Searching for sex-specific DNA markers in the genome of Nile tilapia (*Oreochromis niloticus*): Improving the efficiency of the FluoMEP method

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ABSTRACT

We describe the utilisation of a new method, known as Fluorescent Motif Enhanced Polymorphism, FluoMEP, developed by us (Chang, A., Liew, W.C., Chuah, A., Lim, Z., Lin, Q., Orban, L. 2007. *Electrophoresis*, 28), as applied to searching for sex-specific DNA markers in the genome of the Nile Tilapia, *Oreochromis niloticus*. It combines the rapid mass screening capability of RAPD (random amplified polymorphic DNA) with the automated, high resolution capillary electrophoresis (CE) used in detection of AFLP (amplified fragment length polymorphism) patterns. Three families were screened, yielding a total of four Y-linked markers. We also describe the methods applied to improve the efficiency of the FluoMEP method for detecting polymorphisms by increasing the number of peaks. These methods include varying the PCR and reaction mix conditions, utilising degenerate oligonucleotide-primed PCR (DOP-PCR) primers and modifying the degenerate primers to become repeat-targeting primers.

INTRODUCTION

FluoMEP, a novel method we developed, possesses the rapid mass screening capability of RAPD (Welsh & McClelland, 1990; Williams et al., 1990) combined with the high resolving resolution offered in AFLP (Vos et al., 1995). Here we utilised FluoMEP in the search for sex-specific DNA markers in the genome of the Nile tilapia. Male mono-sex populations, by applying hormone, treatment, are usually preferred due to higher growth rate (Palada-de Vera & Eknath, 1993) and precocious maturation. However, with the increasing resistance to hormone treatment, there is a need to develop a rapid molecular-sexing tool. FluoMEP has already demonstrated its usefulness in searches for sex-specific markers in some preliminary screens performed in *O. niloticus* Family 1 and in guppy, *Poecilia reticulate*. We report the results of the comparative FluoMEP screens performed on the 80 individuals of the Families 2 and 3 obtainediv. We also describe the various methods used to improve the efficiency of the FluoMEP method.

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ii Supervisor
iii This project was undertaken together with Zijie, Lim who will be submitting his own report separately.
iv This project is a commercial collaboration. As such, details of the Y-linked markers, including the RAPD primer, cannot be divulged.
MATERIALS AND METHODS

Fin clip samples were obtained from GenoMar ASA, 30 samples (15 male 15 female) from Family 1 and 40 samples (20 male 20 female) from Families 2 and 3 each. The FluoMEP PCR reactions were performed as recommended in (Chang et al., 2007) and separated by capillary electrophoresis (CE) on the ABI 3730xl DNA analyzer (ABI systems). The results (in the form of peak profiles) were then analysed by the GeneMapper v3.5 software (ABI). The DNA samples of each family were formed into 4 pools (2 male, 2 female). Comparative FluoMEP analyses were then performed on these 4 pools.

Conditions varied for the FluoMEP reaction included lowering the annealing temperature and delayed addition of the common primer, increasing the amount of MgCl2, varying the amount of RAPD primer and increasing the amount of DNA template. Effect of different DNA sample quality was also compared, using HotSHOT (Truett et al., 2000), Genomic DNA Mini Kit (Geneaid) and phenol-chloroform procedure. Degenerate primers, D005 and D006 were designed according to (Telenius et al., 1992). D007 was modified from D006. These primers were first tested against themselves, with RAPD primers, then in FluoMEP reactions with Com5H. Optimisation was attempted for these reactions too. D005 was also modified to target recurring repeats by incorporating repeat sequences into two variants.

Double-common primer FluoMEP reactions were performed. Com2F was used together with Com3F and Com4F in 24 primer combination screenings in Families 2 and 3. 5 pmol of RAPD primer, 10 pmol of the 1st common primer and 10 pmol of the 2nd common primer were used.

RESULTS

FluoMEP screens
In family 1, 96 primer combinations were screened in a previous project. One Y-linked marker, OnY3, with sexing efficiency of 90% was isolated. 504 primer combinations were screened in Family 2, and two (OnY4 and OnY6) were successfully verified to show 92.1% and 89.5% sexing efficiencies, respectively. A total of 480 primer combinations were screened in Family 3, but no Y-linked markers were discovered.

FluoMEP optimisation
All variations of FluoMEP reaction conditions showed little or almost no improvement to the original FluoMEP procedure as recommended by Chang et al., 2007 (data not shown). Agarose gel electrophoresis of the preliminary testing of the degenerate primers showed promising results. Peak profile results for D005 to D007 in combination with Com5H showed that the smears that are obtained when using agarose gel electrophoresis are actually multiple peaks. Optimisation experiments showed no marked difference. Large numbers of peaks were obtained, still restricted to the 0 to 500 bp size range. Only one variant of the repeat-targeting primer modified from degenerate primer worked.
**Combining two common primers**

The increased performance of FluoMEP by using 2 common primers was indeed effective. Family 3 finally yielded one Y-linked marker, OnY7, with sexing efficiency 76.9%.

**DISCUSSION**

The advantage of the FluoMEP method is demonstrated when the use of degenerate primers is employed. With the high resolving capability of CE incorporated into the FluoMEP method which we developed, we are able to resolve what is seen on agarose gels as smears, into individual peaks. Although the number of peaks was dramatically increased by the introduction of degenerate primers, it was observed that the peaks were mostly restricted to the 0 to 500 bp range. In order to favour longer fragments, we have to experiment further with decreasing buffer concentration, decreasing elongation temperature and having initial cycles of higher annealing temperature. Experiments with the repeat-targeting primers seemed to show that the restriction site seems to play a role in the binding of the primers. It is possible that the degenerate primers are targeting the restriction. Potential ways to explore this would include design and compare degenerate primers with rare and frequent restriction sites.

However, it should be not presumed that the low number of Y-linked markers isolated is solely due to the limitations in the FluoMEP method. Other methods have been used, such as RAPD and AFLP, in previous work (Lee, 2007; Lim, 2006; Lin, 2006; Pathak, 2007) and by other groups (Bardakci, 2000; Ezaz et al., 2004; Lee et al., 2003). The reason for the markers having lower than 100% sexing efficiency could be due to the very nature of the sex chromosomes themselves, being still in the primitive stage (Carrasco et al., 1999). The present markers may be targeting the regions that just lie outside the sex determining region, which may be prone to recombination in different families. This is also the reason why the Y-linked markers are not 100% efficient, as the markers might not be targeting the sex determining region proper but the regions surrounding it. It is known that there is suppression of recombination at the sex determining region and the adjacent regions. Therefore, the greater the proximity of a particular locus to the sex determining region, the more it would be protected from recombination. As a result, any markers targeting that locus would have higher sexing efficiency as this region would be more tightly linked to the sex determining region.

**REFERENCES**


resolution for analysis of DNA copy number changes by array comparative genomic hybridization. *Genome Res* **14**: 188-96.


