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

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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...
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., 2012), 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., 2015), and considered an effective marker-assisted selection breeding technique.

In this study, twelve primer pairs were successfully used in developing the multiplex sets. 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 94°C followed by 35 cycles of denaturing 30 s at 90°C, annealing 30 s at 52°C, and prolonging 50 s at 72°C; with a final prolonging at 72°C 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 No. | 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: ATGAACACAACCTTACC | | | | | | | | |
| Cal29 | KC134240 | HEX | F: AACTACCAATGCCCTGATCC | 0.4 | 221-283 | 18 | 0.75 | 0.86 | 0.84 | 0.88 | +0.07 |
| | | | R: TACTGAGTGAGAAACCTTACC | | | | | | | | |
| Cal06 | KC134217 | FAM | F: AGATGTCGGTGTTAGTTTCA | 0.8 | 295-359 | 18 | 0.67 | 0.89 | 0.87 | 0.91 | +0.13 |
| | | | R: GCATATTTCTCTCTCTGATTC | | | | | | | | |
| Cal19 | KC134230 | HEX | F: TTCACGCAACTTCAAACACT | 0.4 | 400-430 | 10 | 0.46 | 0.82 | 0.78 | 0.82 | +0.29 |
| | | | R: GTTAAACACAGAATGGACACAGG | | | | | | | | |
| **Multiplex PCR Panel 2** | | | | | | |
| Cal50 | KF111430 | FAM | F: GAGAGCATTCCAGGAAGCA | 0.6 | 113-167 | 11 | 0.67 | 0.83 | 0.80 | 0.85 | +0.11 |
| | | | R: AAGTAGAGCGAGACAGA | | | | | | | | |
| Cal53 | KF111433 | HEX | F: TCATCAACTCTTACACTCTC | 0.5 | 241-277 | 13 | 0.68 | 0.81 | 0.78 | 0.84 | +0.11 |
| | | | R: CCAATACACGACACTTAACA | | | | | | | | |
| Cal49 | KF111429 | FAM | F: GCAGTCTCTGCTTTTCTCTC | 0.5 | 278-378 | 22 | 0.69 | 0.91 | 0.90 | 0.95 | +0.14 |
| | | | R: TGTTTCGTGATGGAGGAG | | | | | | | | |
| Cal54 | KF111434 | HEX | F: AGACCTCCTCTGCTTCTC | 0.5 | 406-424 | 10 | 0.61 | 0.80 | 0.76 | 0.80 | +0.15 |
| | | | R: TGGCACAACACACATCTACAG | | | | | | | | |
| **Multiplex PCR Panel 3** | | | | | | |
| Cal52 | KF111432 | FAM | F: GAATCTGCGCTTCTCAGTA | 0.8 | 126-158 | 7 | 0.47 | 0.57 | 0.54 | 0.57 | +0.11 |
| | | | R: ACCGTACCCTCTCCATCA | | | | | | | | |
| Cal46 | KF111426 | HEX | F: AAGAGACCTGAACATGGAAGC | 1.0 | 207-245 | 13 | 0.39 | 0.80 | 0.76 | 0.79 | +0.34 |
| | | | R: TTGGACTGAGAGAAGGAAAT | | | | | | | | |
| Cal17 | KC134228 | FAM | F: GCATATGCTCATCATCATCAG | 0.5 | 264-308 | 15 | 0.67 | 0.90 | 0.87 | 0.92 | +0.14 |
| | | | R: ACCGCAACATCTCAAGAC | | | | | | | | |
| Cal01 | KC134212 | HEX | F: TTCATATCTCTCTACCTTTCA | 0.5 | 352-434 | 14 | 0.56 | 0.88 | 0.85 | 0.90 | +0.23 |
| | | | R: CCTGTCTAGTTCTCTCTCACC | | | | | | | | |

*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.

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).

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.

We used pedigree reconstruction with known mating and sampling information to assess the accuracy of parentage assignment (Jerry et al., 2006). All pilot assignments agreed with the true mating designs with 100% accuracy.

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.

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 pilot (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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