to detect genetic variations of fishes for the studies of population structure and phylogeny since it varies greater than the other regions (Aurelle & Berrebi 2001; Hoelzel et al. 1998; Nesbo et al. 1998). Although cytochrome *b* (Cyt *b*) does not show a high-sequence variation, it can provide enough genetic information on the species level and distribution of genetic variability (Bernardi & Crane 1999; Carr &

Marshall 1991; Zhang et al. 1999). The ND5/6 consists of about 2600 nucleotides, which is suitable for restriction endonuclease digestion (Hansen et al. 1999; Lu et al. 1997; Toline & Baker 1995). In this study, these three fragments were used to assess the genetic diversity of *Leiocassis longirostris*.

Leiocassis longirostris is a semi-migratory fish, which mostly lives in the trunk stream and distributaries upstream of the Yangtze River and is rarely found in lakes (Wu 1975). Because of overfishing, pollution and other disturbances this species has been decreasing rapidly and it has almost disappeared in some locations of the Yangtze River (Dai et al. 1994; Luo et al. 2000; Wu et al. 1999). The Yangtze River, the longest river in China, traverses from west to east for more than 6300 km and has a high drop. The Three Gorges appeared after the middle Pleistocene Duga glacial age and were gradually being formed by glaciation during the Duga age in a period of 0.7 – 1.0 million years (Tang 2001). The natural barriers have separated the Yangtze River into two parts, which are different in geomorphological and ichthyofauna characteristics. In addition, the Three Gorges Dam has had effects on migratory fish in the past years (Lu et al. 1997; Zhang 1998). Fisheries biologists have long been concerned with the influences of the Three Gorges Dam on gene exchanges between fishes distributed in upstream and mid-downstream sections of the Yangtze River. With this study we are attempting to answer the question of whether or not *Leiocassis longirostris* inhabiting the upstream and mid-downstream belongs to different populations. The main purpose of this work was to assess the population diversities by PCR–RFLP and sequencing. Also, this may provide useful information for the conservation of this species.

Materials and methods

Sample collection and DNA extraction

Fish samples used in this study were collected from four locations of the Yangtze River (Figure 1). Samples of up-stream were collected from Chongqing city (CQ, $n = 21$) and those of mid-down stream were obtained from the Shishou city (SS, Hubei Province, n = 28), Wuhan city (WH, Hubei Province, $n = 24$), and Jiujiang city (JJ, Jiangxi Province, $n = 16$). Fin tissues of the live fish were cut and soaked immediately in 95% ethanol, then stored in −20 °C. Fin tissues were digested over night in 750 μl extracting buffer (10 mM Tris, 0.1 M EDTA, 0.5% SDS, pH 8.0) and 10 μl proteinase K (20 mg/ml) was added. The standard method of phenol–chloroform extraction and 100% ethanol precipitation was used to extract DNA (Sambrook et al. 1989).

Amplification of mtDNA fragments

Three mtDNA fragments were amplified by PCR using the primer HD (CATCTTAGCATCTTCAGTG) and primer LD (TCACCCCTGGCTCCCAAAGC) (Liu et al. 2002), primer ND5G (CAACGGTGGTTCTTCAAGTC) and ND6L (GGAACCAAAAACTCTTGGTGCAACTCC) (Park et al. 1993), L14724 (GACTTGAAAAAC CTCCGTTG) and L15915 (CTCCGATCTCC

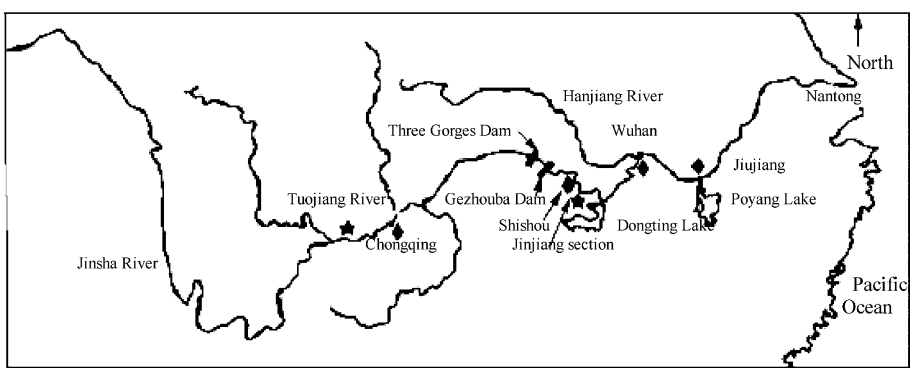

Figure 1. Map of the Yangtze River. Diamonds and pentagons stand for sampling sites and spawning grounds, respectively.

GGATTACAAGAC) (Xiao et al. 2001), respectively. Amplifications were performed in 25 μl reaction mixture, which contained 0.1 mM dNTP, 0.05 μM primers, 1 U Taq polymerase and 2 μl template DNA (200 ng). Amplifications of three fragments were performed in different profiles. PCR profile consisted of initial denaturation for 5 min at 94 °C followed by 40 cycles : denaturation at 94 °C for 30 s, annealing at 48 °C (ND5/6 and D-loop) or 58 °C (Cyt *b*) for 30 s and extending at 72 °C for 90 s (D-loop and Cyt *b*) or 120 s (ND5/6). The final extension at 72 °C was performed for 10 min. PCR products were assessed by 1.0% agarose gel electrophoresis.

Digestions of PCR product

Ten endonuclease enzymes, *Hin*fI, *Dde*I, *Rsa*I, *Msp*I, *Alu*I, *Hha*I, *Hae*III, *Mbo*I, *Taq*I and *Acc*II, were used to digest the three amplified fragments. The 15 μl reaction mixtures contained buffer, 2 U restriction endonucleases and 5 μl PCR products. Reactions were performed at 37 °C or 65 °C (only for *Taq*I) for 5 h. The digestions were assessed by 1.4% agarose electrophoresis and then were scanned.

Purification, cloning of PCR product and DNA sequencing

Five D-loop fragments from Jiujiang, seven fragments from Wuhan, five fragments from Shishou and six fragments from Chongqing were randomly selected. After agarose gel electrophoresis (1.4%), the PCR products were excised from the gel. And they were purified and concentrated using the Glassmilk DNA purification Kit (Biostar International, Canada). The purified DNA was ligated to PMD18-T vector and the plasmids containing the PCR fragments were then used to transform *Escherichia coli* DH5α competent cells. Positive clones were selected then were cultured at 37 °C for 2 h or more. The cultures were sent to being sequenced using the primers M13+ and M13−.

Data analysis

RFLP analysis

Genetic distances (Nei & Li 1979) among the samples were calculated using the Generate and D program of REAP software package (McElroy et al. 1992). Clustering trees were constructed according to the mean genetic distances of four populations using the UPGMA method by Mega Version 2.1 (Kumar et al. 2001). The hierarchical partition of genetic variation among the four populations of *Leiocassis longirostris* was tested by AMOVA software (Analysis of Molecular Variation, Version 1.55, Excoffier et al. 1992) and the statistical significance was tested with 9999 permutations on haplotypes among populations. The input files of AMOVA including distance file and group file were prepared with AMOVA-PREP software.

Sequences analysis

All nucleotide sequences were aligned by computer program Clustal X (Thompson et al. 1997). Genetic distances were calculated and phylogenetic trees were constructed using the UPGMA method by Mega Version 2.1 (Kumar et al. 2001). The genetic structure and AMOVA analysis were performed by Arlequin software (Schneider et al. 2000) and the statistics significance was tested with 9999 permutations.

Results

The amplified fragments of ND5/6, D-loop and Cyt *b* were about 2600, 900, and 1200 bp, respectively. The segments digested from *Msp I, Dde I, Hha I, Mbo I, Taq I* and *Acc II* did not show any polymorphism. A total of 26 segments were obtained from the digestion patterns by five restriction endonucleases and the segments sizes are shown in Table 1. The total sizes added by all digested bands were not identical, which result from undetected little bands and un-separated bands with same size. Fifteen of them (57.69%) were polymorphic. The digestions of ND5/6 fragment by *Alu*I and *Hae*III showed two different patterns. Two patterns were found after the D-loop were digested by *Hin*fI and *Rsa*I. Two patterns were obtained from the digestion product of the Cyt *b* by *Hae*I. The different patterns were marked A and B, respectively. All eight different haplotypes were generated from three fragments and the haplotype frequencies of the four

Table 1. Sizes of restriction fragments for ND5/6, D-loop and Cyt *b of Leiocassis longirostris*. Dashes stand for a particular fragment did not exist in a given pattern.

Restriction endonuclease	haplotype	Total size	Fragment size						
ND5/6									
*Alu*I	A	2565	935	–	568	–	407	345	310
	B	2534	–	679	568	535	407	345	–
*Hae*III	A	2593	710	–	480	432	369	332	270
	B	2493	–	610	480	432	369	332	270
D-loop									
*Hin*fI	A	983	636	–	347	–			
	B	1003	–	564	347	90			
*Rsa*I	A	941	546	395	–	–			
	B	947	546	–	229	172			
Cyt *b*									
*Hae*I	A	1263	815	–	448	–			
	B	1239	–	530	448	261			

populations are shown in Table 2. The frequency of haplotype 1 was the highest among all the observed haplotypes. Haplotypes 5–7 were only found in upstream population and haplotype 2, 3, 4, 9 and 10 were only found in mid-downstream populations. The frequencies of haplotype 4, 7, 9 and 10 were very low. The average genetic distance between four populations ranged from 0.005 to 0.011 and the mean genetic distance of all individuals was 0.008. UPGMA tree (Figure 2) was constructed according to the mean genetic distance between the four populations. The tree was apparently divided into two clades. Wuhan population was clustered with Shishou population and Jiujiang population into one clade, but Chongqing population located on the other clade.

Table 2. Composite haplotypes, haplotype frequencies and sample sizes of the fish.

Haplotype	Composite haplotype	CQ	SS	WH	JJ
1	AAAAA	8	16	18	10
2	AAAAB		7		
3	AAABA		1		6
4	AAABB		1		
5	AABAA	6			
6	AABBA	3			
7	ABBBA	1			
8	BAAAA	3	1	5	
9	BAAAB		1		
10	BAABA		1	1	
N		21	28	24	16

Composite haplotypes are indicated by A and B in the order. ND5/6: *Alu*I *Hae*III;D-loop: *Hin*fI, *Rsa*I; Cyt *b*: *Hae*III. CQ – Chongqing; JJ – Jiujiang; SS – Shishou; WH – Wuhan.

The complete length of D-loop (apart from primers and t-RNA) was 894 bp long. The result of aligned sequence was shown in Table 3 and there were 39 varied cites in all. Eighteen haplotypes were obtained from 23 sequences and average genetic distance was 0.010. It was known that the four populations were divided into two groups from the UPGMA trees (Figures 3 and 4).

The results of variance analysis from RFLP and sequences were uniform basically (Table 4). Most of the genetic variance was distributed within populations. It was 81.77% for RFLP analysis and 69.59% for sequence analysis. Overall Fst was 0.182 for RFLP and 0.304 for sequence. Population pairwise Fsts from RFLP and sequence were shown in Tables 5 and 6, respectively.

Discussion

Genetic variation and population structure analysis

Low levels of genetic variations were detected in this study. In terms of the digestion patterns of ND5/6 fragments, only one individual from Chongqing was different from the other individuals digested by *Hae*III. As for Cyt *b*, polymorphism was only found from the digestion by *Hae*III. Only 10 haplotypes were detected from all three amplified fragments in 89 individuals and it was lower than Chinese farmed carps, bighead carp (19/90), silver carp (28/92), black carp (27/93) and similar to grass carp (7/90) in the Yangtze River (Lu et al. 1997). The reason of the low diversities of grass carp is considered that genetic bottlenecks ever happened (Zhang et al. 2001). The low-genetic diversity of *Leiocassis longirostris* is much more likely to be related with population bottlenecks, low abundance or other factors.

The major factor leading to population homogeneity is the lack of barriers to gene flow (Ward et al. 1994). Mid-downstream of the Yangtze River is continual and the fish can swim up and down without barriers. While there is a natural barrier, the Three Gorges between the upstream and mid-downstream of the Yangtze River and fishes hardly pass though it. The Three Gorges

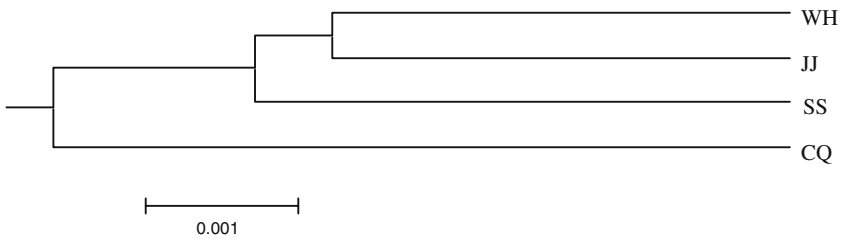

Figure 2. Genetic relationship of the four populations obtained from mean genetic distance on RFLP using UPGMA method.

acted natural barriers in fact. The high-water current and great level of fall inhibited the gene exchange between them. Spawning grounds of *Leiocassis longirostris* are mainly located in Tuojiang River in Chongqing city and Jingjiang section in Hubei Province (Figure 1). So the fish may be divided into two reproductive populations. The similar conclusion was made from the PCR–RFLP and sequencings analysis of mitochondrial fragments. According to the genetic distance, the value of interpopulation genetic distance was higher than that of intrapopulation. Furthermore, clustering tree showed that there was apparent difference between upstream fish and mid-downstream fish (Figures 2–4). The value of population pairwise Fst between Chongqing and the other three populations were higher than that of between the three mid-downstream populations (Tables 4 and 5). There is a little unlikeness of population pairwise Fst between the RFLP and sequencing

Table 3. The varied sites from the aligned D-loop fragment. Dot stands for identity with top reference sites.

	Nucleotide position			
	1122	2222222223	3334455666	667777888
Sample	1112250713	3355666790	1584507044	660057138
	0261885194	5609258011	7683221001	482517594
JJ1	ataaagttag	aagatcttcc	ataattaggt	ttatgaaag
JJ2	........g.	....	.......a..	....
JJ3	.....a..g.	.....tc...	.c....g.a.	....a..ga
JJ4	......c.g.	.....tc...	.c....g.a.	....a..ga
JJ5	........g.	......c...	...t....a.	.....g...
WH1	........g.	.....tc...	.c....g.a.	....a..ga
WH2	....	....	....	....
WH3	.......cg.	......c...	..g......c	....
WH4	........ga	......c...	g.........	....
WH5	........g.	....c.c...	....	....
WH6	.......cg.	......c...	....	....
WH7	........g.	......c...	...t....a.	.....g...
SS1	........g.	.......ct.	....	.......g.
SS2	........g.	.......ct.	....	.......g.
SS3	....	.....tc...	.c....g.a.	.......g.
SS4	........g.	.......ct.	....	.......g.
SS5	..tcc...g.	g.a...-.tt	....c..aa.	ggg...gg.
CQ1	........g.	g.ag..-.tt	......gaa.	......gg.
CQ2	........g.	ggag..-.tt	.......aa.	...c..gg.
CQ3	cc.cc...g.	.......ct.	......g...	.......g.
CQ4	........g.	ggag..-.tt	.......aa.	......gg.
CQ5	........g.	g.ag..-.tt	......gaa.	......gg.
CQ6	........g.	g.a...-.tt	.....c.aa.	......gg.

CQ – Chongqing; JJ – Jiujiang; SS – Shishou; WH – Wuhan.

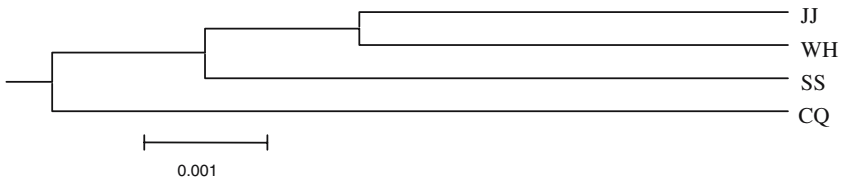

Figure 3. Genetic relationship of the four populations obtained from mean genetic distance on sequence using UPGMA method.

analysis, which may result from the high-variation rate of D-loop. The whole sequences of D-loop could show the variations more sensitively than RFLP analysis. It was also found that there existed limited gene flow according to the haplotype 8. On the one hand, the fish could not be separated completely. Two artificial dams, the Gezhouba Dam and the Three Gorges Dam, may also alter genetic diversities. The Gezhouba Dam was set up for about 30 years and the later was only set up for several years. Even though the *Leiocassis longirostris* consists of two isolated populations, population inhabiting in upstream and mid-downstream, the inhabiting condition and spawning ground will be much changed after the Three Gorges Dam was constructed. What is more, the life cycle time of *Leiocassis longirostris* is from 4 to 5 years. So the effect from dams compared with the Three Gorges could be ignored and the effect from the three Gorges may be primary. But it cannot be ignored in future. On the other hand, Shishou population samples were taken from the hatchery in SS (set up for *Leiocassis longirostris* conservation). They mainly collected the brood stock from mid-stream section of the Yangtze River. Unfortunately, small parts of brood stock were also collected from upstream of the Changjiang River.

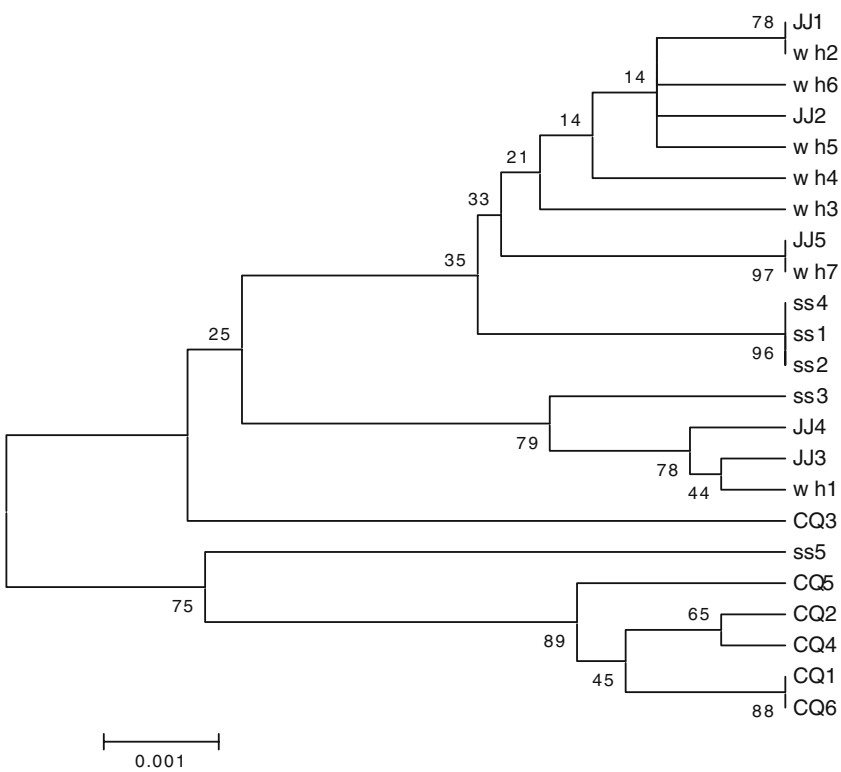

Figure 4. UPGMA tree obtained from sequence pairwise distances.

Table 4. Analysis of variation of *Leiocassis longirostris estimated by AMOVA with 9999 permutations.*

Variance component	Variance		Total(%)		Overall Fst	
	RFLP	Sequence	RFLP	Sequence	RFLP	Sequence
Among populations	0.305	1.358	18.23	30.41	0.182 $p < 0.001$	0.304 $p < 0.001$
Within populations	1.367	3.108	81.77	69.59		

The necessity of conservation

Genetic resources include both the amount of genetic variation as estimated by molecular tools and the absolute numbers of the fish. Low-genetic variations were revealed by this study. Species with low-genetic variations would not be extinct necessarily in future for some genes reserved were highly suitable because of the particular surrounding, but the poorness of genetic diversity is not advantageous for the evolution of species (Zhang 1998). Although it was considered that the spawning grounds mainly located in Tuojiang River and Jingjiang section of the Yangtze River, there may be undetected reproductive populations, which may be extinct with corresponding reduction in interpopulation genetic variation. Owing to over-fishing, water pollution and dam construction, the fish had been decreasing rapidly and only little fish could be obtained in some regions of the Yangtze River. Over fishing decreased the fish abundance and water pollution worsened the living condition. The effects from dams in future are inevitable for river damming is the most dramatic anthropogenic factor affecting freshwater environments, and dams cause habitat loss, change fish reproductive environments and cut off migration routes, resulting in a substantial decline of biodiversity. Measures should be taken to conserve genetic resource of the fish.

Table 5. Population pairwise Fst from AMOVA test with 9999 permutations on RFLP.

	CQ	SS	WH
CQ			
SS	0.2184		
WH	0.1429	0.0939	
JJ	0.2038	0.1660	0.1895

Table 6. Population pairwise Fst from AMOVA test with 9999 permutations on sequences.

	CQ	SS	WH
CQ			
SS	0.2451		
WH	0.5196	0.1901	
JJ	0.4460	0.1184	0.0280

The primary genetic goal of a conservation program is to maintain the existing genetic variation. So the first step taken should be monitoring the genetic composition or variation of brood stock of the Yangtze River including the distributaries, such as Jinshajiang River, Jialingjiang River, Minjiang River. Fu et al. (2003) considered that fishes in the upstream will be seriously affected by the construction of the Three Gorges Dam and action should be taken for priority conservation. Selecting appropriate reserves may be the most adoptable means of preserving fishes in the Yangtze River. If possible, the distributaries could be selected as reserves.

Chinese government has paid great attention on the conservation of freshwater fishes in the Yangtze River. The fishing activities have been prohibited from February to June since 2003 in the Yangtze River. But in fishing seasons, the minimum exploitation size and total yield should be limited to forbid over-fishing. Artificial propagation and restocking were feasible methods of raising the fish abundance. But it was essential that the number of the brood stock should be increased furthest because artificial propagation would reduce genetic diversities. So natural and artificial ecological banks of species resource should be set up to culture more mature fish in the surrounding similar to the Yangtze River and to preserve the quantity of wild fish. Further studies are needed to investigate genetic resources and minimize the disadvantages from the Three Gorges Dam.

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