Genetic Characterization of the Endangered *Brachymystax lenok tsinlingensis* (Salmonidae) Populations from Tsinling Mountains in China using Microsatellite Markers

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A B S T R A C T

Brachymystax lenok tsinlingensis (Salmonidae) is an indigenous and endangered cold-water fish in Tsinling mountain, China, and only is distributed as spot in the Weihe River and Hanjiang River among Tsinling mountain, To access relationship of populations from the two rivers and the genetic diversity of wild population, 11 microsatellite loci were firstly used to evaluate the genetic characterization using 120 samples from 2 tributaries (ZZ and LX populations) of the Weihe river and 2 tributaries (YX and TB population) of Hanjiang river. A total of 59 alleles were found and the number of alleles in each locus ranged from 3 to 8, with an average of 5.4. There were 6 highly polymorphic loci and 4 moderately polymorphic loci. The average expected heterozygosity (H*e*) and observed (H*o*) heterozygosity in four populations ranged from 0.6421-0.6980 and from 0.4841-0.6999. Significant deviations from Hardy–Weinberg were found in the four populations at some loci. Analysis of molecular variance (AMOVA) revealed that relatively little (11.02%) genetic diversity came from individual among population. In contrast, the majority of diversity (88.98%) occurred among individual within a population. Results from Nei's genetic distance indicated that the YX and TB populations were grouped in one cluster, which was clustered with the ZZ population, the LX population was grouped in a separated cluster. Low genetic diversity and strong genetic differentiation have been found in this study. The baseline population genetic information supplied in this study will be vital for the protection and restoration of this endangered species.

INTRODUCTION

*B*rachymystax lenok tsinlingensis (Qinling lenok), as a critically endangered salmonid fish, is only found in Weihe and Hanjing Rivers tributaries(cold freshwater) in Tsinling Mountains, China (Sze-Chung, 1966). In the past few decades, the populations of *B. lenok tsinlingensis* have been declining dramatically because of illegal overfishing and environment changes (Ren and Lang, 2004). In 1988, it has been list as a second class state protected wild animal in China Red Data Book of Endangered Animal (Yang *et al.*, 1999). It is very urgent for researchers to protect this rare cold water salmonid fish resource with effective methods. Microsatellite is one of the molecular markers that are very useful tools to estimate genetic diversity, evolutionary potential, population structure of the endangered species population(Shao*et al.*,2017;Was*et al.*,2002;Liu*et al.*,2014).

The Tsinling Mountain is an east-west trending south vergent mountain, which has been recognized as the geo-ecological boundary between subtropical and warm-temperate zones. *B. lenok tsilinlingensis* is one of two Salmonidae located in the southernmost part of China (the other is hucho taimen) (Xia *et al.*, 2016; Wang *et al.*, 2016). There is no information about the distribution characteristics and genetic diversity of *B. lenok tsilinlingensis* at the northern (Weihe River) and southern (Hanjiang River) of Tsinling Mountain. So, this research aimed at attracting much attention to its genetic diversity, population genetic and the genetic variation assessment of *B. lenok tsilingensis* in the two river systems.

MATERIAL AND METHODS

Sample collection and DNA extraction

One hundred and twenty samples of fin tissues were collected from 4 different tributaries in the northern and southern of Tsinling Mountain between May and September 2015 (Fig. 1, Table I). The fin clips were

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gathered and stored in 95% ethanol until extraction, then these fish were immediately set back to the rivers.

Genomic DNA was extracted from samples of fish fin tissue using a standard traditional phenol-chloroform procedure (Sambrook and Fritsch, 1989) and standardized to a concentration of 50ng/μl and stored in ddH$_2$O.
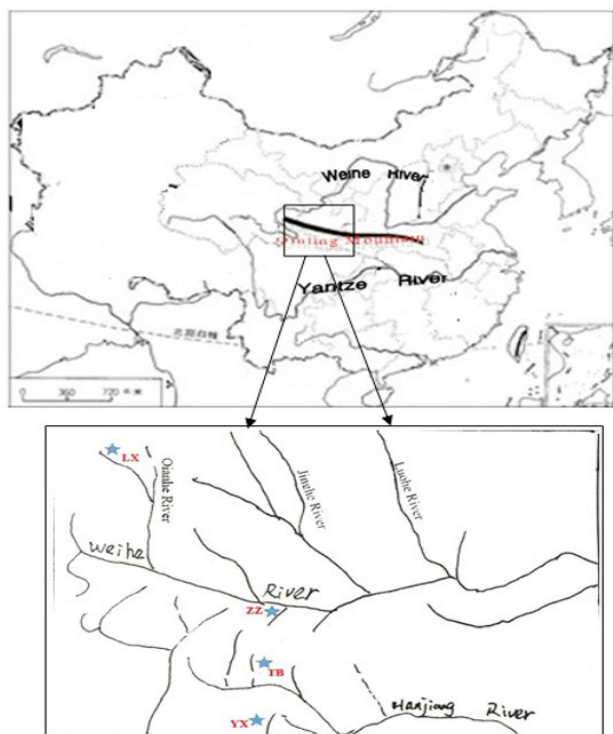

Fig. 1. Locality of sampling of *Brachymystax lenok tsinlingensis* in the study.

Methods

Twenty microsatellite loci were used for the design of the multiplex PCR. The 8 of 18 microsatellite markers were developed in our laboratory using the method of fast isolation by AFLP of sequences containing repeats (FISCO) and primers were designed using Primer 5.0. The other 10 polymorphic primers pairs came from known microsatellite makers in *Brachymytax lenok* and *Hucho bleekeri* (Jia *et al*., 2008). All the primers were synthesized by Nanjing GenScript Biological Engineering & Technology and Service Co. Ltd., Nanjing, China.

For initial testing, the PCR amplifications were carried out in a 10 μl reaction volumes containing the following components: 20 ng of genomic DNA, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.25 μM each primer, 1×Taq buffer and 0.5 U Taq polymerase (Takara). PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation 30 s at

94°C, 30 s at a locus-specific annealing temperature (Table II), and extension 30 s at 72°C, with a final extension at 72°C for 8 min. The PCR amplification produces were separated using 8% non-denaturing polypropylene gel electrophoresis (120V, 4°C for 3-5 h), and then the gel was silver stained and scanned. Only 11 primer pairs that could amplify polymorphism were selected for the further study. Amplification conditions were further optimized by adjusting the relative concentration of ingredient and annealing temperatures.

Table I.- Sampling site and date of *B. lenok tsinlingensis* population.

Population	Location	n	Date
Yangxian (YX)	Xushui river in Yangxian town at the southern foot of Qinling Mountain E 107°17'-107°55'/N 33°19'-33°44'; Elevation 800-3071 m	40	Mar, 2015
LongXian (LX)	Qianhe river in Longxian town at the northern foot of Qinling Mountain E106°26'-107°06'/N34°35'-35°08'; Elevation 1100-2466 m	30	Sep, 2015
Taibai (TB)	Taibai river at huangpoyuan of Taibai town in Baoji Provinces E107°16'-107°42'/N33°38'-33°54'; Elevation 902-1510 m	30	Apr, 2015
Zhouzhi (ZZ)	Heihe river in Zhouzhi town at the northern foot of Qinling Mountain E107°39'-108°19'/N33°41'-33°57'; Elevation 2996 m	20	Apr, 2015

Data analysis

POPGENE32 software was used to estimate the expected heterozygosity:

$$He = 1 - \sum P_i^2$$

Where, P_i is the frequency of the ith allele (Nei, 1973) and observed heterozygosity (Ho=Na/n; Where *Na* is the number of heterozygous genotypes, *n* is the total number of genotypes), fixation index (F_{is} = 1 - H_o/H_e (Wright, 1965)), genetic distance (D) among population, genetic differentiation index (F*st*), and gene flow (*Nm*). We tested the linkage disequilibrium (LD) using GENEPOP program (online version through http://genepop.curtin.edu.au/) and default parameters. The Markov chain method (Guo and Thompson, 1992) was used to estimate the probability of significant deviation from Hardy-Weinberg equilibrium

(HWE).AMOVA was used to analyze the genetic differentiation among the populations. The micro-check software (Van Oosterhout *et al.*, 2004) was used to infer the most probable technical cause of HWE departures, including null alleles, mis-scoring and allelic dropout. Polymorphism information content (*PIC*) (Bostein D *et al.*, 1980) of each locus was analyzed using the following formula:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 P_i^2 P_j^2$$

Where, P_i and P_j are allele's frequencies and n are the locus number.

Table II.- The sequences of 11 pairs of microsatellite primers.

Locus	GenBank Accession No.	Annealing primer (5′-3′)	Product size (bp)	Annealing temp (°C)
GTL05	JN835587	TACATTTCAGTTTGAATCACGCAAC CGTGATGCAGCCCTGCAT	114-150	54.8
GTL08	JN835588	AGGAAATGTTAGGATGATAGAGGCA TTGGTTATTTAATGCGGTTGGG	100-140	50.0
GTL10	JN835590	AGCCTACCTCTTCTGTCTAGTGAGG TGTGCAAATAGTTCAAGAACAAAAG	120-150	63.0
GTL12	JN835591	CTGCAGACTGGATCTTATCAGGAGC GCATACAAGTACGCACGCCGA	170-200	63.0
GTL14	JN835592	AGGCAGACTAACTGTGTGTATC AGTTAGTGTGAACTCTGTTGTGT	160-190	50.0
GTL15	JN835586	AGCGACAGTGTGTGTGAGTAAGTGG CCTCAAACCTGATGACCTCACACA	120-183	55.0
GTL21	JN835594	GAGATTAGATTGGGAGACTATGGTG ATGGTTGTGTTCTCACTCTCTCTTT	120-140	63.0
GTL23	JN835595	TCCAGTGAGACCTGCCCAGTAACAT ATTTGTGCATGAGTGCAGCATGTG	128-210	53.0
HLJZ069*	DQ120978	ACCGACACAACACAAACA ATGAATGCGGGATAAAGT	148–165	45.0
LETET2*	AY84452	TGTCAGAGGCCTTGACTGCGT GCTAGGCTGTTTACTCTAGGT	150-169	60.0
BLE-TET9**	AY486103	ACTGGATAGAAAGACCTGTGG AGATTCTTGGTAAAAGTGAAG	170-230	55.0

*, microsatellite marker of *Brachymystax lenok*; **, microsatellite marker of *Hucho bleekeri.*

Table III.- The genetic diversity indices of eleven microsatellite loci for four *Brachymystax lenok tsinlingensis* populations.

Locus	ZZ				TB				YX				LX			
	Ne	Ho	He	PIC	Ne	Ho	He	PIC	Ne	Ho	He	PIC	Ne	Ho	He	PIC
GTL05	3.8898	0.7000	0.7421	0.6533	3.3235	0.4167	0.6739	0.5904	4.1923	0.9167	0.7609	0.6838	3.7400	0.5556	0.7320	0.6402
GTL08	4.4898	1.0000	0.7421	0.6527	3.8103	0.9167	0.7283	0.6407	4.5000	1.0000	0.7826	0.7031	3.4455	0.4444	0.6993	0.6110
GTL10	3.1667	0.6000	0.6579	0.5781	4.3400	0.8333	0.7717	0.6970	4.6143	0.8333	0.7899	0.7207	4.8784	0.6667	0.8170	0.7340
GTL12	3.0316	0.8000	0.6368	0.5270	4.1923	0.9167	0.7609	0.6838	3.1667	0.8333	0.6522	0.5454	4.0217	0.6667	0.7582	0.6657
GTL14	3.6250	0.6000	0.7158	0.6420	3.4691	0.6667	0.6920	0.5891	3.3515	0.8333	0.6775	0.5961	4.1000	0.6667	0.7647	0.6713
GTL15	2.7627	0.5080	0.5895	0.4992	4.3400	0.5833	0.7717	0.6909	3.1422	0.5333	0.6489	0.5685	2.8143	0.5100	0.6013	0.4889
GTL21	3.4412	0.4000	0.6947	0.6102	3.4388	0.6667	0.6884	0.6308	3.4691	0.7500	0.6920	0.6164	3.1557	0.3333	0.6601	0.5525
GTL23	2.8810	0.6732	0.6105	0.4918	2.5000	0.5517	0.5217	0.3750	2.6654	0.8333	0.5616	0.4316	2.5000	0.6563	0.5294	0.3750
HLJZ069	4.2736	0,7000	0.7737	0.6869	4.0122	0.8333	0.7464	0.6668	3.4091	0.5833	0.6848	0.5887	3.5000	0.3333	0.7059	0.6020
LETET2	2.5619	0.1000	0.5421	0.4244	2.8415	0.2500	0.5978	0.5100	3.4691	0.5000	0.6920	0.5891	2.0283	0.0080	0.3660	0.2859
BLETET9	2.0152	0.0180	0.3579	0.3142	3.7727	0.8333	0.7246	0.6390	3.0487	0.0833	0.6431	0.5470	3.1557	0,1111	0.6601	0.5786
Mean	3.2317	0.5399	0.6421	0.5527	3.6400	0.6789	0.6980	0.6103	3.5480	0.6999	0.6887	0.5991	3.3945	0.4841	0.6631	0.5641

Ne, expected alleles; Ho, observed hererozygosity; He, expected heterozygosity; PIC, polymorphism information contents.

RESULTS

Genetic diversity of analysis

Eleven pairs of polymorphic microsatellite primers were used to analyze genetic diversity of these populations from 4 tributaries. All parameters for genetic variation of each population are summarized in Table III. We amplified a total of 59 polymorphisms loci, the number of alleles per locus ranged from 3-8, with an average of 5.4. The expected (He) and observed (Ho) hererozygosities in the four populations ranged from 0.6421 to 0.6980 and from 0.4814 to 0.6999, respectively. All 11 microsatellite loci were found to be highly polymorphic in four populations, the average of PIC were 0.5527~0.6103.

Hardy–Weinberg equilibrium

The Hardy-Weinberg test on multi-locus based on the Markov chain method showed that there were separately 3, 2, 3, 4 loci deviated from HWE(p<0.01)in each of the four populations, the LX population had the highest level of deviation (four loci). Conversely, the TB population had the least deviation (two loci) The MICRO-CHECKER analysis showed no evidence for scoring error or technical or statistical artifacts, the results of the HWE tests for the individual populations were shown in Table IV.

Table IV.- Test for Hardy-Weinberg equilibrium of genotypes in the four *Brachymystax lenok tsinlingensis* populations.

Locus	ZZ	TB	YX	LX
GTL05	0.00007	0.15613	0.00102	0.04752
GTL08	0.57261	0.00005	0.03502	0.09367
GTL10	0.03363	0.02978	0.11302	0.00265
GTL12	0.01353	0.27674	0.00001	0.03678
GTL14	0.00181	0.29593	0.42283	0.00575
GTL15	0.06548	0.09526	0.05202	0.05437
GTL21	0.07053	0.05277	0.06116	0.07627
GTL23	0.02929	0.02691	0.18728	0.01468
HLJZ069	0.05442	0.41198	0.19910	0.09557
LETET2	0.01059	0.00410	0.01330	0.00071
BLETET9	0.00000	0.04234	0.00000	0.00003

Note: P < 0.01 denotes Hardy-Weinberg disequilibrium.

Genetic structure of four B. lenok tsinlingensis *populations*

Gametal correlation coefficient (F_{ST}) and Gene Flow (Nm) were computed to estimate the differences between populations. The average of Fst was 0.1109, and the average of Nm among populations was 2.4308 at locus (Table V). It indicated genetic variations within the populations were higher than those among the populations, thus genetic flow might be the significant factor between

populations (Table VI, Fig. 2). The AMOVA method was used to analysis the genetic structure of the populations, the result showed that 11.02% of the genetic diversity was distributed among the populations and 88.98% occurred among individuals within the populations.
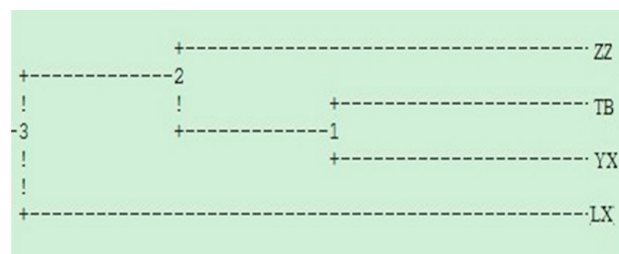

Fig. 2. The phylogenetic tree of 4 *Brachymystax lenok tsinlingensis* populations. Sampling locations: ZZ, Zhouzhi population; TB, Taibai population; YX, Yangxian population; LX, Longxian population.

Table V.- The results of *F*-statistics for each of 11 loci across four *B. lenok tsinlingensis* populations.

Locus	Fis	Fit	Fst	Nm*
GTL05	0.0658	0.1424	0.0819	2.8024
GTL08	-0.1947	-0.0808	0.0953	2.3728
GTL10	-0.0139	0.0532	0.0661	3.5306
GTL12	-0.2024	-0.0464	0.1297	1.6775
GTL14	-0.0191	0.0406	0.0587	4.0119
GTL15	0.2130	0.2324	0.1193	1.5150
GTL21	0.1751	0.2709	0.1161	1.9031
GTL23	-0.2097	-0.2383	0.0063	5.1083
HLJZ069	0.1165	0.2122	0.1082	2.0597
LETET2	0.5946	0.6435	0.2141	0.6179
BLETET9	0.5463	0.6435	0.2141	0.6179
Mean	0.0974	0.1702	0.1109	2.0042

Table VI.- Analysis of molecular variance (AMOVA) within and among the four *B. lenok tsinlingensis* populations.

Source of variation	df	Variance component	Percentage of variation	F_{St}
Among populations	4	0.8166	11.02	0.1102
Within individuals	120	6.4322	88.98	

Clustering analysis

Based on Nei' genetic distance and phylogenetic tree, the highest divergence was between the TB and LX populations (D=0.2640), while the lowest divergence was between TB and YX populations (D=0.0014) which

all located at the foot of the south of Tsinling mountains (Table VII).

Table VII.- Nei's genetic distance of the 4 *B. lenok tsinlingensis* populations.

	ZZ	TB	YX	LX
ZZ	****			
TB	0.0806	****		
YX	0.1232	0.0014	****	
LX	0.1760	0.2640	0.1522	****

DISCUSSION

Results of primer amplification

B. lenok tsinlingensis is endemic to cold-water of Tsinling Mountains and mainly distributed Weihe River and Hanjing River drainage, China. Hanjiang River drainage is southernmost point of *B. lenok tsinlingensis* distribution (the other Salmonidae that distributed southernmost is *hucho bleekeri*). However, the adaptive characters and genetic diversity of these populations have been little studied. We analyzed the genetic diversity using 18 microsatellite primers (Liu *et al*., 2013; Lu *et al*., 2012), but only 3 of 10 microsatellite markers from *Hucho bleekeri* and *Brachymystax lenok* could amplify polymorphic loci in *B. lenok tsinlingensis*. The results showed that the markers obtained from other salmonids are suitable for study of Qinling lenok (Kuang *et al*., 2009), but have relatively low availability.

Hardy-Weinberg and linkage disequilibrium

The global Hardy–Weinberg equilibrium (HWE) of all populations and loci were evaluated, and significant departures from Hardy–Weinberg equilibrium were found for several loci in some locations. The four populations had 3, 2 3 and 4 locus deviation after Bonferroni correction (P<0.01), respectively. Processes causing this non-equilibrium were specific to those populations; those loci did not show consistent deviation cross all locations. Main reasons of deviated HEW were attributed to geography and shrinking of population size. The result also was proved by the other landlocked freshwater Salmondae fish such as Hucho taimen (Kuang *et al*., 2009) and *Oncorhynchus keta walbaum* (Jinping *et al*., 2004) in China.

B. lenok tsinlingensis wild population only distributed the tributaries of Weihe and Hanjing River, which separately located the northern and southern of Tsinling Mountain. The distribution areas of the fish have suffered severe effected even vanish due to local water temperature increasing and excessive fishing in the past forty years, Ren and Lang (2004) once reported that *B. lenok tsinlingensis* population in Qianhe River was not only sharp decrease in number, but also change geographical conditions from altitude 1000 to 1200 m. Thus, the fluctuation of the genotype and gene frequencies is likely due to human influence.

Genetic diversity

The four *B. lenok tisinlingensis* populations exhibited high genetic diversity (H*e*>0.5and PIC>0.5). Dewoody and Avise (2000) computed nearly 40,000 individuals of 78 species freshwater, marine fish and other animal with 524 microsatellite markers, and suggested genetic diversity of freshwater fish (H*e*=0.58 and A*e*=7.1). In the study, the average *He* here was 0.67 and A*e* was 5.4. The *He* was larger, but A*e* was smaller. The reason for accounting for the low Ae is that habitat of these population is in deep water and not be disturb for a long time. Bostein *et al*. (1980) indicated that the locus was high polymorphic when PIC > 0.50, mediate polymorphic when the PIC was between 0.25 and 0.50. The average PIC in the four population here was 0.5642 - 0.6103, which was higher than the polymorphic level, so the four populations were high PIC population. All this indices were higher than study of Yuan and Wang (2009) that suggested lower genetic diversity in the Heihe and Xushui Rivers populations using PAPD methods, the variance maybe was connected with the population size of different tributaries. Xushui River is the first location where *B. lenok tsilinlingensis* were migrated and bred, but the number of the parental fish in Xushui River is relatively few and the germplasm resources were deteriorated owing to no scientific breeding.

Relation and genetic differentiations between populations

There was no significant difference (p>0.01) in the four populations. The average of F*st* of all loci was 0.1109, which was larger than the standard of no differentiation between populations suggested by Wright (1978). According to the expressions of Nm = (1−Fst) / 4 FST, the average of Nm between populations was 2.0042, the differentiations that were caused by genetic drift were stopped among populations. During the Quaternary glaciations (the period from 2.58 million years ago to present), the *B. lenok* originated from Siberia has been flowed to the Yellow sea and the Bohai sea with the role of glacial advance, they ran upstream of the Yellow River to Wei River to spawn as they need breed (Xing *et al*., 2015). Most of the populations backed to primary source as the deglaciation, whereas part of them were stagnated in the high mountain and ravine stream of Wei River basin, and they were sealed off in those regions. After that, the rivers lost the connectivity is aroused by the expansion of

human activities and the construction of many engineering structures, and the blocking of the migration route of *B. lenok* leaded to the point distribution along Weihe River basin, Heredity and aberrance are necessary to adapt to the changing environment, after long time selection, *B. lenok tisilingensis*, as a subspecies of *B. lenok*, survived in the Tsinling Mountains (Yu and Kwak, 2015).

The high genetic identities and low genetic differential of the four populations suggested that *B. lenok tisilingensis* of Weihe River and Hanjiang River derived from the same ancestor. It is said that there was no *B. lenok tsinlingensis* in the southern of Tsinling Mountain before long years ago, and in the 1980s, the fish were migrated to the tributaries of Yangtze River by the local peasant in Zhouzhi town, these isolation habitats induced changes in population genetic structure. The date (Nm>1) suggested that there was gene flow within four populations.

Population genetic research is important to understand population genetic distance and potential species differentiation (Matocq *et al*., 2001). YX and TB populations share the highest genetic identity, indicating the nearest relationship. Populations LX and TB had the lowest genetic identity and the relationship was the farthest. Geographic isolation for a long time and genetic variation help them to adapt to their habitats in the course of living and evolution, thus, these population that live close to each other have higher genetic identities and geographic proximity by the effect of gene flow. The results agreed with Brunner *et al*. (2001) suggestion that *Salvelinus alpinus* among different geographic population showed the significant genetic variation because of its breeding migration and low distribution.

Despite the decline in the fish population size, the genetic diversity of this species is relatively higher than *B. lenok* in other basin in China (Xia *et al*., 2006). *B. lenok tsinglingensis* only distributed in cold-water mountain streams and rivers with elevations ranging from 900-2,300 m above sea level. Though environmental change by dam construction and mining led to the shrinking geographical distribution, the spawning and water source remain healthy due to high altitude, the low density and the discontinuous distribution. In recent year, the nature protected areas of *B. lenok tsinglingensis* have been established by local government to protect the germplasm resources.

In summary, though shrinking geographical distribution of *B. lenok tsinlingensis*, there is high level of genetic diversity in four locations. However, limited number of alleles and deteriotate of habitat may lead to deviate from HWE, and then genetic diversity will sharply decline. Thus, we should be protected the fish from further declines in genetic diversity.

Statement of conflict of interest
The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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