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Developing Microsatellite Multiplex PCR Panels for Topmouth Culter (*Culter alburnus*) and their Application in Parentage Assignment

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Authors' Contribution
JF, SL, ZG and ZD designed research. JF and SL performed experiments and contributed in manuscript writing. WZ, YJ, LW and YY collected the samples and analyzed the data.

Key words
Culter alburnus, Parentage assignment, Microsatellite, Multiplex PCR, Breeding

ABSTRACT

The topmouth culter (*Culter alburnus*) is an economically important fish in China. However, natural stocks have been decreasing rapidly in recent years. Genetic improvement breeding programs of *C. alburnus* offer an essential strategy in developing a sustainable solution. In this study, we developed three multiplex PCR panels, including twelve microsatellite loci for *C. alburnus*, and applied parentage analyses using 36 candidate parents and 136 offspring from four independent artificial breeding pilots. Based on allele frequency analysis using 36 candidate parents, the number of alleles ranged from 7 to 22, observed heterozygosity ranged from 0.39 to 0.75, and expected heterozygosity ranged from 0.57 to 0.91. All loci were highly informative (polymorphic information content, *PIC* >0.5). We detected a significant correlation between exclusion probabilities for parent pairs (*E-PP*) and *PIC* (P<0.01). Simulation analysis revealed that a high assignment rate (>95%) was achieved when the number of candidate parents was less than 200. Meanwhile, real parentage analysis revealed that almost each offspring (135/136, 99.26%) was unambiguously assigned to a parent pair with high accuracy (100%), respectively. In addition, we detected significantly unequal progeny contributions of parents and parent pairs in total or in each artificial breeding pilots (P<0.01). In brief, we developed microsatellite multiplex PCR panels for *C. alburnus* with high capability of monitoring pedigree information, which can be used as a basic tool for family selection breeding of this species.

INTRODUCTION

The topmouth culter (*Culter alburnus*) is a carnivorous pelagic fish belonging to the Cyprinidae family and widely distributed throughout large water bodies in China (Chen, 1998). It commonly feeds on small fish species, which plays an important role in the ecological equilibrium of water ecosystems. Its fast growth and distinctive flavor render it a major economic freshwater fish species in Eastern China, especially in the Lake Taihu area. Fish culture production of this species has expanded significantly over the past few decades (Wang *et al*., 2007), mainly due to the increasing market demand and improvements in artificial breeding and rearing techniques. Unfortunately, over-fishing, changes in living conditions and water pollution have all caused a sharp reduction in resource (Wang *et al*., 2007). Therefore, sustainable aquaculture of this species relies on effective conservation and rational utilization of wild resource. Recently, hybrid (Guo *et al*., 2018) and meiotic gynogenesis (Li *et al*., 2018) breeding methods were introduced to genetically improve the species, and reduce seed and broodstock demands from wild population. However, breeding strains were either limited or undetected in practice stocks.

Artificial selection is commonly conducted in many important fish species (Gjedrem *et al*., 2012) as an effective method to sustainably utilize wild resource and

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maintain culture production. However, as phenotypic selection usually targets a few outperforming individuals without knowledge of their pedigree, this can potentially lead to inbreeding and fitness depression (Fu *et al*., 2013), and subsequently limited genetic improvement. Therefore, accurate pedigree information is imperative in a successful breeding program (Lacy, 2012). Recently, microsatellite markers have shown promising practicability in parentage assignment with few loci and high accuracy (Luo *et al*., 2017; Sudo *et al*., 2018; Wang *et al*., 2018). Moreover, multiplex PCR methods are becoming more utilized for microsatellite loci co-amplification, which has considerably reduced time and costs in parentage analysis procedures. Nowadays, multiplex PCR panels have been successfully developed and utilized for parentage assignment in many aquaculture animals (Fu *et al*., 2013; Nie *et al*., 2012; Popa *et al*., 2015), and considered an effective marker-assisted selection breeding technique.

Despite its ecological and economic importance, studies on topmouth culter genetics remain limited (Guo *et al*., 2018; Liu *et al*., 2014; Wang *et al*., 2007) and selection breeding programs non-existent. Therefore, in this study, we developed three multiplex PCR panels involving twelve microsatellite loci with redesigned primers based on released sequences on GenBank database, and conducted parentage analysis using parent and offspring samples from four independent artificial breeding pilots. This approach not only proved effective in monitoring pedigree information, but also demonstrated great potential as an essential tool when developing breeding programs for *C. alburnus*.

MATERIALS AND METHODS

Fish samples and DNA extraction

Culturing and spawning of *C. alburnus* were carried out at Aquatic Breeding Farm of Zhejiang Institute of Freshwater Fisheries (Zhejiang, China). Thirty-six adult fish (24 females and 12 males) were used for artificial breeding from June 10 to June 15, 2017. Mating designs for four breeding pilots are shown in Table I. Pilot A and Pilot C consisted of eight females and four males, Pilot B and Pilot D consisted of four females and four males, and Pilot A and Pilot B shared four males in practice. Broodfish of four pilots were processed under hormone stimulation and artificial stripping. Milt samples were stripped in advance and stored in an icebox (about 4ºC) for a short time, and fertilized with the eggs stripped out. The fertilized eggs of four pilots were hatched in independent cylinders, and the fry cultured in four different ponds respectively. Thirty-four offspring were randomly sampled from each pilot after one-month nursery rearing.

Fin clips of broodstock and offspring were collected and stored in absolute ethanol until DNA extraction. Genomic DNA was extracted using a traditional phenol-chloroform method. DNA concentration and purity of samples were assessed using the Nano Drop 2000C spectrophotometer (Thermo scientific, US) and 1% agarose gel. Afterward, DNA concentration was adjusted to 20-50 ng/μL and stored at –20ºC for later PCR amplification.

Table I. Mating design of four artificial breeding pilots in *C. alburnus*.

Pilot	No. of females	No. of males
Pilot A	8	4*
Pilot B	4	
Pilot C	8	4
Pilot D	4	4

Note: *, Pilot A and Pilot B shared four males

Microsatellite primer design and multiplex PCRs

The original sequences released on GenBank database (Liu *et al*., 2014) were chosen for designing primers. Novel primers were designed for microsatellite loci of differing product sizes. Amplification efficiency and polymorphism was tested using six parent samples. Primers with different product sizes were used in developing the multiplex sets. In this study, twelve primer pairs were successfully used in the multiplex PCR panels (Table II). Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and each forward primer was labeled with a fluorescent dye (HEX or FAM).

PCR amplification was performed at a total volume of 25 μL, which included: 12.5 μL 2× Taq PCR MasterMix (TianGen Biotech Co., Ltd., Beijing); 4.8 μL, 3.8 μL and 5.6 μL primer mixture (10 μM original concentration of each) for three multiplex PCR panels, respectively (Table II); 2 μL genomic DNA (20-50 ng/μL); and supplemented with DNase/RNase-free deionized water. PCR amplification was conducted under the following conditions: 10 min pre-denaturation at 94ºC followed by 35 cycles of denaturing 30 s at 90ºC, annealing 30 s at 52ºC, and prolonging 50 s at 72ºC; with a final prolonging at 72ºC for 10 min. Reaction products were resolved on Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, USA) and sized relative to an internal size standard (GeneScan-500 ROX) using GeneMapper Version 3.5 software (Applied Biosystems, USA).

Data analysis

Estimates of genetic variation such as the number of alleles (*Na*), observed (*Ho*) and expected heterozygosity

Table II. Information of microsatellite loci and multiplex PCR panels (25 µl) in *C. alburnus*.

Locus	Accession No.	Label	Primer sequence (5'-3')	Volume (10 µM, µL)	Summary statistics of allele frequency analysis (N = 36 parents)						
					Size (bp)	Na	Ho	He	PIC	E-PP	Fn
Multiplex PCR panel 1											
Cal18	KC134229	FAM	F: CAAGGACAAGGATTATG	0.8	127-179	15	0.69	0.89	0.87	0.91	+0.12
		—	R: ATGAACACAACTTACC	0.8							
Cal29	KC134240	HEX	F: AACTACCAATGCCTGATTCC	0.4	221-283	18	0.75	0.86	0.84	0.88	+0.07
		—	R: TACTGAGTGAGAAACCTTTCC	0.4							
Cal06	KC134217	FAM	F: AGATGTCGTGGTTAGTTTCA	0.8	295-359	18	0.67	0.89	0.87	0.91	+0.13
		—	R: GCGTATTCCTCTCCTGATTC	0.8							
Cal19	KC134230	HEX	F: TTCACGCACTTCAAACACT	0.4	400-430	10	0.46	0.82	0.78	0.82	+0.29
		—	R: GTATAACACAGAATGACACAGG	0.4							
Multiplex PCR Panel 2											
Cal50	KF111430	FAM	F: GAGAGCATTCAGGAAGCA	0.6	113-167	11	0.67	0.83	0.80	0.85	+0.11
		—	R: AAGTAGAGCGAGCAGAGA	0.6							
Cal53	KF111433	HEX	F: TCATCAACTCTCACACTCTC	0.5	241-277	13	0.68	0.81	0.78	0.84	+0.11
		—	R: CCATATCCAGCACTCTAACA	0.5							
Cal49	KF111429	FAM	F: GCGGTGTTCTGCTTCTTC	0.3	278-378	22	0.69	0.91	0.90	0.95	+0.14
		—	R: TGTGTCGTGATGGAGGAG	0.3							
Cal54	KF111434	HEX	F: AGACCTCCTCCTCTTCTC	0.5	406-424	10	0.61	0.80	0.76	0.80	+0.15
		—	R: TGGCACAACAACACAGAC	0.5							
Multiplex PCR Panel 3											
Cal52	KF111432	FAM	F: GAATCTGCCGTTCTCACTA	0.8	126-158	7	0.47	0.57	0.54	0.57	+0.11
		—	R: ACCTGTCCACCTCAATCA	0.8							
Cal46	KF111426	HEX	F: AAGAGACTGAACATTGAAGC	1.0	207-245	11	0.39	0.80	0.76	0.79	+0.34
		—	R: TTGGACTGAGAGAAGGAAAT	1.0							
Cal17	KC134228	FAM	F: GCATAGTGACATCATAGCAT	0.5	264-308	15	0.67	0.90	0.87	0.92	+0.14
		—	R: ACCGCAACATTACAAAGAC	0.5							
Cal01	KC134212	HEX	F: TTCCATATCCTCTCATCCTTCA	0.5	352-434	14	0.56	0.88	0.85	0.90	+0.23
		—	R: CCTGCTATTGTTTCTTTCATCC	0.5							

Na, number of alleles per locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *PIC*, polymorphism information content; *E-PP*, exclusion probability of parent pair; *Fn*, null allele frequency.

(*He*), polymorphic information content (*PIC*), exclusion probability for parent pairs (*E-PP*) and null alleles frequency (*Fn*) of twelve microsatellite loci were estimated based on the genotype data of 36 candidate parents via allele frequency analysis using Cervus 3.0 software (Marshall *et al*., 1998; Kalinowski *et al*., 2007). Correlation analysis for PIC and E-PP of loci were carried out using SAS software (SAS Institute, 1996).

Simulation analysis were performed to evaluate the probable performance of the microsatellite loci for parentage identification on *C. alburnus* breeding programs. Simulation analyses based on genotypes of 36 candidate parents were conducted using the likelihood-based approach in Cervus 3.0 software (Marshall *et al*., 1998; Kalinowski *et al*., 2007) and performed using the following parameters: 10,000 offspring and a pool of candidate parents, varying from 10 to 400 parents and 5 to 200 parent pairs; 98% of candidate parents were sampled and loci typed, 1% error rate in likelihood calculations. Real parentage analysis was performed using Cervus

3.0 software (Marshall *et al*., 1998; Kalinowski *et al*., 2007), based on genotype data of parents and offspring from four breeding pilots. Furthermore, both simulation and parentage analysis were carried out based on tested parents (the same candidate parent information was used in the simulation) in different multiplex panel(s), under the methods mentioned above.

Pedigree reconstruction was compared to known parental information from the mating design and sampling, and used to examine the accuracy of parentage assignment. Chi-square test was then applied to detect the different progeny contributions of parents and parent pairs using SAS software (SAS Institute, 1996).

RESULTS AND DISCUSSION

In this study, we developed three multiplex PCR panels with four microsatellite loci in each panel. Information and genotyping results of four multiplex PCR panels are shown in Table II and Figure 1. We used different product size designs and fluorescence dyes (FAM or HEX) that avoided alleles overlapping and optimized the genotyping process, respectively. This was highly effective and efficient in both time and cost compared with scoring each locus with independent PCR protocols.

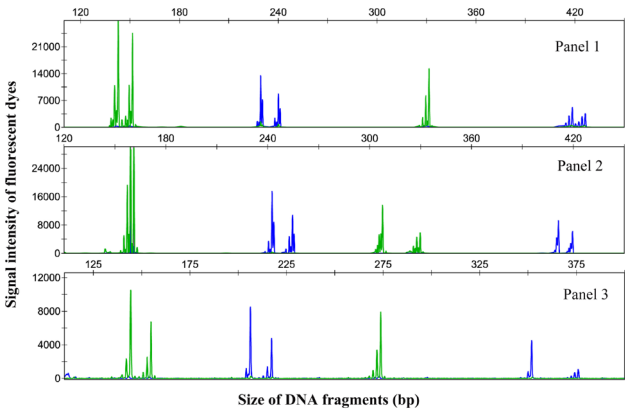

Fig. 1. Genotyping chromatograph of microsatellite loci co-amplification of three multiplex panels in *C. alburnus* individuals, obtained using an Applied Biosystems 3730XL sequencer. *Blue* and *green lines* indicate FAM and HEX labels, respectively.

We evaluated the genetic diversity parameters of twelve microsatellite loci using 36 candidate parents. The summary statistics are shown in Table II. We revealed that *Na*, *Ho* and *He* ranged from 7 to 22, 0.39 to 0.75, and 0.57 to 0.91, respectively. Genetic diversity parameters of microsatellite loci in *C. alburnus* were slightly higher in our study than previously reported (Liu *et al*., 2014), but lower than those reported in another study (Fu *et al*., 2013). It should be noted that the individuals tested and the detection methods were related to the diversity parameters (Liu *et al*., 2009). All twelve microsatellite loci were highly informative (*PIC* > 0.5) (Botstein *et al*., 1980), and exclusion probabilities for the parent pairs (*E-PP*) ranged from 0.57 to 0.92, which significantly correlated with the *PIC* of microsatellite loci ($P<0.01$; Fig. 2). These correlations are also reported in another study (Ma *et al*., 2013). Microsatellites with high genetic diversity parameters and hence relatively high statistical powers are therefore included in parentage assignments (Marshall *et al*., 1998; Tokarska *et al*., 2009).

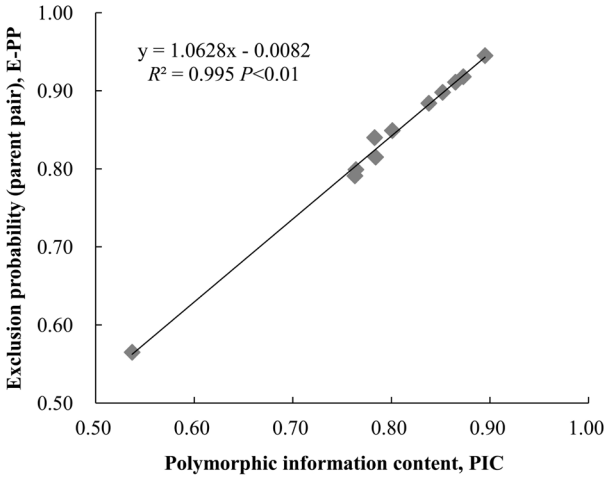

Fig. 2. Scatter diagram of polymorphic information content (*PIC*) and exclusion probability for parent pair (*E-PP*) of microsatellites.

Simulation assignment approaches estimate the theoretical power of microsatellite markers and allow the adjustment of marker-selection toward the best benefit direction (Hauser *et al*., 2011). In this study, we conducted simulation analyses (with sexes known or unknown, and varying number of parents) to assess the resolution capability of these twelve loci. According to the simulation results shown in Figure 3, all assignments for mother alone, father alone, and parent pairs were higher than 95% when the number of candidate parents was less than 200 parents (sexes unknown) or 100 parent pairs (100 father and 100 mother, sexes known). This is far greater than the minimum number of parent pairs recommended to prevent inbreeding and obtain a long-term response in a mass selection program (Bentsen and Olesen, 2002). The real parentage analysis for 36 candidate parents and 136 offspring are shown in Table III. The assignment rates for

breeding pilots ranged from 97.06% to 100% (average was 99.26 %). We checked pedigree reconstruction with known mating and sampling information, which we used to assess the accuracy of parentage assignment (Jerry *et al*., 2006). All pilot assignments agreed with the true mating designs with 100% accuracy.

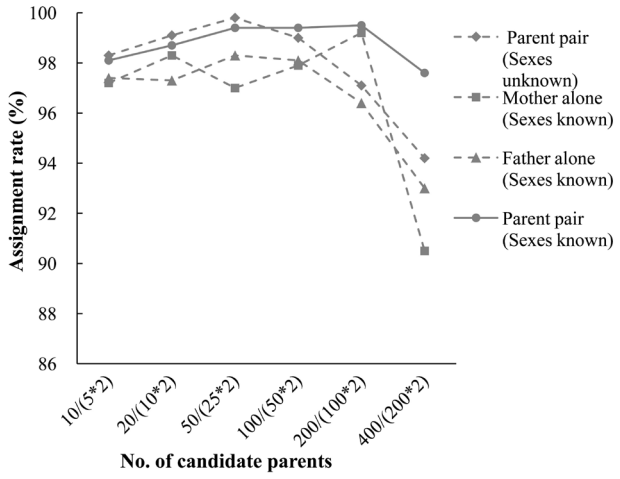

Fig. 3. Assignment rates from simulation analysis for parents (parent pairs) based on different number of candidate parents. These range from 10 (or 5 males and 5 females) to 400 (or 200 males and 200 females) sexes unknown (or sexes known) parents (confidence in 95%).

Table III. Parentage analysis results for four breeding pilots in strict confidence (95%).

Pilot	No. of individuals		Assignment rate (%)	Accuracy rate (%)
	Tested	Assigned		
Pilot A	34	33+1*	97.06	100
Pilot B	34	34	100	100
Pilot C	34	34	100	100
Pilot D	34	34	100	100
Total	136	135	99.26	100

Note: * indicates with ambiguous assignment.

As shown in Figure 4, simulation assignment rates and parentage assignment rates, based on different multiplex PCR panel or panels, followed a similar trend. In general, we detected a higher assignment rate when we used more panels. Although, the parentage assignment rates were based on real data, they were commonly lower than those detected in simulations, which may due to the null alleles observed at many loci (Carlsson, 2008). Even so, our results highlight the use of simulation analyses to evaluate assignment capacity of multiplex PCR panels (or panel combinations) before breeding practice. We showed that the assignment rate of parentage analysis was higher than 80% when two panels were used (Panels 1 and 3, or Panels 2 and 3), and higher than 95 % when three panels were used. Consequently, using three multiplex PCR panels in this study is considered sufficient and efficient for pedigree reconstruction for *C. alburnus*.

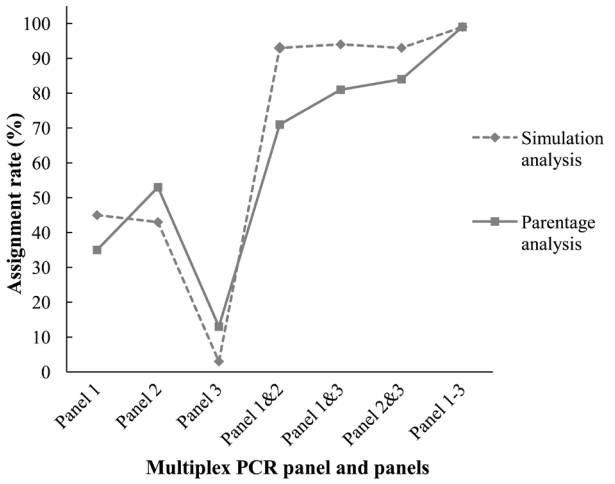

Fig. 4. Assignment rates of simulation and parentage analyses based on 36 candidate parents (24 females and twelve males), using genotype information from different multiplex panels and panel combinations (confidence in 95%).

In aquaculture breeding schemes, equalizing family size is challenging because of the unequal reproductive success of brooders during the breeding practice and differential survival among families (Kong *et al*., 2015). We detected nine full-sib families (from six dams and three sires) in Pilot A. In Fig. 5, we show different progeny contributions (number and percentage) for the parents and parent pairs in Pilot A. We detected significant unequal progeny contribution of parents and parent pairs in total for each breeding pilots (P<0.01). This phenomenon, which has also been observed in other aquatic species (Fu *et al*., 2016; Herlin *et al*., 2008; Loughnan *et al*., 2013; Rhody *et al*., 2014; Sudo *et al*., 2018), may result in higher rates of inbreeding in long-term selection due to a gradual decline of effective population size. Therefore, using molecular markers to monitor pedigree information could be used as an essential tool incorporated into breeding schemes of fish species, including *C. alburnus*.

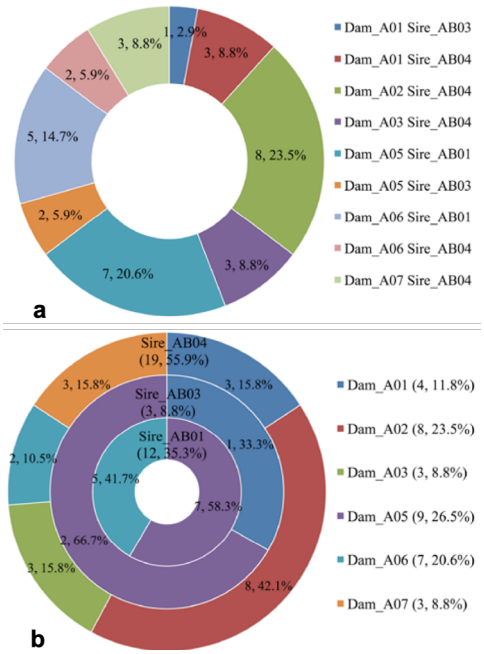

Fig. 5. Pie charts of progeny contributions of parent pairs (a) and parents (b) in breeding Pilot A. (a): the numbers and percentages represent the progeny contributions of nine parent pairs (full-sib families). (b): the numbers and percentages within parentheses represent the progeny contributions of six females and three males, and other numbers and percentages represented the contribution of maternal progeny contribution inside three paternal half-sib families (three circles).

CONCLUSIONS

In this study, we developed a multiplex PCR panels for *C. alburnus* that provides a powerful tool for parentage assignment and population genetic studies of this species. It can be used as an essential tool in developing breeding programs for *C. alburnus*.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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