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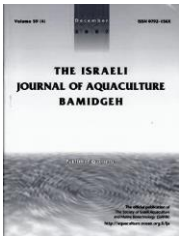

Parentage Assignment in Striped Catfish (*Pangasianodon hypophthalmus*) Based on Microsatellite Markers

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Keywords: parentage assignment; *Pangasianodon hypophthalmus*; microsatellite; multiplex PCR

Abstract

In this study, five microsatellite markers from two multiplex PCR sets were used to determine the pedigree of five striped catfish (*Pangasianodon hypophthalmus*) families that came from a breeding program in Vietnam. The number of alleles per locus and polymorphic information content (PIC) were 4 to 7 and 0.551-0.803, respectively. In the parentage analysis based on five microsatellites, 62.7% of offspring could be allocated unambiguously to their parental pairs using the perfect mismatch setting, and assignment rate improved to 89.3% after a single mismatch was allowed. Parentage analysis when only four microsatellites were used (a locus with a high frequency of null alleles was excluded) increased the assignment rate to 68% for a perfect mismatch and to 90% for assignment with a single mismatch. Results here confirm that a DNA marker-based approach to parentage assignment in *P. hypophthalmus* will be useful in genetic improvement programs for this important aquaculture species in Vietnam.

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Introduction

Vietnam was the fourth largest aquaculture producer worldwide contributing 3.624 million tons to world production in 2016 (FAO, 2018). Among farmed aquatic species, striped catfish (*Pangasianodon hypophthalmus*) known locally as 'ca tra' is one of the most valued indigenous species there, and contributes significantly to improving food security, poverty alleviation, and economic development in Vietnam. While striped catfish has been cultured in the Mekong Delta since 1960, commercial aquaculture production of this species has increased dramatically over the past decade. In 2016, production of striped catfish reached over 1.15 million tons with an estimated value of US$ 1.71 billion (Vietnam Association of Seafood Exporters and Producers, 2017). The striped catfish has been certified as one of a small number of strategically important aquaculture species by the Vietnamese government, however to date, little effort has been directed at genetic improvement of production traits for this catfish. There are several complex traits in striped catfish that would benefit from genetic improvement to assist growth of the industry and enable this species to remain highly competitive across Asia. Key traits include growth rate, fillet yield, fillet quality (colour, fat content, etc.), resistance to some important diseases, feed conversion ratio, and tolerance to raised environmental salinity. Saline water intrusion into freshwater habitats where striped catfish are mainly farmed has become a major impact of climate change across the low topography of the Mekong Delta region in Vietnam.

In farmed terrestrial animals it has been demonstrated many times that systematic stock improvement programs can be an efficient way of enhancing stock productivity. Growth rates doubled in pigs, while milk production in cows and the number of eggs laid per hen have improved by up to 2.5 times over the last 50 years (Gjedrem, 2005a). Aquaculture in general however, lags far behind farmed terrestrial animals with respect to uptake of stock improvement technology, with most fish and shellfish producers still relying on either harvesting wild stocks or farming unimproved stocks that are only a few generations removed from the wild (Gjedrem et al., 2012a). Large scale family-based stock improvement programs, now established as the industry standard for genetic improvement of aquaculture species, were first developed for several high value salmonid species and Nile tilapia in Asia (Gjedrem, 2012b). In 2001, a breeding program was initiated on farmed striped catfish in Vietnam by Research Institute for Aquaculture No. 2 (RIA2) to improve growth rate. The breeding program has resulted in an increase of 13% in body weight per generation (Sang et al., 2012). RIA2 maintains the breeding program for striped catfish to ensure long-term local industry sustainability and expansion of the Vietnamese catfish aquaculture industry (pers. com.). One of current challenges for this breeding program is to develop pedigree information that will be crucial for estimating key genetic parameters accurately and to address any potential effects of high inbreeding rates and consequent declines in performance (Liu et al., 2016). An appropriate tagging system is required to identify broodstock individuals and to maintain pedigree records. Physical tags that include elastomer dye tags or passive transponders are commonly used for this purpose in certain high value species. Use of a physical tag is not practical however, when tagging small juvenile fish or shrimp (Jerry et al., 2004). The method also requires use of many tanks for rearing families separately, a practice that requires very high investment in facilities and trained personnel. This method also carries a risk associated with biasing family values as a result of tank effects if the rearing practices are not sufficiently well standardized or tissue damage, premature tag loss and uncertainty related to juvenile handling vulnerability are problems (Jepsen et al., 2015).

Microsatellites or simple sequence repeats (SSRs) are highly polymorphic genetic markers that are abundant across the genomes of most species and are inherited in a co-dominant Mendelian fashion. As a result, they are widely regarded as the markers of choice for individual identification and for kinship inference. Microsatellites have been used successfully for parentage assignment in a number of breeding programs undertaken on farmed fish (Li-qin et al., 2019; Trong et al., 2013; Yang et al., 2014), crustaceans, and mollusc species (Jerry et al., 2004 and 2006; Wang et al., 2010).

Developing microsatellite markers for a target species offers a number of advantages over physical markers as genetic tags for parentage assignment in allocating offspring to their biological parents. This method allows many families to be reared in a common tank from fertilization to harvest size, a practice that can significantly reduce the cost and labor involved while eliminating any problems with common environmental effects (Gjedrem, 2005b). A major limitation on using this approach however is the economic cost associated with developing and screening molecular markers which are specific in each instance, so an important goal will be to identify the minimal number of loci required for identification and so limit the costs of applying the technology economically where possible. Development of multiplex PCR array allows geneticists to screen multiple microsatellite loci in a single reaction or only a few reactions rather than screening each locus separately. This technique can thereby significantly reduce the time and cost associated with SSR analysis (Sint et al., 2012). Furthermore, multiplex PCR also reduces repeated manipulation of large number of samples during genotyping and therefore reduces risk of handling errors.

A large number of microsatellite markers have been developed for *P. hypophthalmus* in the past (Volckaert et al., 1999; Ha et al., 2009; Na-Nakorn & Moeikum, 2009; Ha et al., 2016) and more widely for pangasid taxa (Hogan and May, 2002). This resource can provide valuable tools for developing parentage analysis in genetically improved striped catfish stocks. The specific objectives of the current study therefore were to trial and develop multiplex PCR panels from published microsatellite data sets and to evaluate their power for accurate parentage assignment of *P. hypophthalmus* individuals in the RIA2 stock improvement program in Vietnam. Advantages of a genetic identification approach over physical tagging of individuals can improve estimation of key genetic parameters including heritability estimates for key production traits and breeding values for broodstock individuals and/or families used in the breeding program.

Materials and Methods

Sample collection and DNA extraction. Samples came from the breeding program for *P. hypophthalmus* implemented at the Southern National Breeding Centre for Freshwater Aquaculture (An Thai Trung Village, Cai Be District, Tien Giang Province) under RIA2. A total of 85 fin clip samples were collected, including 10 parents of 5 full-sib families and 15 offspring from each family. All fin samples were stored in 95% ethanol until DNA extraction that followed a modified salt DNA extraction method first described by Miller et al. (1988).

Microsatellite amplification.

Five microsatellite loci primer sets developed and specific for *P. hypophthalmus* were selected based on their individual levels of polymorphism and allele size range. Selected loci included two primer sets (CB18, CB19) published by Ha et al. (2016), a single primer set (PSP-G579) published by Hogan and May (2002), and two primer sets (Phy01, Phy07) described by Volckaert et al. (1999). Forward primers were labelled with fluorescent dyes (FAM, HEX or TAMRA).

Determination of optimal annealing temperature for each microsatellite primer set was tried on a panel of five individuals using a gradient temperature approach (range from 49.7-69.0°C). A 25 µL PCR reactions contained approximately 100 ng DNA template, 0.5 U Taq DNA polymerase (Bioline, UK), 0.5 µM forward and reverse primers, 0.8 mM dNTP (Bioline, UK), 1.25 mM $MgCl_2$ and 1X reaction buffer (Bioline, UK). Amplification started with initial denaturation at 95°C for 4 min, followed by 30 cycles: 30s denaturing at 95°C, 45s at annealing temperature (49.7-69.0°C), 90s extension at 72°C, then a final 5 min extension at 72°C.

The five microsatellite marker loci screened here were divided into two multiplex PCR sets according to allele size range and optimum annealing temperatures. Multiplex PCR set 1 included CB18, CB19, PSP-G579, while multiplex PCR set 2 included Phy01 and

Phy07 (Table 1). Primer concentrations, annealing temperatures, DNA template concentrations and PCR cycles were optimized for five sample individuals. A 25 µL PCR reactions contained approximately 100-200 ng template DNA, 1 U Taq DNA polymerase (Bioline, UK), 0.2-0.5 µM forward and reverse primers, 2 mM dNTP (Bioline, UK), 1.25 mM $MgCl_2$ and 1X reaction buffer (Bioline, UK). The following PCR conditions were used for multiplex PCR set 1: 4 min initial denaturation at 95°C, 30 cycles of 30s denaturation at 95°C, 30s annealing at 60.5°C, and 30s elongation at 72°C, then a final extension at 72°C for 10 min. Multiplex PCR set 2 started with an initial step at 94°C for 5 min, followed by 34 cycles of 40s denaturation at 94°C, 30s annealing at 62°C, and 90s elongation at 72°C, then a final extension at 72°C for 20 min. PCR products were analysed using the ABI3730XL DNA Analyzer (Thermo Fisher Scientific, USA). Fragment sizes were determined using GeneMapper v4.0 software.

Table 1. Characteristics of five microsatellite primers from the published microsatellite sequences for *P. hypophthalmus*

Locus	*Primer sequence (5'-3')*	T_a (°C)	*Predicted product size (bp)*	*Observed product size (bp)*
PCR multiplex set 1:				
CB18[a]	F: **FAM**- AGAAGGAACGCTGGACTGAGG	53.0	350	337-409
	R: TATACCTGCTGGGAGAATGGATG			
CB19[a]	F: **TAMRA**- TCGAGAGTCTGAAGCACAAACC	57.0	250	255-303
	R: CAGACATGAGGAGGATGAAGACG			
PSP-G579[b]	F: **FAM**-GAGAGGGGGTGAAATAATGATAGG	54.6	200	192-210
	R: ATGGTTCTCCTGCAAGCAATGTCT			
PCR multiplex set 2:				
Phy01[c]	F: **TAMRA**- GTAAACAGAGCCACCTGCGG	65.0	154-160	167-189
	R: CAGATCCACACCCACAACACC			
Phy07[c]	F: **HEX**-GATCAGTCACTTCAGCACCTGCC	68.0	214-270	222-279
	R: TCCAAATCTCTGTGATGGTGAGCC			

[a] Ha et al., 2016; [b] Hogan & May, 2002; [c] Volckaert et al., 1999; T_a: annealing temperature

Data analysis.

CERVUS 3.0 software (Kim and Sappington, 2013) was used for analysis of genetic diversity at each microsatellite locus. Data included number of alleles per locus (N_a), observed (H_o) and expected (H_e) heterozygosity, polymorphic information content (PIC), null allele frequency, and departures from Hardy-Weinberg equilibrium (HWE). Parentage analysis for five full-sib families was estimated using an exclusion method in VITASSIGN 3.1 (Vandeputte et al., 2006). Statistical analyses were performed first including all five microsatellites and then in a second analysis that excluded data from locus PSP-G579 as this locus showed a relatively high null allele frequency in the sampled individuals.

Results

Genetic diversity of microsatellite markers.

A total of 75 offspring and 10 parents from five full-sib families were genotyped for the five genetic markers. Table 2 presents genetic diversity estimates for the five microsatellite markers. Number of alleles per locus ranged from 4 (PSP-G579) to 7 (Phy01). PIC values ranged from 0.551 for PSP-G579 to 0.803 for Phy01. Estimated values demonstrated that all five microsatellite loci were relatively highly informative (PIC > 0.5) (Botstein et al., 1980). Observed and expected heterozygosities ranged from 0.329 to 0.976 and 0.605 to 0.830, respectively. Tests for Hardy-Weinberg Equilibrium found that two out of five loci showed significant disequilibrium ($P < 0.01$). Null allele

frequency estimates per locus analyzed with CERVUS ranged from 1.43 to 34% across loci.

Table 2. Genetic diversity of microsatellite markers in *P. hypophthalmus*

Locus	*N*	*N_a*	*H_o*	*H_e*	*PIC*	*HWE*	*Null allele frequency*
CB18	85	5	0.647	0.665	0.626	NS	+0.0143
CB19	85	6	0.788	0.773	0.731	**	-0.0209
PSP-G579	85	4	0.329	0.605	0.551	***	+0.3419
Phy01	85	7	0.976	0.830	0.803	NS	-0.0862
Phy07	85	6	0.741	0.775	0.739	NS	+0.0183

N: number of individual, N_a: number of alleles, H_o: observed heterozygosity, H_e: expected heterozygosity, PIC: polymorphic information content, HWE: Hardy-Weinberg test

Parentage analysis.

Parentage analysis was performed on a total of 85 individuals (75 offspring and 10 parents) to verify parentage between parent-offspring pairs. A number of different outcomes were possible for each sampled individual based on results from VITASSIGN software. An individual could be assigned to the correct parental pair and no others, or to more than a single parental pair, or to no parental pair, or to an incorrect single parental pair (Vandeputte et al., 2006). The first analysis (1) that included all five loci resulted in 62.7% of offspring being assigned unambiguously to their true parental pairs with perfect exclusion. When a single mismatch was allowed, this yielded improved assignment, with 89.3% of offspring correctly assigned to their true parents. In the second analysis (2) where data from the locus with the highest null allele frequency was excluded, results showed that 84% of offspring were correctly allocated to their true parents with perfect exclusion. Parentage assignment in this case was increased to 90.7% of all offspring when a single mismatch was allowed (Table 3).

Table 3. Parentage assignments of 75 offspring from five full-sib families from the breeding program for *P. hypophthalmus* using a panel of four or five microsatellites

Parameters	*Analysis 1 (5 microsatellites)*	*Analysis 2 (4 microsatellites)*
Number of dam	5	5
Number of sire	5	5
Number of offspring	75	75
0 mismatch		
% of offspring assigned to right parents	62.7	68.0
% of offspring assigned to multiple parents	0.0	0.0
% of offspring unassigned to any parental pair	37.3	32.0
1 mismatch		
% of offspring assigned to right parents	89.3	90.7
% of offspring assigned to multiple parents	0.0	0.0
% of offspring unassigned to any parental pair	10.7	9.3

Discussion

In principle, any variable genetic markers that are transmitted in a stable fashion from parents to offspring can be used to assign parentage. Microsatellite markers that are highly polymorphic therefore, offer useful tools for determining parentage in the RIA2 striped catfish stock. The five loci screened here showed levels of diversity and allele size ranges that are similar to those published elsewhere for striped catfish, except for locus Phy01 that had a higher number of alleles compared with an earlier study (Ha et al., 2016; Hogan and May, 2002; Volckaert et al., 1999).

In our study, locus PSP-G579 showed a relatively high frequency of null allele (34.2%) in the samples tested. Null alleles have been reported in genetic analyses of a number of aquaculture species. Microsatellite loci in some species show relatively high null allele frequencies ranging from 27.8 up to 36.1% when multiplex assays have been used for parentage assignment, for example in the eastern oyster *Crassostrea virginica,*

(Wang et al. 2010). Similarly, null allele frequencies in SSRs from Pacific oyster *Crassostrea gigas*), also ranged from 11-22% (Li et al., 2009). Presence of null alleles has been identified as a common explanation for less than 100% assignment of tested offspring to available parents in earlier parentage assignment studies (Castro et al., 2007). However, they may not necessarily affect the overall outcome of assignment other than reducing overall detection power. In addition, the discriminatory power of a marker locus in parentage assignment studies depends on the distribution of alleles at a locus among the parents, but not necessarily on the presence of null alleles (Wang et al., 2010). Based on these observations, we decided not to exclude any loci in the current study from further analyses. In fact, when we removed data from the locus with the highest null allele frequency (PSP-G579), the assignment rate was improved to 68.0% for zero mismatch and 90.7% for a single mismatch (Table 3). From this it can be seen in the current study that presence of null alleles at marker loci can clearly impact assignment power.

Results of Hardy-Weinberg analyses showed that two out of five SSR loci did not conform to Hardy-Weinberg equilibrium expectations. This outcome is not unexpected in parentage assignment studies and can be caused by a number of 'non-fatal' factors namely presence of null allele as the major cause, small samples size (Li et al., 2007), asymmetrical distributions in offspring numbers from parental pairs and non-random mating (Jerry et al., 2004). In the current study, we observed evidence for high null allele frequencies at two of the SSR loci tested (Table 2) that also showed significant departures from Hardy-Weinberg equilibrium. Secondly, we acknowledge that the sample size used in our study was quite small. Lastly, and probably of most relevance here is that HWE deviations are not unexpected, because samples used in the current study came from a breeding program in striped catfish that employed a partly factorial mating approach among selected broodstock.

Parentage assignment in the current study was established using two datasets of five (analysis 1) and four (analysis 2) SSR loci, respectively. Assignment success rate based on five microsatellites was higher than that reported for offspring of the GIFT strain of Nile tilapia using two multiplex sets of total 11 SSR loci (Trong et al., 2013). While assignment rate in the analysis 2 reached 68.0%, our rate was relatively lower than those reported in some fish (Yang et al., 2014; Sudo et al., 2018), oysters (Wang et al., 2010; Li et al., 2010), and crustaceans (Jerry et al., 2006; Liu et al., 2018). According to Yang and his co-workers (2014), successful parentage analysis requires either a small set of highly polymorphic markers or a very large number of markers with low to moderate levels of polymorphism. Jerry et al. (2006) reported that only 4-5 highly polymorphic SSR markers (10-26 alleles per locus) were required to assign over 95% of offspring to parents in a *Penaeus monodon* stock and they could achieve complete assignment of all offspring using only seven loci. Similarly, total assignment success reached 100% with six informative microsatellites in Pacific oyster, *Crassostrea gigas* (Li et al., 2010) and with 8-9 microsatellites in eastern oyster, *Crassostrea virginica* (Wang et al., 2010). In the current study we used only five or four polymorphic microsatellite loci of moderate allelic variability; so if more highly polymorphic markers can be identified then assignment power should increase. Adding loci with only low variation however, results in very little extra discriminatory power and essentially is a waste of multiplexing power. Furthermore, correct assignment is largely determined by the characteristics of individual marker loci, with some loci better suited for inclusion in DNA parentage marker suites than others (Jerry et al., 2006). Thus total number of alleles per locus and a high PIC value in isolation are not sufficient criteria to select loci for inclusion in a multiplex panel (Trong et al., 2013).

The principle of exclusion, that checks incompatibilities between parent and offspring genotypes following Mendelian rules of inheritance, is simple and straightforward. According to this method, a parent and offspring will share at least one allele per locus for a co-dominant marker. So that a putative parent is rejected as a true parent if both alleles at one locus mismatch with that of an offspring (Trong et al., 2013), making this

method extremely sensitive to genotyping errors or mutation. This is a major drawback of the exclusion method, therefore, several assignment programs allow a given number of mismatches between offspring and parents. The exclusion-based approach allows one or two mismatches between putative parents and offspring that is typical for microsatellites (Vandeputte et al., 2006). Allowing a single mismatch in the current study improved assignment rates in both analyses conducted (1 and 2) to 89.3 and 90.7%, respectively. Allowing two mismatches, did not however further improve assignment rates (data not shown). These results concord with those of Vandeputte and his co-workers (2006) who reported a correct assignment rate of 35.1% (with perfect mismatch) that increased to 54.4% with a single mismatch and only 57.1% with two mismatches. Single mismatches between parents and offspring can be due to genotyping errors or presence of mutation in some offspring (Castro et al., 2006; Vandeputte et al., 2006). In fact, microsatellites are sensitive to genotyping error, particularly when automated multiplex systems are employed. The common error rate for microsatellites in laboratories worldwide has been reported at 1% (Trong et al., 2013) and error rates approaching 2% have also been described in several SSR studies (Castro et al., 2006). Even when the genotyping error rate is lower than 1% there can be problems with parental assignment (Trong et al., 2013). We were not able to estimate the genotyping error rate by re-genotyping all individuals in the current study, so this remains a limitation here. Furthermore, presence of un-assigned offspring sometimes has been attributed to various factors, including presence of null alleles, relatively low resolution of markers, and contamination from other batches of offspring (Sudo et al., 2018).

In conclusion, we believe that we have developed a suitable and robust microsatellite-based tool for parentage assignment in our *P. hypophthalmus* farm stock even given that our loci showed only moderate assignment rates. The SSR panel developed here has potential to replace traditional procedures for identification of families in the current striped catfish breeding program. We recommend that further studies should seek more highly polymorphic microsatellite markers among the published sets available currently or investigate new markers from whole genome analyses. Furthermore, application of the current markers to a larger number of families is likely to improve overall assignment rate as more rare alleles are screened in a larger number of individuals.

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