Progress in Biological Sciences
Vol. 1, No.1, 49-54, Winter/Spring 2011

DNA markers in hybrids of female Caspian kutum *Rutilus frisii kutum* and male grass carp *Ctenopharyngodon idella*: possible production of gynogenic progeny

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Hybrids were produced by crossing female Caspian kutum *Rutilus frisii kutum* with male grass carp *Ctenopharyngodon idella*. The genome of eight larvae and parents were studied using microsatellite markers for genetic evaluation and verification. After DNA extraction from parent fish and progeny, hybrid heritability of two loci was assessed using two pairs of microsatellite primers. Hybridizied offspring showed as similar banding pattern to that of their maternal parent, without heritability of the paternal genome. © 2011 Progress in Biological Sciences, Vol. 1, No.1, 49-54.

KEY WORDS: Kutum, Grass carp, Hybridization, Microsatellite markers.

INTRODUCTION

At the beginning of the nineteenth century, hybridization of fish gave new opportunities in experimention and culture, and development of artificial insemination techniques simplified fish breeding. The number of natural and artificial hybrids is unknown, but studies have shown that 5,000-6,000 hybrids have been produced (Chevassus, 1983). Interspecific hybridization in fish was developed, and species with in a genus can be crossbred (Verspoor, 1988; Zhang and Tiersch, 1997) creating hybrids that may be fertile (Verspoor and Hammar, 1991). Interspecific hybridization can affect genetic characterization, behavior, diet variation, heterosis, sterile population production, monosex populations, polyploidy induction, and chromosome manipulation, or create a new morph (Krasznai, 1987).

Hybrids are useful for phylogenetic studies because their metaphase shows haploid sets of both parents (Perez et al., 1999), but distinguishing hybrids from their parents by morphological characters may be difficult, since some may resemble the female parent and some the male parent. Recognition of hybrids of Atlantic salmon *Salmo salar* and brown trout *Salmo trutta* is problematical, particularly at the juvenile stage (L'Abee-Lund, 1988). This is also the case in other salmonids (Youngston et al., 1992; Beall et al., 1997). In these situations only biochemical genetic markers (Vuorinen and Piironen, 1984) or DNA analyses (Grosset et al., 1996; Elo et al., 1997) are appropriate for hybrid recognition.

Hybrids can be verified via morphological, cytological, biochemical methods, and with DNA analysis. In cytological and biochemical hybrid identification, it is necessary that fish be killed. The DNA technique is advantageous since the small amount of tissue necessary to discriminate an individual can be taken without physical harm to the fish (Perez et al., 1999).

DNA polymorphism analysis is widely applied in evaluation of genetic diversity in aquatic organisms (Sanchez et al., 1996; David and Jarne, 1997; Norris et al., 1999; Taniguchi and Perez-Enriquez, 2000). Microsatellite markers used as genetic markers can be easily distinguished by PCR because of differences in repeat times

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(Moor et al., 1991). Microsatellite analysis has widespread applications because of its sensitivity to genetic diversity within and between populations (DeWoody and Avise, 2000; Selkoe and Toonen, 2006). Compared to molecular markers such as allozymes, RAPD, and RFLP markers, microsatellite markers are codominant and contain more information for population analysis and identifying differences in and among populations (Yan et al., 2005).

Reciprocal mating has been carried out between kutum *Rutilus frisii kutum* and roach *Rutilus rutilus*, but the hybrids were not economically viable (Hosseini, 1993). The Guilan Research Fisheries Station, carried out hybridization between female kutum and male grass carp (Hosseini, 1996) with the goal of producing a fish with the flavor of kutum and growth rate of grass carp. Verification of this hybridization using cytogenetic methods indicated that all hybrid progeny showed a karyotype similar to the maternal (kutum) karyotype, suggesting that the hybrids were gynogenic (Nowruzfashkhami et al. 2001).

The aim of the present study was to track microsatellite markers to explore the contribution of the parental genomes to progeny of female kutum and male grass carp.

MATERIAL AND METHODS

The study was conducted at the Shahid Ansari Bony Fishes Stock Rehabilitation and Breeding Center, Rasht, Iran using a breeding female kutum caught from the Lemir River in western Guilan province and a cultured male grass carp obtained from an earthen pond. A small piece of each fish's caudal fin was clipped for DNA extraction. Hormone stimulation was carried out in the kutum by pituitary gland (2 mg/kg). The male grass carp was injected with a combination of LHRh-a + pituitary gland (12.5 µg + 0.5 mg/kg). The eggs and milt were obtained by pressure on the fish abdomen. Three procedures were conducted: hybrid (female kutum x male grass carp), control kutum (female x male kutum), and control grass carp (female x male grass carp). Each treatment used 15 g eggs. Dry insemination was conducted with 0.15 ml fresh sperm. The experiment was done in triplicate. Fertilized eggs were placed in 21°C water in Weise incubators for embryogenesis. At 4 h post-insemination, 100 eggs were removed randomly from the incubator and cleavage or cell division assessed. Hatching took place over a period up to 84 h. Eight larvae were killed for DNA extraction, and the whole body placed in 96% ethanol. The DNA was extracted using a phenol-chloroform technique (Pourkazemi, 1996). For evaluation of DNA quality, both specterophotometry and agarose gel (1%) were employed. Recognition of hybrids was carried out via microsatellite markers at the International Sturgeon Research Institute (Rasht, Guilan, Iran). To distinguish hybrid heritability, two microsatellite markers (Ca3 and Ca5) of the cyprinid *Campostoma anomalum* were used (Dimsosky et al. 2000). The GenBank Accession numbers are AF277575 (Ca3) and AF277577 (Ca5). The primer sequences for amplification of two microsatellite markers were as follows:

Ca3 F (5′-GGACAGTGAGGGACGCAGAC-3′)Ca3 R (5′-TCTAGCCCCCAAATTTTAC GG-3′) Ca5 F (5′-TTGAGTGGATG GTGCTT GTA-3′) Ca5 R (5′-GCATTGCCA AAAGTTA CCTAA-3′).

The standard mix of PCR consisted of 1.5 mM MgCl2, 0.2 mM dNTPs, 10X PCR Buffer, 0.75 u Taq DNA polymerase, and 10 pmol of each primer to a final volume of 20 µl (CinaGene Company, Tehran, Iran). PCR conditions for Ca3 markers were 94°C for 3 min (1 cycle), then 94°C for 30 sec; 56°C for 30 sec; 72°C for 40 sec (30 cycles); and a final single cycle at 72°C for 5 min. PCR conditions for the Ca5 marker were 94°C for 3 min (1 cycle), 94°C for 30 sec; 58°C for 30 sec; 72°C for 30 sec (30 cycles); and a final cycle at 72°C for 5 min. The PCR product was run on polyacrylamide gel (6%) and silver stained.

RESULTS

Numbers of hatched and surviving hybrid larvae were low, and in only one of the three hybrid repeats did any (eight) individuals survive. In the kutum control group, no larvae hatched. In the grass carp control group, 4870 larvae successfully hatched. The results of fertilization, hatching, and survival rate are summarized in Table 1.

Table1. Fertilization and hatching rates of hybrids (female kutum × male grass carp), kutum control, and grass carp control

Groups	Weight of eggs g	eggs g-1	Fertilization %	Hatching %
1 (female kutum × male grass carp)	15	275	10.00 (412/4125)	0.00
2 "	15	275	22.00 (907/4125)	0.88 (8/907)
3 "	15	275	8.50 (350/4125)	0.00
Control (female × male kutum)	15	275	5.00 (206/4125)	0.00
Control (female × male grass carp)	15	785	74.50 (8772/11775)	55.50

The results of analysis of Ca5 microsatellite markers in hybrids and their parents showed that progeny inherited the maternal allele and showed no contribution from the paternal allele (Fig. 1).
The results obtained with Ca3 microsatellite markers showed that hybrid progeny inherited the maternal allele only (Fig. 2).

DISCUSSION

Since sexual maturation takes place March-April in kutum and in May for grass carp, the end of the reproduction season in kutum coincides with the beginning of the season in grass carp. Hence, the eggs of kutum were not of good quality. The reproduction of grass carp takes place at warmer temperatures than does kutum reproduction. In general, there is no simultaneity in sexual maturation of grass carp and kutum. Thus for production of the hybrid (kutum × grass carp) the quality of milt and eggs of kutum would be generally poor, unless the grass carp milt is saved via cryopreservation, which is not generally used for grass carp in Iran.

Various types of chromosome and gene heritability occur in interspecific hybridization. Therefore progeny obtained from hybridization can manifest gynogenesis, androgenesis, diploidy, triploidy, and tetraploidy (Chevassus, 1983). As a result, clonal hybrids of Atlantic salmon and brook trout may be gynogenetic just after one generation (Galbreath et al., 1997).
Morphological discrimination of fish species is difficult in fish such as Atlantic salmon and brown trout, particularly at juvenile stages (L'Abee-Lund, 1988). Their hybrids can present a salmon-like (Youngson et al., 1992) or a trout-like (Beall et al., 1997) phenotype, the only reliable identification being through the analysis of genetic patterns. Using DNA markers is the best method to identify Atlantic salmon × brown trout hybrids and to differentiate the species (Gross et al., 1996; Elo et al., 1997). DNA analysis showed that the locus positions in Atlantic salmon and brook trout were distinct from each other, but the hybrids inherited the loci of both parents. In the present study, phenotypic similarity was observed between kutum and hybrids

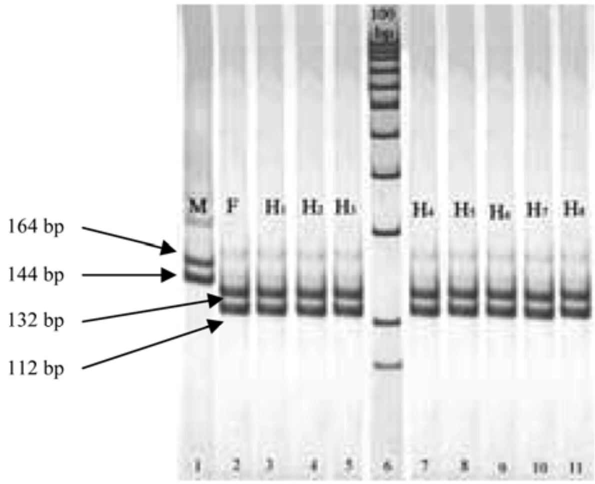

Fig.1. Banding pattern in male grass carp (column 1), female kutum (column 2), and their progeny (columns 3 to 5 and 7 to 11) (column 6, marker 100 bp) using Ca5 microsatellite markers.

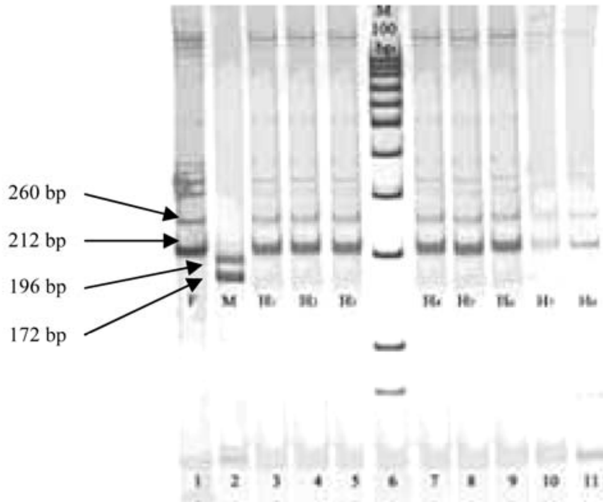

Fig. 2. Banding pattern in female kutum (column 1), male grass carp (column 2), and their progeny (columns 3 to 5 and 7 to 11) (column 6, marker 100 bp) using Ca3 microsatellite markers.

(kutum ♀ × grass carp ♂) (Fig. 3). Even cytological methods could not identify differences between hybrids and the maternal parent. Therefore, DNA markers are necessary to verify the hybrid genome and its heritability. Evaluation of genetic diversity with DNA showed that microsatellites are adequately sensitive for the study of homozygosity with inbreeding. Therefore, it is suitable for differentiating populations. A few microsatellite loci having more than 20 alleles are required for distinction of a given parent's offspring in mixed populations, although loci with lower numbers of alleles may be suitable for population genetics and phylogeny (O'Connell and Wright, 1997; Estoup and Angers, 1998).

Our findings suggest that microsatellite markers are preferable for distinguishing hybrids obtained from female kutum and male grass carp, since phenotypic discriminating of hybrids is difficult due to the similarity of a few meristic traits with female kutum (Khara, 1998). Elo et al. (1997) suggested that DNA markers are suitable for the study of hybrids. In the present study, we used two microsatellite loci in eight hybrid individuals. Mia et al. (2002) used three microsatellite loci for distinction of progeny resulting from the mating of silver carp *Hypophthalmichthys molitrix* and bighead carp *Aristichthys nobilis*. They analyzed five individuals of

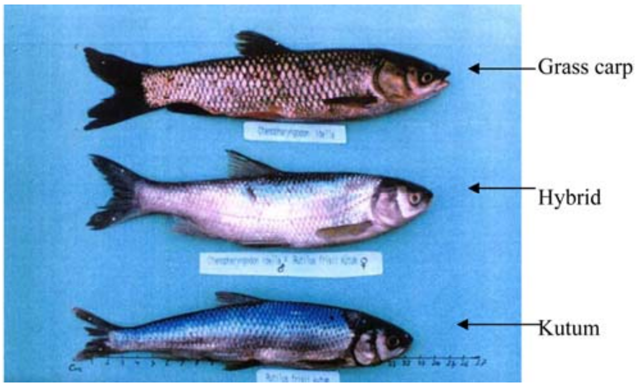

Fig 3. Phenotypic comparison of grass carp (above), hybrid (kutum ♀× grass carp ♂) (middle), and kutum (bottom).

doubtful hybrids, three of which were heterozygote in the three loci and recognized as hybrids. Nowruzfashkhami et al. (2001) showed that hybridization of female kutum (2n = 50 chromosomes) and male grass carp (2n = 48 chromosomes) (Hosseini, 1996) resulted in chromosome numbers of hybrids equal to that of their maternal (kutum) parent. They suggested that the similarity of the hybrid karyotype and phenotype (a few meristic traits) to the female kutum demonstrated gynogenesis.

According to our findings, the two microsatellite markers used were adequate to compare genomic heritability of hybrids to that of their parents. The similarity of the hybrids with their maternal parent suggests gynogenesis.

Marian and Krasznai (1978) reported sterile triploid fingerlings from mating female grass carp and male bighead carp. In their study, diploid gynogenesis and mosaic individuals in hybrids were found as well. In hybridization of female goldfish *Carassius auratus gibelio* and male grass carp, gynogenetic individuals were produced (Chevassus, 1983). This might be due to the insemination of ovum with degraded genome sperm or to lack of insertion of sperm nuclei into the ovum.

Unfavorable ambient conditions, such as warm or cold water or chemical conditions can impair separation of the second polar body in oogenesis to produce diploid eggs and gynogenetic fish (Kirpichnikov, 1981); hence gynogenesis may occur naturally in a few fishes, such as Amazon molly *Poecilia formosa* (Hubbs and Hubb, 1932) and goldfish (Kirpichnikov, 1981). Results of our study confirmed previous morphometric and cytogenetic studies and showed that the studied hybrid progeny have a genome similar to their maternal parent and seem to be gynogenetic.

Acknowledgements

The authors thank Mr. Mohammad Hossein Toluiee, Head of Shahid Ansari Bony Fishes Stock Rehabilitation and Breeding Center and his deputy, Mr. Reza Khomeirani. We are also grateful to the personnel and staff of their center and to the staff of the Genetics Department of International Sturgeon Research Institute

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