DNA sex identification in the three-spined stickleback

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Abstract

We describe the AFLP technique that can be used to identify sex-linked DNA markers in fish with genetical sex determination. To demonstrate, we isolate two sex linked markers in the three-spined stickleback (*Gasterosteus aculeatus*) and develop a sex identification test.

The ability to carry out DNA sex identification on a species depends on gender determination. Mammals have sex determination (GSD) where the decisive male switch is a conserved Y-linked gene, SRY (Foster *et al.*, 1992). This type of GSD is a well established mechanism and has lead to the evolution of separate X and Y sex chromosomes (XY:male, XX:female; Charlesworth, 1991). Consequently because the Y only occurs in one sex, any individual that contains a DNA marker unique to this chromosome must be male (Griffiths, In Press).

In teleosts, the situation is different. As Bull (1983) describes, several different forms of sex determination operate. In a species with environmental sex determination the genomes of individuals are identical but a trigger such as temperature or social dominance dictate whether male or female gender develop. In polyfunctional sex determination, combinations of genes scattered amongst the chromosomes elicit gender whilst in other species the ratio of X chromosomes to autosomes provide a trigger. Gender is adequately determined by these mechanisms but there is no DNA sequence that is unique to one gender. This makes DNA sex identification either difficult or impossible.

Fortunately in teleosts, such as the three-spined stickleback (*Gasterosteus aculeatus* L.), GSD does operate. In some species, sex chromosomes have fully developed. In others a pair of autosomes act as sex chromosomes but their morphology remains similar. As a consequence the genders are difficult recognize karyotypically (Ansari *et al.*, 1988; Sola *et al.*, 1981) although sex identification can be achieved using molecular genetical methods.

One method to identify a sex-specific DNA sequence is AFLP (Amplified Fragment Length Polymorphism, although this is a poor description of the procedure; Griffiths & Orr, 1999; Reamon-Buttner *et al.*, 1998; Vos *et al.*, 1995). In simple terms, the technique starts with genomic DNA which is cut with two restriction enzymes, for example *Mse*I and *Eco*RI. Adaptors, which are synthesized sections of DNA, are ligated to each cut site. A polymerase chain reaction (PCR) is then carried out, using primers complementary to the adaptors, but extending three bases into the genomic fragment. Their sequence extension varies eg E-CAT and M-TTG and this selects and amplifies a small portion of the genome. Different primers are used in separate reactions to amplify distinct parts of the genome.

The PCR products are displayed on a polyacrylamide gel and silver staining or primer radiolabelling reveals about 100 bands. Consistent experimental conditions are required to ensure that an individual always displays the same array using a given set of primers. Once a sex-specific

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marker is found it can either be employed to identify gender directly with AFLP or it can be sequenced and used to design primers for a standard PCR based sexing technique. We took the latter course which provides a sexing technique that is faster and cheaper than AFLP (Griffiths & Orr, 1999).

To identify sex-linked bands, pre-sexed individuals have to be used. For initial AFLP screening three-spined sticklebacks were taken from a population in Edinburgh, Scotland (n=16). For large-scale, geographic screening samples were collected from Milngavie, Scotland (n=5), Silverdale, England (n=8), Vikhog, Sweden (n=8), west coast, Canada (n=6) and various populations in Japan (n=10). Interspecific testing included the nine-spined (*Pungitus pungitus* L.; n=8; Skåne; Sweden) and fifteen-spined stickleback (*Spinachia spinachia* L.; n=6; Gullmarsfjord, Sweden). The fish were sexed by external morphology or gonadal structure (Wootton *et al.*, 1995). DNA was purified by incubating the tail and 5 mm of the caudal peduncle in 250 µl of 2xCTAB (0.1 M TRIS pH 8.0, 1.4 M NaCl, 0.02 M EDTA pH 8.0, 2% CTAB, 1% PVP) extraction buffer with 2% beta-mercaptoethanol and 5-10 µl of Proteinase K (10 mg/ml) at 37 °C overnight. Two rounds of phenol:chloroform and a chloroform extraction were performed, followed by alcohol precipitation (protocol 9: Milligan, 1998). The DNA was resuspended in 20-150 µl of TE (10 mM Tris (pH7.6), 1 mM EDTA).

AFLP was carried out as described by Griffiths & Orr (1999) and Vos *et al.*, (1995) although pre-selective amplification was omitted. Identifying sex-linked markers was difficult as it was uncertain which was the heterogametic sex. Whilst the four-spined stickleback has heterogametic females (WZ:female, ZZ:male; Chen & Reisman, 1970), *G. aculeatus* need not follow this pattern. From breeding experiments Beatty (1964) suggested such a WZ system although karyotyping found no sex chromosomes (Klinkhardt & Buuk, 1990). Five AFLP primer combinations were screened in 3 male and 3 female individuals. Any primers that produced bands solely in one sex were re-screened on 6 more individuals to decrease the probability of false identification of a true sex linked marker to $p = 2.4 \times 10^{-4}$ ($p=q^m.(1-q)^f$ where $q=m/m+f$ and m and f are the number of males and females (Lessells & Mateman 1996)). With the primer pairs *E-AGG* (5'-CGACTGCGTACCAATTC*AGG*-3') and *M-CAA* (5'-GATGAGTCCTGAGTAA*CAA*-3') and *E-AGG* and *M-CAG* 5'-GATGAGTCCTGAGTAA*CAG* -3' two male specific bands were produced. Consequently three-spine sticklebacks have primitive X and Y chromosomes in heterogametic males despite their close relative, the four-spine stickleback, having female heterogamety.

Guided by the autoradiograph, the sex-specific bands were cut from dried AFLP acrylamide gel, re-amplified and sequenced directly. Two sets of primers were designed (Oligo Lite: Rychlik & Rhoads, 1989): Ga1F 5'-CTTCTTTCCTCTCACCATACTCA-3' and Ga1R 5'-AGATGACGGGTTGATAAACAG-3'; Ga2F 5'-CACATTATTACAACATACGGACA-3' and Ga2R 5'-ACAGACGCTGAATGACGAAG-3'. The PCR reactions were carried out in 10 µl, containing 10ng/ul of each primer, 200 µM of each dNTP, 5-10 ng/ul target DNA, 0.35 units Taq polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM TrisHCl (pH8.8), 0.1 % Triton X-100 (where the last 3 are in a Promega buffer). An UnoII thermal cycler (Biometra) denatured the reaction at 95 °C for 1 min 30 s, then performed 30 cycles of 48 °C for 20 s, 72 °C for 20 s and 94 °C for 30 s with a final run of 48 °C for 1 min and 72 °C for 5 mins. The PCR products were sized in a 3% agarose gel which revealed male specific bands of 371 bp (Ga1F/Ga1R) and 297 bp (Ga2F/Ga2R).

Although Y-linked markers were available, a sexing test also requires a positive control to confirm amplification in each PCR reaction. This would usually require a second set of primers but PCR with Ga1F/Ga1R produces a second band of about 600 bp in both sexes (Fig1). To act as a true control this band should amplify less well than the sex-linked product (unlike Fig1). However, because the control is larger than the sex linked band, its relative amplification efficiency can be changes by adjusting the length of the PCR extension period. As a consequence we can adjust the PCR conditions to produce an optimal sex identification test.

One problem with the PCR techniques is that it requires the correct annealing of the primers. A mutation in the priming site may prevent amplification so no product would ensue. This is termed a 'null allele'. Because the sex-linked markers are probably non-functional DNA which evolves at a faster rate than the coding-DNA, their mutation rate is relatively high. Furthermore, the Y chromosome is transmitted by sperm, which unlike ova, are continually being manufactured. As Ellegren & Fridolfsson (1997) have shown, frequent copying increases the speed of mutation thus increasing the frequency of null alleles to falsify the sexing test. To check reliability a large number

(>100) of known males could be tested for null alleles (Lessells & Mateman 1998). Alternatively, individuals from populations that cover the geographic range of the species may be screened. Hence, fewer samples will cover more possible mutations than intensely screening a single population. Our analysis of the three-spined stickleback used the latter approach as the species has evolved independently in anadromous and marine situations and a variety of land-locked, geographically-isolated populations.

Sex identification based on Ga1F/Ga1R was tried on six Atlantic and Pacific populations that span the stickleback range. The sex of all fish was identified correctly (n=53; Fig 1). Moreover, the sequence of the PCR product from Ga1F/Ga1R was ascertained once in each population and no mutations were identified (Genbank: AF267743, AF267744). However the test is limited to a single species as neither nine-spined nor fifteen-spined sticklebacks produced a Y-linked band (Fig 1).

One aim of the Ga1F/Ga1R test was to allow sex identification but cause such minor damage that the fish can be released. We used tissue samples from dead Japanese sticklebacks in this trial. The first was a pectoral spine, whose removal has little consequence to mobility and mate choice (Ward & Fitzgerald, 1987). The weight of the spines was 0.016 g (n=10). The second was a clipping taken from the tail fin (<0.0001 g; n=10). DNA extraction was carried out as described earlier and the PCR gave correct identification in every case. To complete the range of tests, sex identification was carried out on freshly emerged fry (<1 day old). Although there was no morphological corroboration with these results, unambiguous banding patterns identified both males and females in an approximately 50/50 ratio (n=244, F=118, M=126)

Our study shows that the AFLP technique can be used to identify sex-linked markers successfully in some teleosts. It cannot be used throughout the class because sex determination mechanisms vary between species (Bull, 1983; Sola *et al.,* 1981). In species with genetical sex determination, AFLP can recognize whether the XY or WZ sex chromosome system is in use. This is normally carried out karyotypically (Klinkhardt & Buuk, 1990) or through breeding (Beatty 1964) which can be time consuming procedures. Perhaps most importantly our study demonstrates the sensitivity of the AFLP procedure. The technique has been carried out to sex birds (Griffiths & Orr, 1999) but this is the first time that it has been used on a species where the sex-specific region of DNA is so small that it cannot be identified cytologically. What is more, we have shown that the Ga1F/Ga1R primers are so sensitive that they can work on tiny amounts of tissue which could allow live animals to be sexed successfully. As the three-spined stickleback is used extensively in the fields of behavioural ecology, developmental biology, ecotoxicology and neuroendocrinology, the availability of a test that can give unambiguous gender data, even of newly-hatched fry or from very small non-destructive tissue samples, should open up new avenues for research.

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FIG. 1. Sex identification of alternate male and female DNA samples from disparate populations of the three-spined stickleback using Ga1F and Ga1R PCR primers (lanes 2-9). The male specific band is of 371 bp and the larger, positive control band occurs at 600 bp. The technique fails to sex male and female nine-spined (lanes 10 & 11) or fifteen spined sticklebacks (lanes 12 & 13) so is taxonomically restricted. Lane 1: 1 Kb marker (BRL), 2 and 3: England, 4 and 5: Sweden, 6 and 7: Japan and 8 and 9: Canada